# ANTI-NEUROINFLAMMATORY ACTIVITIES OF LIPID COMPONENTS FROM SCLEROTIA OF *Lignosus rhinocerotis* AND STROMA OF *Cordyceps militaris* IN MICROGLIA BV2 CELLS

University of Malaya

**NEERANJINI A/P S.NALLATHAMBY**

**FACULTY OF SCIENCE**
**UNIVERSITY OF MALAYA**
**KUALA LUMPUR**

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NEERANJINI A/P S.NALLATHAMBY

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INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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Name of Candidate : Neeranjini A/P S. Nallathamby

Registration/Matric No. : SHC 110107

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Field of Study : Mycology (Biology & Biochemistry)

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# ABSTRACT

Inflammation in the brain, which is characterised by activation of microglia cells, plays an important role in the progression of many central nervous system (CNS) diseases. Many medicinal and edible mushrooms have been studied intensively for the treatment of neuroinflammatory conditions. In this study, an assay guided approach was adopted to identify the active ingredients of *L. rhinocerotis* and *C. militaris* responsible for its anti-neuroinflammatory activity. Preliminary *in vitro* antioxidant investigations of the extracts revealed that the ethanol extract of *L. rhinocerotis* had higher antioxidant capabilities compared to the aqueous extracts in all the three assays tested viz., DPPH, TEAC and FRAP; however, the aqueous extract of *C. militaris* exhibited higher antioxidant activities compared to the ethanol extract. The MTS assay revealed that both the (ethanolic and aqueous) extracts of the mushrooms did not have cytotoxic effects on the BV2 cells up to 100 μg/mL concentrations. The ethanol extracts of both mushrooms decreased NO production of LPS stimulated BV2 cells by more than 60% compared to their aqueous extracts at 100 μg/mL. Further fractionation of the ethanol extract to the hexane and ethyl acetate fractions showed that the ethyl acetate fractions of both mushrooms had a significant effect on NO production inhibition (>50%) compared to their hexane fractions at 10 μg/mL. Two bioactive subfractions, CE2 and CE3 from *C. militaris* and TE3 from *L. rhinocerotis* from the ethyl acetate fraction were obtained through vacuum liquid chromatography. These subfractions also showed more than 45% reduction in NO production at 100 μg/mL. Twenty two lipid components were identified by GCMS analysis whereby eight (8) components from TE3, eleven (11) components from CE2 and three (3) components from CE3. All these components had synergistic effect to reduce neuroinflammation. The qPCR results showed that all

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subfractions down regulated the proinflammatory genes (iNOS, COX2 and IL-1β) and only TE3 and CE2 up-regulated the HO-1 and NQO-1 anti-inflammatory genes. Subfractions TE3 and CE2 inhibited neuroinflammation via NRF2 and NFκB pathways while the CE3 subfraction inhibited neuroinflammation via NFκB pathway in LPS triggered BV2 cells.

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# ABSTRAK

Neuroinflamasi adalah keradangan sistem saraf dalam otak yang disebabkan oleh jangkitan, kecederaan otak yang trauma, metabolit-metabolit toksik atau *autoimmunity* memainkan peranan penting dalam perkembangan pelbagai penyakit neurodegenerasi mempunyai ciri-ciri pengaktifan sel-sel microglia. Dalam kajian ini, pendekatan berpandukan bioasei aktiviti antioksidan dan antineuroinflammasi secara *in vitro* terhadap ekstrak-ekstrak *L. rhinocerotis* and *C. militaris* telah dikaji. Aktiviti antioksidan ekstrak, fraksi dan subfraksi aktif *L. rhinocerotis* and *C. militaris* disaring menggunakan esei DPPH, TEAC dan FRAP. Kedua-dua cendawan ini menunjukkan 20-60% perencatan aktiviti DPPH, 10-42% perencatan aktiviti TEAC dan 13-690 mg FE/ g ekstrak penggurangan aktiviti FRAP. Semua ekstrak dan fraksi tidak memberikan kesan sitotoksik terhadap sel-sel BV2 apabila diuji secara individu melalui esei MTS pada kepekatan-kepekatan yang dikaji. Aktiviti anti-neuroinflammasi ditentukan menerusi keupayaan ekstrak-ekstrak mengurangkan produksi nitrik oksida dalam sel mikroglia BV2 yang dipengaruhi oleh LPS. Pemfraksian terhadap ekstrak etanol *L. rhinocerotis* dan *C. militaris* yang mempunyai aktiviti antineuroinflamasi melebehi 60% pada kepekatan 100 µg/mL telah dilakukan dengan menggunakan pelarut heksana dan etil asetat. Fraksi etil asetat *L. rhinocerotis* and *C. militaris* menunjukkan pengurangan produksi NO sebanyak 50% pada kepekatan 10 µg/mL. Kesan sitoksik subfraksi bioaktif CE2 and CE3 daripada fraksi etil asetat *C. militaris* dan subfraksi TE3 daripada fraksi etil asetat *L. rhinocerotis* diuji terhadap sel-sel BV2 tanpa dan dengan pengaruh LPS. Didapati bahawa ia tidak memberi kesan sitotoksik malah boleh melindungi sel-sel BV2 daripada produksi NO yang dipengaruhi oleh LPS. Pada kepekatan 1 µg/mL - 100 µg/mL, subfraksi-subfraksi bioaktif menunjukkan pengurangan produksi NO melebihi

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45%. Komposisi kimia subfraksi-subfraksi bioaktif dikenalpasti melalui analisis GCMS. Sejumlah 22 komponen lipid dikenalpasti dalam subfraksi TE3, CE2 dan CE3 yang masing masingnya terdiri daripada lapan, sebelas dan tiga komponen. Setiap komponen yang dikenalpasti mempunyai kesan sinergi terhadap penggurangan neuroinflamasi. Berdasarkan analisis qPCR, ketiga-tiga subfraksi tersebut berupaya menurunkan ekspresi gen proinflamasi (iNOS, COX2 and IL-1β), manakala TE3 dan CE2 mampu meningkatkan gen antiinflamasi (HO-1 and NQO-1). Subfraksi-subfraksi TE3 dan CE2 telah mengurangkan neuroinflammasi melalui laluan NRF2 dan NFκB manakala subfraksi CE3 menggurangkan neuroinflammasi melalui penghalangan pengaktifan NFκB.

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# TABLE OF CONTENT

ORIGINAL LITERARY WORK DECLARATION ii

ABSTRACT iii

ABSTRAK v

ACKNOWLEDGEMENT vii

TABLE OF CONTENTS viii

LIST OF FIGURES xii

LIST OF TABLES xiv

LIST OF SYMBOLS AND ABBREVIATION xvi

LIST OF APPENDICES xix

**CHAPTER 1: INTRODUCTION** 1

1.1. Objectives of the study 6

**CHAPTER 2: LITERATURE REVIEW** 7

2.1. Inflammation 7

2.2. Mushrooms 15

2.3. Medicinal mushrooms 17

2.4. *Lignosus rhinocerotis* 19

2.5. *Cordyceps militaris* 24

2.6. Anti-inflammatory activity of mushrooms 27

2.7. Lipids and essential fatty acid components in mushroom 29

2.8. Bioassay investigations 33

2.8.1. Antioxidant activities 33

2.8.2. MTS cytotoxicity assay 35

2.8.3. Nitric oxide determination assay 36

2.8.4. qRTPCR gene expression 37

University of Malaya

**CHAPTER 3: MATERIALS AND METHODS** 39

3.1. Mushroom samples 39

3.2. Preparation of crude extracts from *Lignosus rhinocerotis* and *Cordyceps militaris* 39

3.2.1. Hot water extraction 39

3.2.2. Ethanol extraction 40

3.3. *In vitro* antioxidant activity of *Lignosus rhinocerotis* and *Cordyceps militaris* 40

3.3.1. DPPH radical scavenging activity 40

3.3.2. Trolox equivalent antioxidant activity 41

3.3.3. Ferric reduction antioxidant power 42

3.4. *In vitro* BV2 cell culture 42

3.4.1. Cell culture 42

3.4.2. Cell count 43

3.5. *In vitro* studies of *Lignosus rhinocerotis* and *Cordyceps militaris* on BV2 cells 43

3.5.1. Cytotoxic effect 43

3.5.2. Anti-inflammatory activity 44

3.6. Bioassay guided fractionation of *Lignosus rhinocerotis* and *Cordyceps militaris* 45

3.6.1. Fractionation of ethanol extracts 45

3.6.2. Isolation of Lipid Components 46

3.6.2.1. Sample preparation 46

3.6.2.2. Vacuum Liquid Chromatography (VLC). 46

3.6.2.3. Thin-Layer Chromatography (TLC). 46

3.6.3. Identification of major bioactive constituents by GC-MS 47

University of Malaya

3.7. Gene expression studies of *Lignosus rhinocerotis* and *Cordyceps militaris* bioactive constituents 48

3.8. Statistical analysis 51

**CHAPTER 4: RESULTS** 52

4.1. Aqueous and crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies 52

4.1.1. Physical characterisation of aqueous and crude ethanol extracts 52

4.1.2. *In vitro* antioxidant activity of aqueous and crude ethanol extracts 54

4.1.3. *In vitro* cytotoxic effect of aqueous and crude ethanol extracts 56

4.1.4. *In vitro* anti-inflammatory activity of aqueous and crude ethanol extracts 58

4.2. Bioassay guided fractions from crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies 61

4.2.1. Physical characterisation of hexane and ethyl acetate fractions 61

4.2.2. *In vitro* antioxidant activity of hexane and ethyl acetate fractions 63

4.2.3. *In vitro* cytotoxic effect of hexane and ethyl acetate fractions 65

4.2.4. *In vitro* anti-inflammatory activity of hexane and ethyl acetate fractions 68

4.2.5. GC-MS profiling of bioactive ethyl acetate fraction 71

University of Malaya

4.3. Bioactive subfractions from the ethyl acetate fraction and their *in vitro* studies 73

4.3.1. Physical characterisation of the bioactive subfractions 74

4.3.2. *In vitro* antioxidant activity of bioactive subfractions 75

4.3.3. *In vitro* cytotoxic and neuroprotective effect of bioactive subfractions 76

4.3.4. *In vitro* anti-inflammatory activity of bioactive subfractions 80

4.3.5. GC-MS profiling of bioactive subfractions 82

4.3.6. Gene expression study of bioactive subfractions 83

**CHAPTER 5: DISCUSSION** 92

5.1. Aqueous and crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies. 95

5.2. Bioassay guided fractions from crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies. 101

5.3. *In vitro* anti-inflammatory and gene expression studies of *Lignosus rhinocerotis* and *Cordyceps militaris* bioactive subfractions. 109

**CHAPTER 6: CONCLUSION** 127

6.1. Future investigation 129

**LIST OF PUBLICATIONS AND PROCEEDINGS** 130

**REFERENCES** 131

**APPENDICES** 164

University of Malaya

**LIST OF FIGURES**


| | | |
|---|---|---|
| Figure 2.1: | Mechanism of inflammation | 10 |
| Figure 2.2: | The physical appearance of *L. rhinocerotis* and its scientific classification | 19 |
| Figure 2.3: | The physical appearance of *C. militaris* and its scientific classification | 24 |
| Figure 2.4: | Names and structures of fatty acids. | 30 |
| Figure 2.5: | Names and structures of essential fatty acids | 31 |
| Figure 2.6: | Names and structures of sterols. | 32 |
| Figure 4.1: | Flow chart for aqueous and ethanol extracts of *L. rhinocerotis* | 53 |
| Figure 4.2: | Flow chart for aqueous and ethanol extracts of *C. militaris* | 53 |
| Figure 4.3: | Effects of aqueous and ethanol extracts of *L. rhinocerotis* on the viability of BV2 cells. | 57 |
| Figure 4.4: | Effects of aqueous and ethanol extracts of *C. militaris* on the viability of BV2 cells. | 58 |
| Figure 4.5: | Effects of *L. rhinocerotis* extracts on LPS – induced nitric oxide (NO) production by BV2 microglia cells. | 59 |
| Figure 4.6: | Effects of *C. militaris* extracts on LPS – induced nitric oxide (NO) production by BV2 microglia cells. | 60 |
| Figure 4.7: | Flow chart showing fractionation of ethanol extract of *L. rhinocerotis* | 62 |
| Figure 4.8: | Flow chart showing fractionation of ethanol extract of *C. militaris* | 63 |


University of Malaya

Figure 4.9: Effects of hexane and ethyl acetate fractions of *L. rhinocerotis* on the viability of BV2 cells. 66

Figure 4.10: Effects of hexane and ethyl acetate fractions of *C. militaris* on the viability of BV2 cells. 67

Figure 4.11: Effects of *L. rhinocerotis* hexane and ethyl acetate fractions on LPS – induced nitric oxide (NO) production by BV2 microglia cells. 69

Figure 4.12: Effects of *C. militaris* hexane and ethyl acetate fractions on LPS – induced nitric oxide (NO) production by BV2 microglia cells. 70

Figure 4.13: Cytotoxic and neuroprotective effects of bioactive subfractions on the BV2 cells viability. 79

Figure 4.14: Effects of bioactive subfractions on LPS – induced nitric oxide (NO) production by BV2 microglia cells. 81

Figure 4.15: Effect of bioactive subfractions on the proinflammatory gene expression in LPS induced BV2 microglia cells. 86

Figure 4.16: Effect of bioactive subfractions on the anti-inflammatory gene expression in LPS induced BV2 microglia cells. 88

Figure 4.17: Effect of bioactive subfractions on the NRF2 and NFκB2 transcriptional factor and its anti-inflammatory gene expression in LPS induced BV2 microglia cells. 90

University of Malaya

# LIST OF TABLES


| | | |
|---|---|---|
| Table 2.1: | The effect of inflammation based on the intensity and type of inflammation | 12 |
| Table 2.2: | The edible and medicinal mushroom species in Malaysia | 16 |
| Table 2.3: | Mushrooms used for medical purpose by Temuan people in Ulu Kelang Village | 18 |
| Table 2.4: | Mushrooms and its medicinal properties | 18 |
| Table 2.5a: | Nutritional profile (g/100g dry weight) and energy value (kcal/100g dry weight) of sclerotium of *L. rhinocerotis* | 21 |
| Table 2.5b: | Mineral content in sclerotium of *L. rhinocerotis* (mg/100g dry weight). | 21 |
| Table 2.5c: | Concentrations of toxic metals content in sclerotium of *L. rhinocerotis* (mg/kg). | 21 |
| Table 2.6: | Medicinal properties of *L. rhinocerotis* | 22 |
| Table 2.7: | Synonyms names of *Cordyceps* spp. mushroom. | 25 |
| Table 2.8: | Medicinal properties of *Cordyceps militaris* | 27 |
| Table 3.1: | List of genes investigated | 50 |
| Table 4.1: | Antioxidant activities of hot aquoeus and ethanol extracts of *L. rhinocerotis* and *C. militaris* | 55 |
| Table 4.2: | Antioxidant activities of hexane and ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* | 65 |
| Table 4.3: | Lipid constituents (%) of ethyl acetate fraction of *L. rhinocerotis* | 72 |
| Table 4.4: | Lipid constituents (%) of ethyl acetate fraction of *C. militaris*. | 73 |


University of Malaya

Table 4.5: Yield and physical appearance of bioactive subfractions of *L. rhinocerotis* and *C. militaris* 74

Table 4.6: Antioxidant activities of bioactive subfractions of *L. rhinocerotis* and *C. militaris* 75

Table 4.7: Identified lipid constituents in bioactive subfractions of *L. rhinocerotis* and *C. militaris* 83

University of Malaya

## LIST OF SYMBOLS AND ABBREVIATION

| | |
|---|---|
| AA | Ascorbic acid |
| ABTS | 2,2,-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| AD | Alzheimer's disease |
| ANOVA | Analysis of variance |
| Asp | Aspirin |
| β- | beta |
| *C. militaris* | *Cordyceps militaris* |
| cDNA | Complementary deoxyribonucleic acid |
| $CO_2$ | Carbon dioxide |
| COX-2 | Cyclooxygenase-2 |
| CT | Cycle threshold |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulfoxide |
| DPPH | Diphenyl-1-picryl-hydrazyl |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal Bovine Serum |
| FE | $FeSO_4.7H_2O$ equivalent |
| $FeSO_4.7H_2O$ | Ferrous Sulphate Heptahydrate |
| FFA | Free fatty acid |
| FRAP | Ferric reducing antioxidant power |
| g | gram |
| GA | Gallic acid |
| GAE | Gallic acid equivalent |

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| | |
|---|---|
| $H_3PO_4$ | Phosphoric acid |
| HO-1 | Heme oxygenase 1 |
| $KH_2PO_4$ | Pottasium phosphate monobasic |
| $K_2HPO_4$ | Pottasium phosphate dibasic |
| IL-1β | interleukin-1beta |
| iNOS | inducuble Nitric oxide synthase |
| L-NAME | NG-Nitro-L-Arginine Methyl Ester |
| LPS | Lipopolysaccharide |
| *L. rhinocerotis* | *Lignosus rhinocerotis* |
| L | Litre |
| μg/mL | Microgram per mililitre |
| μL | Microlitre |
| μM | Micromolar |
| mM | Millimolar |
| min | Minute |
| $MgSO_4.7H_2O$ | Magnesium sulphate heptahydrate |
| MTS | (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) |
| Nm | Nanometre |
| $Na_2CO_3$ | Sodium carbonate |
| NCD | non-communicable diseases |
| NF-κB | Nuclear factor kappa Beta |
| NO | Nitric oxide |
| NRF2 | nuclear factor (erythroid-derived 2)-like 2,/NFE2L2 |
| NSAID | non-steroidal anti-inflammatory drug |
| NQO-1 | NAD(P)H dehydrogenase [quinone] |

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| | |
|---|---|
| $O_2\cdot$ | Superoxide anion radical |
| OH· | Hydroxyl radical |
| % | Percentage |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| Q-RT PCR | Quantitative-Real time Polymerase chain reaction |
| RNA | ribonucleic acid |
| ROS | Reactive oxygen species |
| RT | Reverse transcription |
| SD | Standard deviation |
| *spp.* | Species |
| STAT 3 | Signal transducer and activator of transcription 3 protein |
| TEAC | Trolox equivalent antioxidant capacity |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumor necrosis factor-alpha |
| TPTZ | Tripyridyltriazine |
| °C | Degree Celcius |
| w/v | weight over volume |
| WHO | World Health Organization |
| WPR | Western Pacific Region |

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## LIST OF APPENDICES

Appendix A: Standard curve of antioxidant assays 164

Appendix B: Standard curve of cell based assays 166

Appendix C: Preparation of reagents, buffers and solutions 167

Appendix D: Statistical analysis 170

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# CHAPTER 1: INTRODUCTION

The history of inflammation dates back to the ancient Egyptian and Greek cultures. Inflammation was first defined as 'to set the fire' in Latin during the 1st century AD by a Roman physician Cornelius Celsus. The description of inflammation given later by Hippocrates in the 5th century BC was continued to be practised until the 19th century AD. Inflammation is a natural physiological process in response to infections, injury, trauma and exposure to foreign matters, irritants or pollutants (Medzhitov, 2008). Classic symptoms of inflammation are redness, pain, heat, swelling and loss of functions (Rocha de Silva, 1978). The primary functions of acute inflammation are to rapidly destroy and isolate any source of the disturbance, remove damaged tissue, and restoration of tissue homeostasis (Akiyama *et al.*, 2000; Mitchell *et al.*, 2003).

There are two stages of inflammation, acute and chronic. Acute inflammation is an initial or early stage of inflammation that is mediated through activation of innate immune cells in response to any disturbance of tissue homeostasis (Phillip *et al.*, 2004). This inflammatory response helps the body to prevent infections and persists only for a short time period. Acute inflammation is also known as therapeutic inflammation as its progress can lead to chronic inflammation. During the progression of chronic inflammation, various inflammatory cells are activated and initiates response by releasing cytokines, chemokines and reactive oxygen and nitrogen species that forms a powerful defense against injuries (Lin *et al.*, 2007). Yet, the downside of this response is that persistent activation of inflammatory cells can result in continued tissue damage underlining variety of diseases. Thus, chronic inflammation has been actively researched now because it has been one of the most potent but unknown causes for various kinds of

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diseases such as hay fever, atherosclerosis, arthritis rheumatism, cancer and Alzheimer's diseases (Stankov, 2012).

According to World Health Organisation (WHO), (2014), inflammation is also included in the noncommunicable diseases (NCD). NCD are diseases that are non-infectious or non-transmissible. NCDs can refer to chronic diseases which last for long periods and progress slowly. The most common NCDs are autoimmune diseases, cardiovascular diseases, chronic respiratory and renal diseases, neurological diseases and cancers (Camps, 2014). Every year, 38 million people (28 million in developing countries) die from NCDs, nearly 16 million of them prematurely (before they reach the age of 70). The number of NCD deaths has increased worldwide and the most in every region since 2000. Globally, NCD deaths are projected to increase by 15% between 2010 and 2020 with an estimated 12.3 million deaths in the Western Pacific Region including Malaysia. It is one of the top five factors contributing to the high fatality rate, including chronic respiratory diseases, heart diseases and diabetes in Malaysia (WHO, 2013). It is recorded in health reports that 67% of the fatality in Malaysia are due to NCDs (WHO, 2011). NCDs are in increase in countries or communities with lower income. This invisible epidemic is not recognised because of the socioeconomic conditions of many countries.

Currently, nonsteroidal anti-inflammatory drugs (NSAIDs), including both traditional nonselective NSAIDs and the selective cyclooxygenase (COX)-2 inhibitors are widely used in the treatment of inflammatory diseases as these drugs are effective in the management of pain and edema caused by inflammatory mediator release (Suleyman *et al.*, 2007). However the disadvantages of NSAIDs drugs are their side effects when ingested for prolonged periods or in high doses. The most common side effects of these

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drugs are gastrointestinal toxicity (Abramson & Weaver, 2005) and renal failure (Lacroix, 2004). According to U.S. Food and Drug Administration (FDA) medication guide for NSAIDs (2014), some of the severe side effects of NSAIDs are heart attack, stroke, heart failure from fluid retention, anaemia, life-threatening skin and allergic reactions besides renal and gastrointestinal problems (FDA, 2014). Other than that, common or mild side effects are stomach pain, constipation, diarrhoea, gas, heartburn, nausea, vomiting and dizziness.

These adverse effects of steroidal and NSAID medications, has brought greater interest in natural products. Natural products have been used for centuries as dietary supplement and herbal remedies to reduce inflammation (Reynolds, 1995). Studies done on some plants, herbs, roots and fruits extracts have shown potential to prevent and cure chronic diseases (Yang *et al.*, 2015). Uses of natural products are effective in traditional remedies for example; turmeric (Krishnaswamy, 2008) in the treatment of rheumatic disorders and ginger in ameliorating arthritic knee pain (Tapsell *et al.*, 2006). Traditional medicine refers to the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2013). Traditional medicine covers a wide variety of therapies and practices which vary from country to country and region to region. In some countries, it is referred to as alternative or complementary medicine. They have been used extensively in Chinese medicine and Ayurveda medicines. According to articles by Dr. Mercola and Susan Bratton (2015), some of the most potent anti-inflammatory natural products include turmeric, ginger, green tea, herbs and spices; and mushrooms.

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According to a survey done by Professor Gerald Muench, of the Molecular Medicine Research Group at the University of Western Sydney, mushrooms are in the top six most potent anti-inflammatory foods accompanied by onions, oregano, red sweet potato, cinnamon and cloves (Gunawardena *et al.*, 2014). In this last decade, the influence of mushroom particularly has been increasing among people. Mushrooms should be a human best friend as they are high in fibre, low in calorie, fat-free and contain vital nutrients such as selenium, potassium, and vitamins (Breene, 1990). Mushrooms have been valued as nutritional food in many countries. They are appreciated not only for the texture and flavour, but also for their chemical and nutritional characteristics (Breene, 1990; Chang, 1991; Manzi *et al.,* 1999).

In Asia, mushrooms have long been used as traditional foods and medicines (Zhang, 2006). They are also known for their therapeutic values (Ying *et al.*, 1987) in preventing cancer (Schmidt *et al.*, 2007) and tumours (Zhang *et al.*, 2006). Some components from mushrooms show other quite significant medicinal properties, such as immunomodulatory, cardiovascular, liver protective (Kim *et al.*, 2012), anti-inflammatory (Hirota *et al.*, 2002; Queiroz *et al.*, 2010), anti-diabetic (Kanagasabapathy *et al.*, 2012). There have also been reports on its effect on nerve regeneration (Wong *et al.*, 2011) and neurite outgrowth (Eik *et al.*, 2012).

The anti-inflammatory properties of mushrooms and its mode of action against inflammation are intensively studied to reduce the neurodegenerative diseases, cardiovascular diseases and cancer that is on the rise. Some of the common active compounds from mushrooms used at present are terpenoids, lipids and sterols, polysaccharides and phenolic compounds (Lindequist *et al.*, 2005). There are many potential pathways used to suppress inflammation by mushrooms. Many mushrooms

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such as *Pleurotus* sp. (Jenidak *et al.*, 2011), *Antrodia camphorata*, *Phellinus linteus* (Huang *et al.*, 2012) and *Ganoderma lucidum* (Yoon *et al.*, 2013) suppressed inflammation via NF-κB, MAPK and STAT3 regulated inflammatory pathway. The bioactive extracts/components of various edible mushrooms remarkably reduced the expression of proinflammatory cytokines such as iNOS, TNF-α, IL-1β and IL-6 in the activated cells (Moro *et al.*, 2012). *Antrodia camphorata* (Tsai *et al.*, 2011) and *Phellinus linteus* (Kim *et al.*, 2007) also suppressed inflammation through NRF pathway by activating and upregulating phase II antioxidant enzymes; HO-1, NQO-1 and GST.

Many mushrooms are well recorded for their anti-inflammatory potentials against chronic diseases. In this study, two medicinal mushrooms, *Lignosus rhinocerotis* (Cooke) Ryvarden and *Cordyceps militaris* (L) Link were investigated for their anti-inflammatory effects.

Based on its ethnic knowledge, one of the most sought-after species used for wound healing purpose is the wild Tiger's Milk mushroom (*Lignosus rhinocerotis* (Cooke) Ryvarden). These mushrooms are widely used for medicinal purposes, especially by local traditional Chinese medicine practitioners and the native people (Orang Asli). According to indigenous folklore, the Tiger's Milk mushroom is believed to grow when a drop of a tigress' milk has fallen to the ground, hence its name. Known as *cendawan susu rimau* or *cendawan susu harimau* in Bahasa Malaysia, they grow on soil, deep in the tropical jungles (Tan *et al.*, 2010). The Orang Asli are the main people who gather these mushrooms, which are not easy to find. The indigenous and Chinese communities use these mushroom to treat cough, asthma, fever, indigestion and food poisoning, and cancer (Chang & Lee, 2004). The powder is also sometimes mixed with

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Chinese rice wine and applied topically to treat lumps, sores and boils (Chang & Lee, 2004).

The *Cordyceps militaris* (L.: Fr.) Link mushroom is commonly known as orange caterpillar fungus. Cordyceps grows in the larva of insects. *Cordyceps militaris* is an Ascomycete; it parasitizes insect larvae, grows, and gradually turns into a mature fruiting body. The parasitic complex of fungus and caterpillar has been used in tonics and medicinal purposes for centuries in eastern Asia (Stone, 2010). According to the review by Das *et al.,* (2010), *Cordyceps militaris* is commonly used to treat renal and pulmonary dysfunction; and heart disease. It also has several bioactivities, such as anti-metastatic (Nakamura *et al.*, 1999), immunomodulatory, antioxidant (Ng & Wang, 2005) and antitumor (Khan *et al.*, 2010).

Therefore, this study aimed to investigate the effect of *L. rhinocerotis* and *C. militaris* extracts on a) antioxidant properties, b) the cytotoxicity and inhibition of nitric oxide production in BV2 cells, c) gene expression in anti-inflammatory pathways and d) identification of chemical components responsible for the anti-neuroinflammatory activities.

1.1 The objectives of the study were to:

a) determine anti-inflammatory activities of crude and fractionated extracts of *L. rhinocerotis* and *C. militaris*
b) identify components in the bioactive fractions that inhibit inflammation in the *in vitro* model.
c) investigate the *in vitro* biological and molecular mechanisms of anti-inflammatory actions of potential active constituents/ fractions.



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# CHAPTER 2: LITERATURE REVIEW

## 2.1 Inflammation

Inflammation is designed to limit invasions and damage after injury, a process which is essential for the survival of human in the absence of medication. Inflammation is a biological reaction on a disrupted tissue homeostasis. It is a pervasive form of defence also known as a nonspecific response to tissue malfunctions that is employed to both innate and immune system to combat pathogens. The perturbations of homeostasis are triggered by infections, injuries and exposure to contaminants or irritants that are foreign to the body to combat harmful pathogens, and damaged cells and tissues (Ashley *et al.*, 2012). Inflammation cascade is also triggered by a complex network of immunological, environmental, physiological and behavioural origins such as burns, toxin ingestion, frostbite, blunt force trauma, high levels of free radicals, stress and alcohol.

The key symptoms of inflammation are redness, heat, swelling, pain, and loss of functions. Redness and heat result from the increased blood flow to the site of injury. Swelling results from the accumulation of fluid at the injury site, a consequence of the increased blood flow. Finally, swelling can compress nerve endings near the injury, causing the characteristic pain associated with inflammation. Pain is also important to make the organism aware of the tissue damage. Additionally, inflammation in a joint usually results in impairment of function, which has the effect of limiting movement and forcing the rest of the injured joint to aid in healing (Stankov, 2012).

Inflammation consists of a regulated cascade of immunological, physiological and behavioural processes that is coordinated by immune signalling molecules

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(cytokines). The first step involves the recognition of the infection or damage (Figure 2.1b). It is normally detected by pathogen- associated molecular patterns (PAMPs) or damage- associated molecular patterns (DAMPs). Advantages of detecting these signals are the reduced inadvertent targeting of host cells. Many damage signals are recognised by receptors such as toll-like receptors (TLRs), intracellular nucleotide binding domain and leucine rich repeat containing domains (NOD and NLRs).

Once the recognition of ligands occurs, TLRs activate the common signalling pathways which in turn activate nuclear factor kappa B cells (NF-κB) as in Figure 2.1c. This transcription factor is found in all cells and is in an inactivated state when bound to inhibitor protein IκB. Upon transduction of the signal, NF-κB is released from IκB and translocated to the nucleus. Thus the transcription is upregulated through binding to target genes. Intracellular NLRs respond to the increased DAMPs that alerts the immune system to cell injury. Transcription and translation of genes lead to the third stage of the inflammatory cascade which is the inducible expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α (Figure 2.1d). In conjunction with chemokines, the proteins facilitate the recruitment of monocytes and neutrophils (Figure 2.1e). Neutrophils create a toxic environment by releasing chemicals from the cytoplasmic granules (degranulation process). These toxic chemicals induce high reactive oxygen and nitrogen species (ROS and RNS) and proteineases. These reactive species are destructive to both the host and pathogens and induce liquefaction of surrounding tissues to starve the microbial metastasis which is the major contributor to host collateral damage.

This net effect of these interactions results to cardinal signs of inflammation such as heat, swelling, redness, pain and loss of function. The effector functions are further regulated by the adaptive immune system (Figure 2.1f). The last phase of inflammation is



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a coordinated program of resolution is set by the tissue resident to recruit macrophages (Figure 2.1g). During acute inflammation, these cells produce proinflammatory mediators but block further neutrophil recruitments to help in wound healing.

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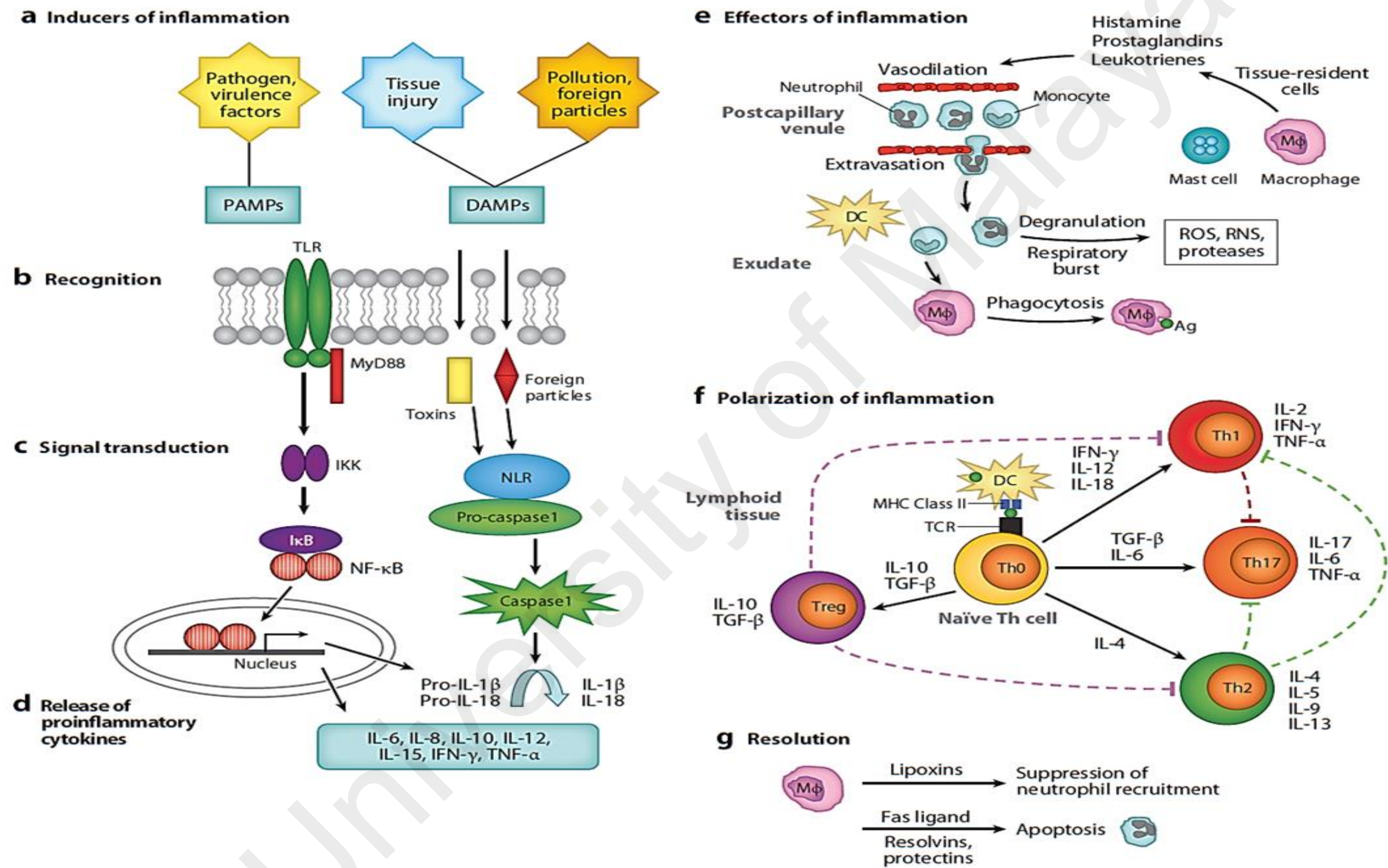


**Figure 2.1:** Mechanism of inflammation.

Source: Ashley *et al.*, 2012

Inflammation is divided into two, acute and chronic inflammation. Inflammation, when regulated properly is putatively adaptive (acute inflammation). The macrophages may prevent the spread of infectious agents and damage to nearby tissues; help to remove damaged tissue and pathogens; and assist the body's repair processes. Acute inflammation is a temporary occurring process encompass the immediate and early response that usually appears within minutes and ceases upon the removal of the foreign bodies. Acute inflammation involves the recruitment of blood derived products such as plasma, fluid and leukocytes. It alters the local dilation of blood vessels as well as increased vessel permeability and vasodilation to improve blood flow to the injured area (Medzhitov, 2008). It is characterized histologically by the presence of neutrophils, basophils and eosinophils that have emigrated from blood vessels into the injured tissue. Neutrophils are short-lived, thus are only involved in the early stages of inflammation. Neutrophils release pro inflammatory cytokines that induce the liver to synthesise various acute phase reactant proteins and also induce systemic inflammatory responses for example, fever and leucocytosis (Mitchell *et al*., 2003).

Conversely, when the immune relevant gene disruption leads to continuous inflammatory response without the presence of any stimuli, the excessive inflammation is not regulated properly, resulting in excessive collateral damage and pathology (chronic inflammation) (Nathan, 2002). Chronic inflammation may progress from acute inflammation as the injuries persist for a long period. It can also be triggered by cellular stress and dysfunction, such as that caused by excessive calorie consumption, elevated blood sugar levels, and oxidative stress (Karin *et al.*, 2006). In the attempt of repairing affected tissues; various chemical mediators were released by macrophages, lymphocytes and plasma cells. These mediators perpetuate the pro-inflammatory response from further

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damage, infiltrated and destruct tissues (Mitchell *et al*., 2003). There are several risk factors such as age, obesity, diet, low sex hormones, smoking, stress and sleep disorders which increase the likelihood of establishing and maintaining a low-level inflammatory response (Trakada *et al*., 2007; Gilliver, 2010; Singh *et al*., 2011; Peairs *et al*., 2011; Lee *et al*., 2011).

The Table 2.1 shows the effect of various inflammations in the body based on the intensity, duration and magnitude of the inflammation. The chronic and high grade inflammatory responses in immune privileged organs lead to extensive damage, whereas acute and low level inflammation in localised tissue has minimally intrusive to the individual. But the low-level chronic inflammation is the most life threatening because its silent nature belies its destructive power.

**Table 2.1:** The effect of inflammation based on the intensity and type of inflammation.

| Intensity | Type | |
|---|---|---|
| | **Acute** | **Chronic** |
| Low | Para inflammation<br>Metaplasia | Inflammatory diseases (diabetes atherosclerosis)<br>Autoimmune disorders<br>Neurodegenerative diseases<br>Tumour growth |
| High | Acute phase response<br>Release of cytokines<br>Neutrophil migration<br>Recruitments of macrophages and microglia<br>Localised tissue damage | Tissue disruption<br>Cancer<br>Sepsis<br>Cytokine storm |

It can persist undetected for years or decades that can accumulate slowly and spread throughout the body. Some of the diseases related to chronic inflammation are cardiovascular diseases (CVD), diabetes, cancer, age related macular degeneration (AMD), chronic kidney diseases (CKD), osteoporosis, depression, cognitive decline,

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dementia, rheumatoid arthritis and pancreatitis (Arizona Centre for Advanced Medicine, 2015).

The brain: a long deliberated immune privileged site because of the presence of BBB and the deficiency of the lymphatic system is also capable of inflammatory response. Invading pathogens, trauma and infection can trigger the activation of glial cells (mainly microglial and astrocytes), production of ROS/ RNS inflammatory mediators, neurotoxic free radicals and other immune factors that may contribute to brain inflammation/ neuroinflammation. (Akiyama *et al*., 2000; McGeer & McGeer, 2004). Neuroinflammation is a two edged sword as it must be tightly regulated because both deficient and excessive response will result in pathological disorder. Nonetheless, the characteristic inflammatory features such as swelling, heat, and pain are not present in the brain. In acute system, inflammatory mechanisms limit injury and promote healing. However, in chronic system, neuroinflammation can seriously damage viable host tissues. The loss of specific neuronal population by intraneuronal and extracellular accumulation of fibrillary materials is indicated to promote neuroinflammation. Emerging evidence indicates that neuroinflammation is closely associated with neurodegenerative disorders in both acute (stroke, injury) and chronic (multiple sclerosis, Alzheimer's, Parkinson) (McGeer & McGeer, 2004; Tansey *et al.*, 2007). Regulation of chronic inflammation of microglial is essential for maintaining the environment in nervous tissues and preventing the onset of neurodegenerative diseases.

In the central nervous system, microglial cells are the resident phagocytes of the innate immune system. Microglia is distributed ubiquitously in the nervous system, serves as a pathological sensor and activates in response to harmful stimuli. They represent the first line of defence against invading pathogens or other types of brain



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tissue injury. The activation of microglia cells leads to a cascade of events that is characterised as an inflammatory process. This cascade is mediated at first by overexpressed proinflammatory cytokine through various pathways. It causes neuronal death and activates more microglia, which release more proinflammatory cytokines to self-sustain and return to equilibrium. Over a long time period, this slow and smouldering inflammation in the brain destroys sufficient neurons and causes neurodegenerative diseases (Griffin, 2006). In summary, microglia cells become activated, increase in the number, migrate, and surround damaged or dead cells, and subsequently clear cellular debris when under pathological situation such as neurodegenerative diseases such as Alzheimers, Parkinson and stroke (Kim & Vellies, 2005). This action is similar to the one performed by phagocytic active macrophages of the peripheral immune system (Fetler *et al*., 2005).

In some situations, the role of microglia has been found to be beneficial, as it can reduce Aβ accumulation by increasing its phagocytosis, clearance, and degradation. Microglia can also secrete a number of soluble factors such as the glia-derived neurotrophic factor (GDNF), which are potentially beneficial to the survival of neurons (Liu *et al*., 2003). Microglia possesses dual functions of neuroprotection and neurotoxicity because it is able to produce cytotoxic proinflammatory factors that induces neuronal cell death and as well as neurotrophic factors, which supports the survival of neurons. In this study, BV-2 cell a type of microglia cell that is derived from mouse is used. BV-2 cells are a useful cell line because they can be maintained in culture, yet they keep many of the functions and feature that microglia expresses *in vivo* (Henn *et al.*, 2009).

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## 2.2 Mushrooms

Mushrooms are macro fungi with fruiting bodies that are large enough to be viewed by human eyes (Chang & Miles, 1992). Mushroom is a fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. They are generally categorised as Basidiomycetes and Ascomycetes because they have a stem (stipe), a cap (pileus), and gills (lamella) or pores on the underside of the cap. Mushrooms consist of 14 000 to 22 000 known species. The estimated number of mushroom species on the earth is about 140 000, suggesting that only 10% are known. Though, not many mushrooms from the 10% of known species are the well investigated. Assuming the useful mushrooms among the undiscovered and unexamined mushrooms were only 5%, which implied there are still 7000 species undiscovered that can benefit to mankind (Hawksworth, 2001).

The use of mushrooms dates back to Paleolithic times. Even anthropologists comprehend the influence of mushrooms in affecting the course of human evolution. Mushrooms have played pivotal roles in ancient Greece, India and Mesoamerica. The oldest archaeological evidence of mushroom use discovered so far is based on the dental record of a prehistoric woman nicknamed The Red Lady of El Mirón. The archaeologists have found spores of several mushroom species embedded in her teeth (Gray, 2015). For thousands of years, Eastern cultures have valued mushrooms' health benefits. It has long been celebrated as a source of powerful nutrients. China is one of the earliest countries to utilise mushroom as tradition and food. Currently there are 950 species of domesticated mushrooms, among which about 50 species have been successfully cultivated (Chang, 1999). China also stands out as the major producer and exporter of medicinal mushrooms.

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Mushroom consumption in Malaysia however, is less compared to the Eastern countries. Recently, imported, dried and canned mushrooms are commercially available in urban areas. Only a limited variety of cultivated mushrooms are available in supermarkets and wet markets (Lee *et al*., 2009). Several species with medicinal properties sold in Chinese medicine shops are mainly imported from China and Taiwan. This probably was introduced by early Chinese settlers. According to summary done by Lee *et al*. (2009), there are a total of 83 species of edible mushrooms found in Malaysia but only 31 species are consumed and 14 species were used for medicinal values by the indigenous people of Malaysia (Semai, Temuan, Che Wong, Jakun and Bateq communities) (Table 2.2).

**Table 2.2:** The edible and medicinal mushroom species in Malaysia

| Edible mushroom species | | Medicinal mushroom species |
| --- | --- | --- |
| *Amanita hemibapha* ssp. *similis*<br>*Amanita princeps*<br>*Amauroderma subresinosum*<br>*Auricularia* sp.<br>*Boletus aureomycelinus*<br>*Calvatia* sp.<br>*Cantharellus* sp.<br>*Clavulina* sp.<br>*Clitopilus* cf. *orientalis*<br>*Cookeina sulcipes*<br>*Coriolus hirsutus*<br>*Craterellus cornucopioides* var. *mediosporus*<br>*Cymatoderma* sp.<br>*Dacryopinax spathularia*<br>*Hygrocybe conica* | *Lactarius gerardii*<br>*Laetiporus sulphureus*<br>*Lentinus* cf. *sajor caju*<br>*Lentinus squarrosulus*<br>*Lenzites acuta*<br>*Lenzites vespacea*<br>*Macrocybe/Lyophyllum* sp.<br>*Panus giganteus*<br>*Russula* sp.<br>*Scleroderma* sp.<br>*Schizophyllum commune*<br>*Termitomyces microcarpus*<br>*Termitomyces* sp.<br>*Thelephora* cf. *fuscella*<br>*Tremella fusciformis*<br>*Volvariella* sp. | *Amauroderma* sp.<br>*Amauroderma subresinosum*<br>*Cookeina sulcipes*<br>*Coriolus hirsutus*<br>*Coriolus versicolor*<br>*Daldinia concentrica*<br>*Lentinus* cf. *sajor caju*<br>*Lenzites acuta*<br>*Lignosus* sp.<br>*Microporus xanthopus*<br>*Phellinus* sp.<br>*Pycnoporus sanguineus*<br>*Thelephora* cf. *fuscella*<br>*Xylaria polymorpha* |

Source: Lee *et al.,* 2009

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## 2.3 Medicinal mushrooms

The use of mushrooms as medicine can be traced back to thousands of years in cultures around the world. In China, usage of *Ganoderma lucidum* was first documented around 500 BC. In the Italian Alps region, the frozen body of Otzi (who died approximately 3,300 BC) was found in 1991 carrying *Piptoporus betulinus* on his tool belt, potential as a treatment for internal parasites. Wild mushrooms are becoming more important in our diet for their nutritional (Manzi *et al.*, 1999; Barros *et al.*, 2007), organoleptic (Maga, 1981) and medicinal (Lee *et al.*, 2009) characteristics. They are appreciated not only for the texture and flavour but also for their chemical and nutritional characteristics (Manzi *et al.,* 1999). Mushrooms are considered to be a good source of proteins (10-40%), carbohydrates (3-21%) and dietary fibre (3-35%). Mushrooms contain all the essential amino acids (Breene, 1990; Chang 1991), the main classes of lipid and are importantly low in calories. They are excellent sources of vitamins such as thiamine (vitamin $B_1$), riboflavin (vitamin $B_2$), nicotinic acid (vitamin $B_1$), biotin and ascorbic acid (vitamin C). Edible mushrooms are high in nutrients, good dietary for vegetarians, diabetic and heart patients (Breene, 1990).

In Asia, mushrooms have long been used as traditional food and medicines (Zhang, 2006). Some components from mushrooms show significant medicinal properties, such as immunomodulatory, cardiovascular, liver protective (Kim *et al*., 2012), anti-fibrotic, anti-inflammatory (Hirota *et al.*, 2002; Queiroz *et al.*, 2010), anti-diabetic (Kanagasabapathy *et al.*, 2012), anti-viral, antimicrobial activities (Ooi, 2000; Wasser, 2002); anti-cancer and anti-tumour (Bobek & Galbavy, 1999). Some of the mushrooms used for medical purpose by indigenous people of Malaysia and other parts of the world are shown in the Table 2.3 and Table 2.4 respectively.

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**Table 2.3:** Mushrooms used for medical purpose by Temuan people in Ulu Kelang Village

| Species | Local names | Ailments treated |
|---|---|---|
| ***Amauroderma* sp.** | Cendawan sawan | Epilepsy, baby cries |
| ***Auricularia auricularia-judae* (Bull.)** | Cendawan memeh | Febrifuge |
| ***Coprinus* sp.** | Cendawan kaki satu | Pruritis and *Tinea versicolor* |
| ***Lignosus rhinocerotis* (Cooke) Ryvarden** | Cendawan susu rimau | Postpartum |
| ***Microporus xanthopus* (Fr.) Kuntze** | Cendawan pengering | Contraceptive |
| ***Pyconoporus sanguineus* (L.) Murill** | Cendawan Be'reng | Wound and sore |
| ***Termitomyces clypeatus* R. Heim** | Cendawan susu pelanduk | Lassitude and ferrifuge |

Source: Azliza *et al.*, 2012

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**Table 2.4:** Mushrooms and their medicinal properties

| | **Anti-bacterial** | **Anti-inflammatory** | **Antioxidant** | **Anti-tumour** | **Anti-viral** | **Anti-diabetes** | **Cardiovascular** | **Immune system** | **Kidney tonic** | **Liver tonic** | **Lungs/ respiratory** | **Nerve tonic** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Agaricus bisporus*** | | | | X | X | X | | X | | | | |
| ***Auricularia judae*** | | | X | | | | | | | | | |
| ***Boletus edulis*** | | | | X | | | | | | | | |
| ***Cordyceps sinensis*** | X | X | X | X | X | X | X | X | X | X | X | X |
| ***Ganoderma lucidum*** | X | X | X | X | X | X | X | X | X | X | X | X |
| ***Grifola fondosa*** | X | X | | X | X | X | | X | | | X | X |
| ***Hericium erinaceus*** | X | X | | X | | | | | | | | X |
| ***Inonotus obliquus*** | X | X | | X | X | X | | X | | X | | |
| ***Lentinula edodes*** | X | | | X | X | X | | X | X | X | | |
| ***Phelinus linteus*** | X | X | | | X | | | | | | | |
| ***Pleurotus ostreatus*** | X | X | | X | | | X | X | | | | |
| ***Polyporus umbellatus*** | X | X | | X | X | | | X | | X | X | |
| ***Schizophyllum commune*** | | | | X | X | | | | | | | |
| ***Trametes versicolor*** | X | | X | X | X | | | X | X | X | | |

Source: Stamets, 2000

## 2.4 *Lignosus rhinocerotis*

*Lignosus rhinocerotis* (Cooke) Ryvarden, the tiger milk mushroom, belongs to the Polyporaceae family. It is a unique “National Treasure” that can only be found in a small geographic region encompassing South China, Thailand, Malaysia, Indonesia, Philippines, Papua New Guinea, New Zealand, and Australia (Tan, 2009). *Lignosus rhinocerotis* (Cooke) Ryvarden is taxonomically synonymous with *Polyporus rhinocerus* (Cooke), *Fomes rhinocerotis* (Cooke), or *Scindalma rhinocerus* (Cooke) as cited in MycoBank (http://www.mycobank.org). This mushroom is also known locally in Malaysia as “cendawan susu rimau” which means tiger milk mushroom because it is believed that the mushroom emerges at the spots where the milk of tigress has dropped on the ground of humus rich soil deep in the tropical jungles. The Figure 2.2 below shows the physical appearance of *L. rhinocerotis* and its scientific nomenclature.

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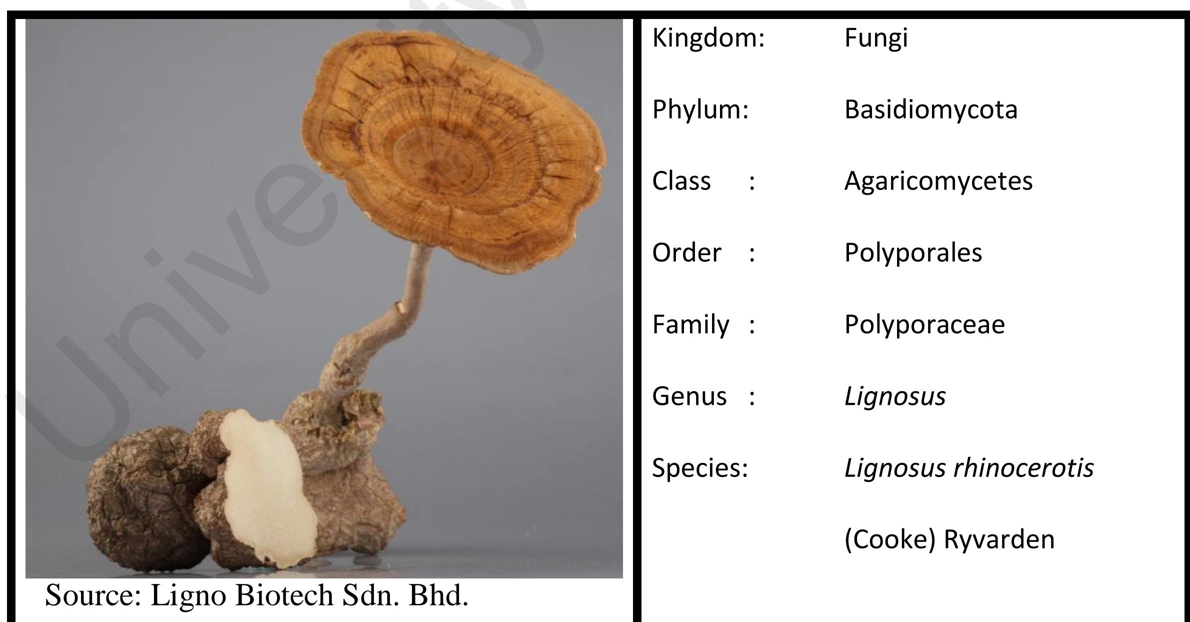


Source: Ligno Biotech Sdn. Bhd.

**Figure 2.2:** The physical appearance of *L. rhinocerotis* and its scientific nomenclature

This mushroom consists of three distinct parts: cap (pileus), stem (stipe) and tuber (sclerotium). Its morphology is unusual for polypores because the fruiting body (cap and stem) grows from the tuber in the ground rather than from the usual wood substrate. Sclerotia are a compact mass of hardened fungal mycelium containing food reserves for survival in extreme environments. The irregular shaped sclerotium which remains underground for months is always solitary and unnoticeable, makes the collection of the mushroom difficult and limiting studies done on this national treasure. The sclerotium of the mushroom possesses medicinal properties. The sclerotium is white in colour and appears like milk when grind with water. It also tastes like milk (Tan, 2009).

Recently, Tan (2009), reported successful cultivation of the mushroom, which would overcome the supply problem and make possibilities for more investigation to be done on *Lignosus rhinocerotis*. Other researchers also domesticated this mushroom using mycelium and submerged culture techniques. The optimisation of substrate formulation was done for cultivation of *L. rhinocerotis* mycelium. Based on the optimised formulation, the pilot cultivation was conducted and sclerotia and sporophores were successfully produced (Abdullah *et al.*, 2013). The growth profile of submerged cultures was studied by assessing the biomass weight, glucose concentration and pH level of medium. The effects of various carbon and nitrogen sources were observed in the submerged cultures (Rahman *et al.*, 2012). Nutritional values, mineral content and toxic metal content have been profiled for the sclerotium of this mushroom using samples from various parts of Malaysia as shown below in Table 2.5 (a-c). Amino acid contents of wild and cultivated *L. rhinocerotis* sclerotia has been analysed and documented by Yap *et al.* (2013).

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**Table 2.5a:** Nutritional profile (g/100g dry weight) and energy value (kcal/100g dry weight) of sclerotium of *L. rhinocerotis*

| Parameters | Source | | | |
|---|---|---|---|---|
| | Kenaboi Forest Reserve Negeri Sembilan[a] | Kuala Lipis Forest, Pahang[b] | Cameron Highland, Pahang[c] | TM02 from Ligno Biotech Sdn Bhd.[c] |
| Energy | 362.83±0.76 | | 218±1.2 | 321.90±0.71 |
| Carbohydrate | 82.6±0.01 | 51.30±0.1 | 88.4±0.35 | 77.6±0.04 |
| Protein | 7.02±0.2 | 3.04±0.59 | 3.8±0.03 | 13.8±0.02 |
| Fat | 0.49±0.00 | 0.46±0.32 | 0.3±0.01 | 0.8±0.00 |
| Moisture | 8.12±0.1 | 39.82±2.22 | - | - |
| Ash | 1.79±0.09 | 4.38±2.71 | - | - |
| Fiber | 22.6±0.35 | - | - | - |
| Sugar | - | - | 0.7±0.04 | 3.0±0.1 |

**Table 2.5b:** Mineral content in sclerotium of *L. rhinocerotis* (mg/100g dry weight).

| Minerals | Source | | |
|---|---|---|---|
| | Kenaboi Forest Reserve Negeri Sembilan[a] | Cameron Highland, Pahang[c] | TM02 from Ligno Biotech Sdn Bhd.[c] |
| Calcium (Ca) | 76.73±2.38 | 3.7±0.17 | 19.3±0.6 |
| Potassium (K) | 225.00±6.92 | 132.20±0.56 | 203.2±2.53 |
| Sodium (S) | 3.96±0.47 | 8.50±0.07 | 8.80±0.09 |
| Magnesium (Mg) | 64.77±6.13 | 75.8±0.37 | 147.9±0.31 |
| Iron (Fe) | 12.93±0.21 | - | - |
| Zinc (Zn) | 1.19±0.04 | - | - |

**Table 2.5c:** Concentrations of toxic metals content in sclerotium of *L. rhinocerotis* (mg/kg).

| Elements | Source | |
|---|---|---|
| | Kuala Lipis forest, Pahang[b] | TM02 from Ligno Biotech Sdn Bhd[c] |
| Mercury (Hg) | 0.05±0.03 mg/kg | ≤ 0.05 ppm |
| Cadmium (Cd) | 0.06±0.03 mg/kg | ≤ 0.3 ppm |
| Lead (Pb) | 1.03±0.01 mg/kg | ≤ 10 ppm |

Source: [a] Lau *et al.*, 2013, [b] Lai *et al.*, 2013, [c] Yap *et al.*, 2013

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These mushrooms are widely used for medicinal purposes for more than 400 years, especially by local traditional Chinese medicine practitioners and the native people (Orang Asli) of Malaysia. In the 1700s, Tuan Haji Mat Yusop, a Malay man in Pahang had reported the mushroom to be potent for medicinal purposes. The Chinese communities use *Lignosus rhinocerotis* mushrooms to treat cough, asthma, fever, gastritis, indigestion and food poisoning (Chang & Lee, 2004). It also has been utilized for more than 15 medicinal purposes by the indigenous people, namely cancer, fever, cough, asthma; starve off hunger, food poisoning, general tonic, wound healing and others (Tan *et al.*, 2010). In China, the sclerotium of *Polyporus rhinocerus* was used to treat liver cancer, chronic hepatitis and gastric ulcer (Wong *et al.*, 2008). Table 2.6 below shows some of the medicinal uses of *L. rhinocerotis* from recent researches. Up to date, there is no detailed report on the anti-inflammatory activities of *L. rhinocerotis* in BV2 microglia cells and its responsible chemical constituents and pathways.

**Table 2.6:** Medicinal properties of *L. rhinocerotis.*

| Medicinal properties | Active extracts | Cell line | References |
|---|---|---|---|
| **Anti-cancer** | cold aqueous | - MCF 7 and A549 | Lee *et al.*, 2012 |
| | aqueous | -HL-60, K562 and THP-1 | Lai *et al.*, 2008 |
| **Anti-inflammatory** | cold aqueous, hot aqueous and methanol | RAW 264.7 | Lee *et al.*, 2014 |
| | hot aqueous, ethanol, hexane, ethyl acetate | BV2 | Nallathamby *et al.*, 2016 |
| **Antioxidant** | cold aqueous, hot aqueous and methanol | - | Yap *et al.*, 2013 |
| **Immunomodulating** | Polysaccharides | Immune cells | Wong *et al.*, 2011. |
| **Neurite outgrowth** | hot aqueous | PC 12 | Eik *et al.*, 2012 |
| | hot aqueous | N2a and BALB/3T3 | Phan *et al.*, 2013 |
| | hot aqueous, ethanol and crude polysaccharide | PC 12 | Seow et al., 2015 |

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Sub-acute and chronic preclinical toxicology analysis was evaluated on the effect of *L. rhinocerotis* towards Sprague Dawley rats. The sub-acute toxicology evaluation was conducted for 28 days. The body weight was measured and behavioural change was observed daily. Oral administration of the slerotia powder (TM02) had no adverse effect on growth rate, haematological and clinical biochemical parameters (including renal and liver function). Histological studies showed treatment dose up to 1000 mg/kg did not induce any pathological changes to the liver, kidney, heart, spleen and lung of the rats (Lee *et al.*, 2011). Subsequent evaluation of chronic toxicology, its anti-fertility and teratogenic effects as well as genotoxicity was done for 180 days consecutively by the same group (Lee *et al.*, 2013). The administration of sclerotia powder had no adverse effect on the general clinical observations, body weight as well as related organ weight, nor induced histological changes to organs (lungs, liver, kidney, spleen, brain, testes/ovary, and epididymis/uterus). It also did not affect the fertility of the rats nor induce teratogenic effects on their offspring. In the reverse mutation Ames test, the treatment of 5000 ug/mL with or without metabolic activation did not cause gene mutation in the genome (Lee *et al.*, 2013). The evaluation done by Chen *et al.* (2013), however, suggested that *L. rhinocerotis* was devoid of mutagenicity up to 100 mg/mL dose. There was also no significant ($p<0.05$) genotoxicity was noted.

The total genome of *L. rhinocerotis* is 34.3Mb encoding 10,742 putative genes. Among them, 79.25% were involved in metabolism of amino acid and carbohydrate, followed by 8.79% of genetic information processing and 5.61% for cellular processes. Interestingly, there are 535 enzymes that are exclusive to *L. rhinocerotis* and not present in other Basidiomycota (Yap *et al.*, 2014).

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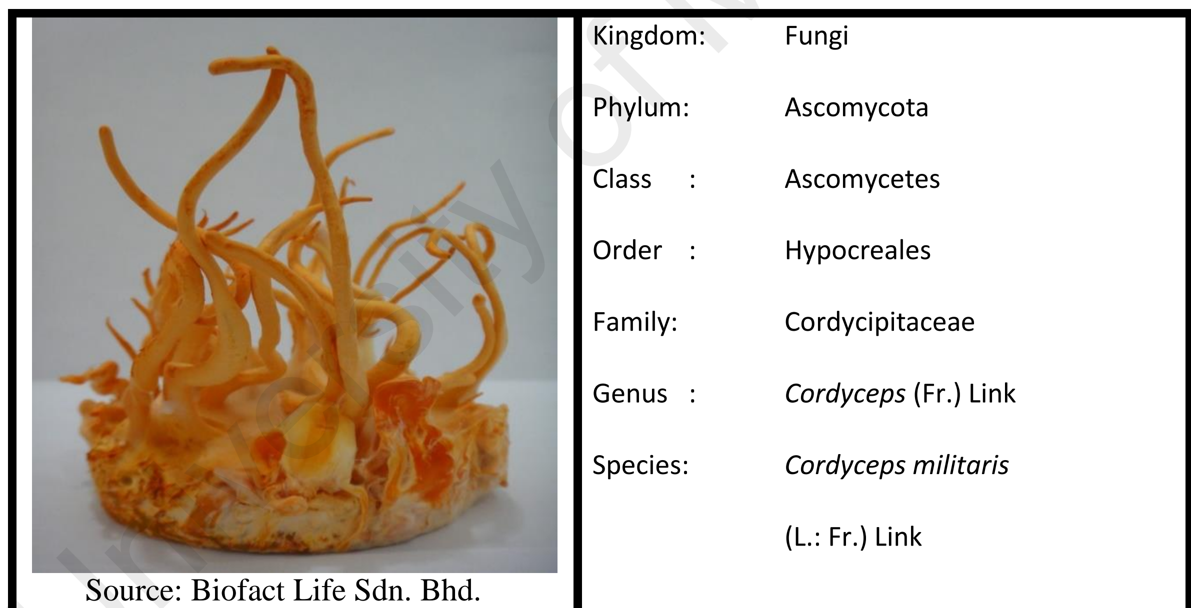
## 2.5 *Cordyceps militaris*

The existence of *Cordyceps* genus has been known for its medicinal values since 2000 B.C. There are more than 400 *Cordyceps* sp. and 90 species are from China (Liang, 2001). A common *Cordyceps* sp., *Cordyceps militaris* belongs to the class Ascomycetes. It is widely used for a long time in traditional Chinese medicine in Asian countries for remedies and as folk tonic (Ying *et al.*, 1987). The name Cordyceps was generated from the Latin words "cord" and "ceps" that means "club" and "head". This describes the appearance of the club fungus (Holliday *et al.*, 2005). The physical appearance of cultivated and scientific nomenclature is shown in Figure 2.3.



| | |
|---|---|
| Kingdom: | Fungi |
| Phylum: | Ascomycota |
| Class : | Ascomycetes |
| Order : | Hypocreales |
| Family: | Cordycipitaceae |
| Genus : | *Cordyceps* (Fr.) Link |
| Species: | *Cordyceps militaris* (L.: Fr.) Link |

Source: Biofact Life Sdn. Bhd.

**Figure 2.3:** The physical appearance of *C. militaris* and its scientific nomenclature

The fungus parasitizes insects and colonizes the dead or living Hepialus (Lepidoptera) caterpillars. Its spores germinate inside the caterpillars, hyphae fill the caterpillar body, and produces a stalked fruiting body (stroma) (Li *et al.*, 1998). A stroma is a compact, somatic structure of ascomycetous fungi in which the fruiting body is formed. The stroma carries asexual spores at the tips of the hyphae. The *C. militaris*

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stroma is cleft, club or cylindrical shaped with a lower sterile stipe and upper fertile clava (apical part or head), and yellow or orange in colour (Kirk *et al.*, 2001).

Cordyceps is known as "Dong Chong Xia- Cao" in Chinese language because the larvae are infected, consumed by mycelia and turn into stiff worm in the winter. The following summer, the stroma emerges from the ground, growing from the larvae which explain its name "winter worm summer grass". Some of popular/ common names of *Cordyceps* are shown in Table 2.7. Unlike *C. sinensis*, *Cordyceps militaris* is distributed worldwide from 0 - >2000 m a.s.l. (Kobayasi, 1941; Ma *et al.*, 2007). This is contributed by its morphological diversity and adaptation to a wide range of host insects from Coleoptera, Diptera, Hymenoptera and Lepidoptera orders. Many *Cordyceps* species are morphologically similar to *C. militaris* such as *C. kyusyuensis*, *C. cardinalis*, *C. pseudomilitaris*, *C. roseostromata* and others (Wang *et al.*, 2008). According to Wang *et al.*, 2008, *C. kyusyuensis* is not a different species but is a synonym of *C. militaris.*

**Table 2.7:** Synonyms names of *Cordyceps spp.* mushroom.

| **Common name** | **Caterpillar fungus, *Cordyceps*, cetepiler mushroom** |
|---|---|
| **Latin/ English name** | *Cordyceps militaris*, *Cordyceps* mushroom, deer fungus, caterpillar fungus |
| **Chinese name** | Dong Chong Xia- Cao, winter worm summer grass, Hia tsao tong tchong |
| **Korean name** | Tong ch'ug ha ch'o |
| **Nepali name** | Yarsagumba, Jeebanbuti, Sanjivani, Kiranghans |
| **Tibetian name** | Yarchakunbu |
| **Other name** | Chong cao, Dong chong cao, Aweto. |

Source: Das *et al.*, 2010

The full genome sequence in *C. militaris* was encoded. The total genome is 32.2Mb. A total of 9684 protein coding genes have been predicted (13.7% species specific genes and 16% related to pathogen-host interactions). More than 63% of genes

are expressed during mycelial growth and fruiting body formation (Zheng *et al.*, 2011). This will help in elucidating the genetic background to synthesize bioactive components. The mushroom is safe to consume as there were no orthologs of known human mycotoxins.

More than 36 species out of 400 *Cordyceps* species have been artificially cultivated but only *C. militaris* has been commercially cultivated and developed in the short production period because of its excellent pharmaceutical activities. The artificial growth and stroma production of *C. militaris* has been studied in the laboratory on various insects since 1980s. Since insects are expensive and prone to contamination, alternative organic substrates such as rice have been used (Lin *et al.*, 2006). It has been cultivated in liquid and solid media, and submerged cultures. Compared to *C. sinensis*, the price of *C. militaris* is affordable in large scale production. The main uses of these cultures are that the fruiting bodies can be consumed directly and it can be incorporated in health products and drugs (Wang & Yang, 2006).

It is commonly used to treat fatigue, renal and pulmonary dysfunction, hyperglycemia, hyperlipidemia, and heart disease like arrhythmia. Cordyceps has also multiple pharmacological activities, such as anti-tumour, antimetastatic, immunomodulatory, and anti-oxidant effects (Ng & Wang, 2005). It is currently being used in many clinical cases for coughing, sputum, dizziness, memory failure, night sweat, toothache, insomnia, pain in joints, nervous prostration, sexual impotence, anaemia and slow recovery from illness (Das *et al.*, 2010). Table 2.8 below shows the medicinal properties of *C. militaris* reported in literature. The major bioactive compounds, cordycepin, adenosine, cordycepic acid, D-mannitol and ergosterol have many pharmacological properties.

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**Table 2.8:** Medicinal properties of *Cordyceps militaris.*

| Biological activity | References |
|---|---|
| **Anti-cancer** | Penman *et al.*, 1970 |
| **Anti-diabetic** | Choi *et al.*, 2004 |
| **Anti-fibrotic** | Nan *et al.*, 2001 |
| **Anti-HIV** | Mueller *et al.*, 1991 |
| **Anti-inflammatory** | Yu *et al.*, 2004, Won & Park, 2005 |
| **Anti-leukemic** | Kodama *et al.*, 2000 |
| **Anti-metastatic** | Liu *et al.*, 1997, Shih *et al.*, 2007 |
| **Antioxidant/ anti-aging** | Yu *et al.*, 2007, Chen *et al.*, 2003 |
| **Anti-proliferative** | Liu *et al.*, 1997 |
| **Anti-tumour** | Liu *et al.*, 1997, |
| **Hypoglycaemic** | Choi *et al.*, 2004, Yu *et al.*, 2007 |
| **Immunomodulatory** | Mao & Zhong, 2006 |
| **Liver-protective** | Jung *et al.*, 2004, Yu *et al.*, 2007 |
| **Neuroprotective** | Ribeiro, 1995, |
| **Pneumo-protective** | Yu *et al.*, 2007 |
| **Reno-protective** | Zhao-Long *et al.*, 2000, |

## 2.6 Anti-inflammatory activity of mushrooms

Persistent inflammation is at the root of all known chronic health conditions such as cardiology diseases, arthritis and Alzheimer disease. It is in existence since ancient times. Researchers have been studying on ways to reduce and suppress the effects of inflammation for decades. Lately, the interest has focused towards the utilisation of natural products in anti-inflammatory work. Many extracts from a range of plant and fungus especially mushrooms, have been reported to possess anti-inflammatory activity when tested with macrophage cells.

Some of the earlier work done on anti-inflammation was with inhibition of croton oil-induced ear edema in mice using butanol (BuOH) fraction of *Phellinus linteus* mushroom (Kim *et al.*, 2004). This fraction was found to be most effective compared to other fractions in inhibition of both ear plug weight and thickness of the mice. There was

also another study done in India by Nitha *et al.* (2007); where the ethanolic extract of cultured mycelia of *Morchella esculenta* significantly inhibited acute inflammation induced by carrageenan and dextran; and chronic inflammation induced by formalin at concentrations of 250 and 500 mg/kg body weight. β-glucan-rich extract from *Geastrum saccatum* mushroom also showed good anti-inflammatory activity when tested against carrageenan paw edema at 50 mg/kg. The extract was further tested with cell line and managed to decrease NO levels by 41.5% compared to the control group (Guerra Dore *et al.*, 2007).

In the subsequent years anti-inflammatory assays progressed to cell line models by determining the reduction of NO levels, continued by gene expression studies on proinflammatory mediator genes. An aqueous mycelium stock from *Antrodia camphorata* resulted in a significant, dose and time-dependent reduction of NO production. It also significantly decreased TNF-α and IL-1β levels in a concentration-dependent manner (Hseu *et al.*, 2005). Cordycepin the major compound from butanol fraction of *Cordyceps militaris* inhibited NO production and decreased the iNOS expression of LPS-stimulated RAW 264.7 cells in a dose dependent manner (Kim *et al.*, 2006), whereas the aqueous extract possessed a dose dependent reduction of NO production in murine macrophages (Wol *et al.*, 2010). Triterpenes isolated from the mushroom *Ganoderma lucidum* also inhibited TNF-α production and suppressed IL-6 production by LPS-stimulated RAW264.7 cells. However, it did not affect the morphology nor demonstrated any sign of toxicity (Dudhgaonkar *et al.*, 2009).

*Sarcodon aspratuss* extract was further purified to isolate the pure compounds ergosterol, ergosterol peroxide, and 9, 11-dehydroergosterol peroxide. These compounds were potent to inhibit LPS-induced TNF-α, NF-kB, IL-1β and IL-1α production in the



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cells (Kobori *et al.*, 2007). Similar compounds were found in extracts of *Amauroderma rugosum,* with potential anti inflammation properties whereby the ethanol and its hexane fraction (HF) were used to determine the production of NO levels. The HF significantly inhibited NO production at all concentrations tested and inhibited it completely at 100$\mu$g/ml. The compounds found in HF that are most probably responsible for reduction are ethyl linoleate and ergosterol (Chan *et al.*, 2013).

Other than these types of compounds, some polysaccharides and glucans are also effective in inhibiting inflammations. The polysaccharide of Golden needle mushroom was used in a study on burned rats resulting in significantly lower IL-10 concentrations compared to the control (Wu *et al.*, 2010). Glucans from *Caripia montagnei* also showed a high reduction of the levels of important mediators, such as the cytokine, IL-6 and NO (Queiroz *et al.*, 2010). Some of the uncommon potential anti-inflammatory compounds are exopolysaccharide, phenolic compounds, hexadecanoic acid, 5-hydroxy-2-pentanone, lactic acid, xylarinic acid (Kim *et al.*, 2010) and many more. The compounds found mainly in mushrooms as mentioned earlier are beneficial to inhibit inflammation. Thus, mushrooms as a whole is a good functional food for anti-inflammatory effects.

## 2.7 Lipids and essential fatty acid components in mushroom

Lipids are large and diverse group of naturally occurring organic compounds that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), fatty acids, monoglycerides, diglycerides, triglycerides, phospholipids, and others. Lipids are mainly hydrophobic or amphiphilic small molecules. The main functions of lipids are storing energy and acting as structural components of cell membranes.

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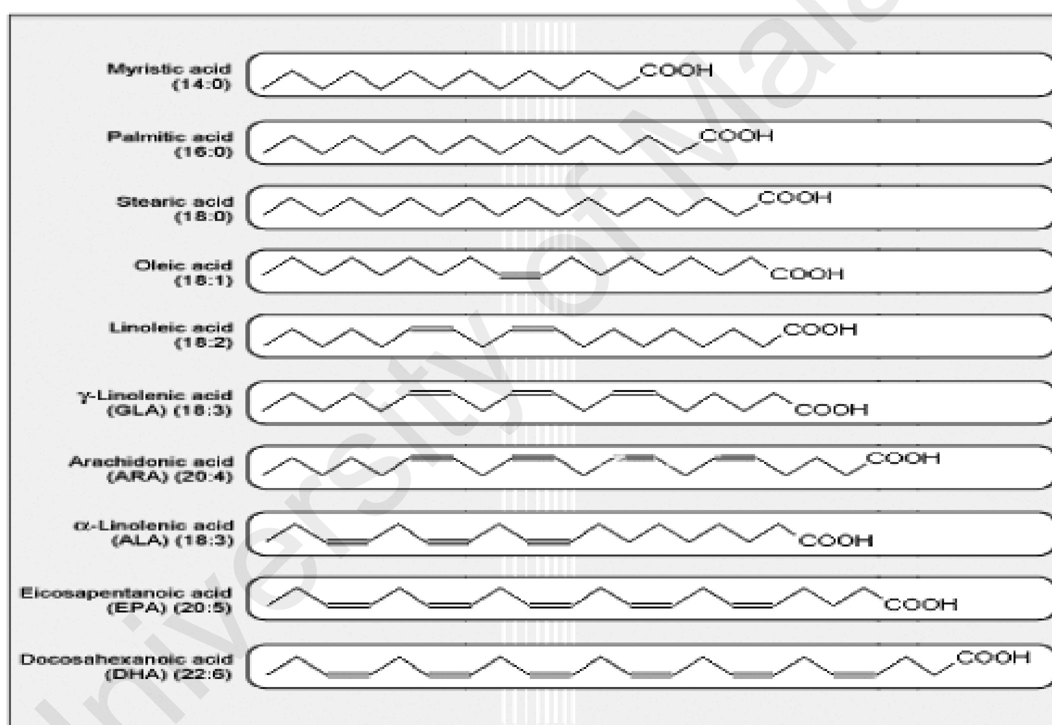
Fatty acids can be considered the defining components of lipids. Most naturally occurring fatty acids possess either saturated or unsaturated chain and have an even number of carbon atoms, from 4 to 28 (Christie, 2012). The names and structures of these fatty acids are shown in Figure 2.4. Fatty acids are usually derived from triglycerides or phospholipids. Most edible mushrooms, including the commercial *Agaricus bisporus* and *Lentinus edodes* show a high proportion of unsaturated fatty acids compared to saturated fatty acids (Miles & Chang, 2004). Generally, mushrooms contain various fatty acids but the common fatty acids are C16 and C18 compounds such as palmitic, oleic, linoleic and linolenic acid (Weete, 2012).




Source: Bauer, 2008.

**Figure 2.4**: Names and structures of fatty acids.

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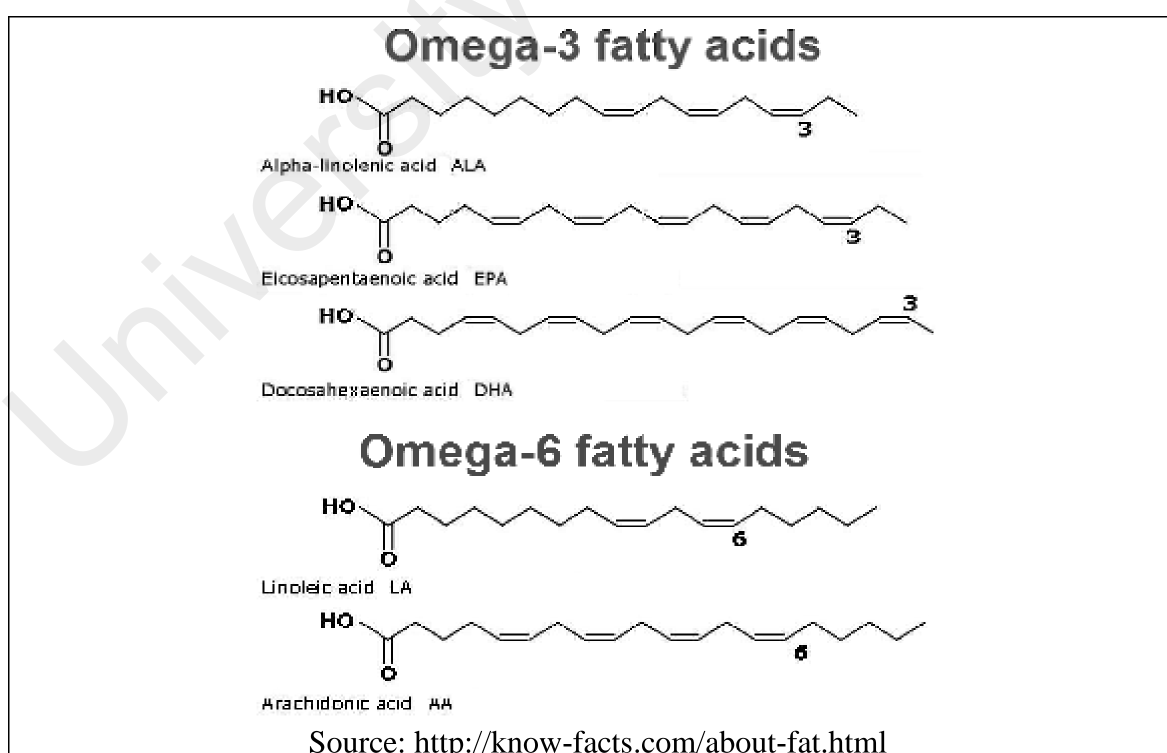
Although humans and other mammals use various biosynthetic pathways to both break down and synthesize lipids, some essential lipids cannot be made this way and must be obtained from the diet. The two polyunsaturated fatty acids, linoleic and linolenic, are designated "essential" because their absence in the human diet has been

associated with health problems, such as scaly skin, stunted growth and increased dehydration. Some other fatty acids are sometimes classified as "conditionally essential," meaning that they can become essential under some developmental or disease conditions; examples include docosahexaenoic acid (an omega-3 fatty acid) and gamma-linolenic acid (an omega-6 fatty acid).When the two essential fatty acids were discovered in 1923, they were designated "vitamin F", but in 1929, research on rats showed that the two essential fatty acids are better classified as fats rather than vitamins (Burr *et al.*, 1930). The essential fatty acids, linoleic and linolenic acids and their longer-chain polyunsaturated metabolites, such as arachidonic acid, are the biosynthetic precursors of the prostaglandins and other eicosanoids, including the leukotrienes, thromboxanes, lipoxins and resolvins. Some of them are occasionally found esterified to phospholipids (and glycosyldiacylglycerols), although their short half-lives may preclude long-term storage in this form. These essential fatty acids are shown in the Figure 2.5.



Source: http://know-facts.com/about-fat.html

**Figure 2.5**: Names and structures of essential fatty acids.

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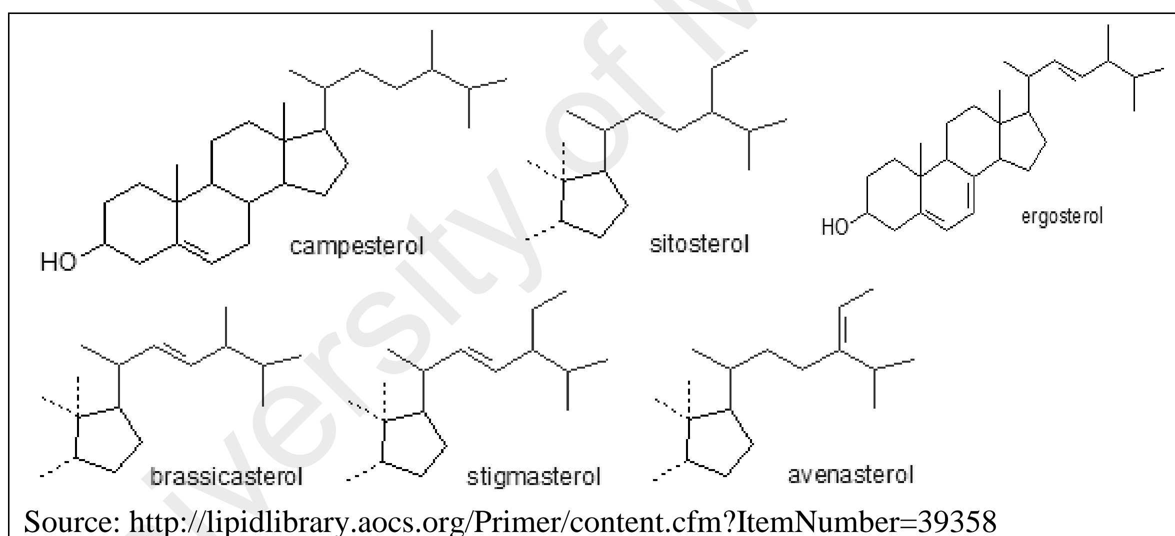
Another important class of lipids called sterols, also known as steroid alcohols, are a subgroup of the steroids. It is an important class of organic molecules steroids that are actually metabolic derivatives of terpenes, but they are customarily treated as a separate group. In plants, phytosterols such as sitosterol, stigmasterol, avenasterol, campesterol and brassicasterol, and their fatty acid esters are usually found, and they perform a similar function as cholesterol in animals. Most phytosterols are compounds with 28 to 30 carbon atoms and one or two carbon-carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain (The Cyberlipid Centre). Ergosterol is a sterol present in the cell membrane of fungi that functions as a biological precursor of vitamin D2. Some of these sterols are shown in Figure 2.6 below.




Source: http://lipidlibrary.aocs.org/Primer/content.cfm?ItemNumber=39358

**Figure 2.6**: Names and structures of sterols.

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## 2.8 Bioassay investigations

### 2.8.1 Antioxidant activities

Antioxidants are defined as substances that are capable of counteracting the damaging effects of the physiological process of oxidation in animal tissue. There are thousands of different substances (such as vitamins, mineral or enzymes) that can act as antioxidants. The most common ones are vitamin C, vitamin E, beta-carotene, and other related carotenoids, along with the minerals selenium and manganese. They're joined by glutathione; coenzyme Q10, lipoic acid, flavonoids, phenols, polyphenols, phytoestrogens, and many more (Sies, 1997).

Antioxidants are widely used in dietary supplements and have been investigated for preventing the development of chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, and cataracts (Valko *et al.*, 2007). An antioxidant is actually behavioural of a compound that can donate electrons and counteract free radicals. Free radicals are produced by oxidation reactions. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. These radicals can start chain reactions in the cell and cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by oxidising themselves, so antioxidants are often reducing agents.

Oxidative stress reflects an imbalance between the systemic indicator of reactive oxygen species and the ability of a biological system to detoxify the reactive intermediates or repair the damages. This condition can cause toxic effects through the

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production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress is damaging to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. The base damage is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. superoxide radical ($O_2-$), hydroxyl radical (OH) and hydrogen peroxide ($H_2O_2$) (Chandra *et al.*, 2015). Reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens (Segal, 2005). Short-term oxidative stress may also be important in the prevention of aging. The use of antioxidants as treatments for stroke and neurodegenerative diseases is intensively studied in pharmaceutical and pharmacology sectors. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases. But generally oxidative stress can cause disruptions in normal mechanisms of cellular signalling by being involved in the development of Parkinson's disease, (Hwang, 2013) Alzheimer's disease (Pohanka, 2014), atherosclerosis (Bonomini, 2008) and others.

There are various *in vitro* methods developed to screen and determine the antioxidant activities of substances. In this study, three antioxidant activities that were commonly used, namely DPPH free radical scavenging system, Trolox equivalent antioxidant capacity (TEAC) and ferric reduction antioxidant power (FRAP). Each of these assays tests different radicals using different mechanisms and time points.

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, stable at room temperature is reduced in the presence of an antioxidant molecule. The antioxidant molecule neutralises the violet colour to pale yellow or

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colourless. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry (Huang *et al.*, 2005), thus it is useful to assess numerous sample simultaneously.

The Trolox Equivalent Antioxidant Capacity (TEAC) assay is based on the scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS•) converting it into a colourless product. In the assay, the decolourisation of ABTS• by a compound-that reflects the amount of ABTS• that has been scavenged- after a fixed period of time is related to that induced by trolox, giving the TEAC value (Re *et al.*, 1999).

The FRAP assay is a simple test measuring the ferric reducing ability of plasma. It is presented as a method for assessing antioxidant power. The reduction of ferric to ferrous ion at low pH causes a colored ferrous-tripyridyltriazine complex [Fe (II) TPTZ] to form. This complex is deep blue colour and FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures (Benzie & Strain, 1996).

**2.8.2 MTS cytotoxicity assay**

Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce the tetrazolium product into an aqueous and soluble formazan product. At death, cells rapidly lose the ability to reduce tetrazolium products. Therefore the production of the coloured formazan product is proportional to the number of viable cells in culture (Berridge, 2005).

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The MTS assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. Tetrazolium dye, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in the presence of phenazine methosulfate (PMS) produces a formazan product that has an absorbance maximum at 490-500 nm (Gauduchon, 2005). MTS tetrazolium is similar to the widely used MTT tetrazolium, with the advantage that the formazan product of MTS reduction is soluble in cell culture medium and does not require the use of a Solubilisation Solution. The MTS assay is often described as a 'one-step' MTT assay, which offers the convenience of adding the reagent straight to the cell culture without the intermittent steps required in the MTT assay. MTS assay is usually done in the dark since the reagent is sensitive to light.

### 2.8.3 Nitric oxide determination assay

Nitric oxide (NO) is a major secretory product of mammalian cells that is involved in many physiological and pathological processes (Hou *et al.*, 1999). It is a powerful vasodilator with a short half-life of a few seconds in the blood. Low levels of nitric oxide production are important in protecting organs such as the liver from ischemic damage. NO is an important biological regulator and is therefore a fundamental component in the fields of neuroscience, physiology, and immunology. It is a key vertebrate biological messenger that plays a role in various biological processes. It is a known bioproduct in almost all types of organisms, ranging from bacteria to plants, fungi, and animal cells (Roszer, 2012).

Nitric oxide, known as the 'endothelium-derived relaxing factor' (EDRF) is biosynthesised endogenously from L-arginine, oxygen, and NADPH by various nitric



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oxide synthase (NOS) enzymes. Reduction of inorganic nitrate may also serve to make nitric oxide. The endothelium (inner lining) of blood vessels uses nitric oxide to signal the surrounding smooth muscle to relax, thus resulting in vasodilation and increasing blood flow. Nitric oxide is also generated by phagocytes such as monocytes, macrophages, and microglial as part of the human immune response (Green *et al.*, 1990). Phagocytes are armed with inducible nitric oxide synthase (iNOS) which is activated by interferon-gamma (IFN-γ) as a single signal or by tumour necrosis factor (TNF) along with a second signal (Green *et al.*, 1993). In this way, the immune system may regulate the effect of phagocytes that play a role in inflammation and immune responses. Nitric oxide is secreted as free radicals in an immune response and is toxic to bacteria and intracellular parasites.

### 2.8.4 qRTPCR gene expression

A real-time polymerase chain reaction or quantitative polymerase chain reaction (qPCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It is used to amplify and simultaneously detect or quantify a targeted DNA molecule in a given sample. Various research areas such as gene expression, gene quantification, allelic discrimination, biomarker discovery, pathogen detection; and discovery and validation of drug targets, frequently use qPCR as the basic diagnostic technique. Advancements in the standard qPCR protocols and instrumentation have led to highly sophisticated and accurate diagnostic tests and experiments are designed (Saunders *et al.*, 2013).

Real-time PCR (qPCR) permits the analysis of the products while the reaction is actually in progress. This is achieved by using various fluorescent dyes which react with

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the amplified product and can be measured by an instrument. This also facilitates the quantitation of the DNA. It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. PCR is therefore often used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample (Filion, 2012). It is not necessary to perform electrophoresis or other procedure after the DNA amplification reaction as the real-time PCR is rapid.

The advantages of real time PCR are:

a) Speed: the amplified DNA is being detected during the PCR reaction so there is no need for a separate detection.
b) Throughput: qPCR is able to process large numbers of samples in short time because it is compatible with liquid handling automation stations.
c) Sensitivity: qPCR is able to distinguish two fold differences in quantity of target DNA molecule and detect down to a few or even one copies of DNA.
d) Lower amounts of starting material: qPCR required as low as 1/1000 the amount for conventional PCR.
e) Broad dynamic range of quantification: quantification can be performed over several orders of magnitude, up to 107-fold dynamic range
f) High repeatability.

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# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Mushroom Samples.

Freeze dried powder of the sclerotium of *Lignosus rhinocerotis* cultivar (TM02) was purchased from Ligno Biotech, Selangor, Malaysia. Freeze dried powder of the stroma of *Cordyceps militaris* was purchased from Biofact Life Sdn. Bhd. The mushroom powder was kept in an air tight bottle at 4 ± 2°C prior to extraction.

## 3.2 Preparation of crude extracts from *Lignosus rhinocerotis* and *Cordyceps militaris*.

### 3.2.1 Hot water extraction

*L. rhinocerotis* and *C. militaris* mushroom powder were soaked in distilled water at a ratio of 1:20. The mushroom powder was kept overnight room temperature shaker at 150 rpm speed. After 24 h, the soaked mushroom powders were double boiled in the water bath for 30 minutes. Boiled mushroom powders were left to cool down and filtered with Watman No. 1 filter paper. The collected filtrate was freeze dried (Christ freeze dryer) at -50 ± 2°C for 48 h and stored in air tight bottles at 4 ± 2$^{o}$C (Eik *et al.*, 2012). Prior to assay, the required extract was dissolved in distilled water and filter sterilised using a micro pore filter of 0.2 microns. The filtered extracts were further diluted to the required concentrations with DMEM media.

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### 3.2.2 Ethanol extraction

The freeze dried powder (1kg) was soaked in a mixture of aqueous ethanol (80%) (Systerm) (10L) for two days at room temperature. The supernatant was decanted, filtered and concentrated in a reduced pressure rotary evaporator (Buchi, Switzerland) at 40°C. The extraction process was repeated five times; the solvent containing extract was then combined and concentrated (Kanagasabapathy *et al.*, 2011). The concentrated extract was stored in an airtight bottle at $4 \pm 2^{o}$C. Prior to assay, the required extract was dissolved in 90% dimethyl sulfoxide (DMSO, D5879, Sigma-Aldrich, St. Louis, MO, USA) and filter sterilized in 0.2µM nylon filter. The filtered extract was further diluted to the required concentrations with DMEM media.

## 3.3 *In vitro* antioxidant activity of *Lignosus rhinocerotis* and *Cordyceps militaris extracts*

### 3.3.1 DPPH radical scavenging activity

The 1,1-diphenyl-2-picryhydazyl (DPPH) free radical scavenging activities of *L. rhinocerotis* and *C. militaris* extracts were measured according to Brand-Williams *et al.*, (1995). Samples were prepared by dissolving five (5) mg of each extract in 90% DMSO (1 mL). Extracts (5µL) were then mixed with 195µL of the methanolic DPPH solution. The mixture was shaken vigorously and left to incubate for 3hr in the dark. The absorbances were measured at 515 nm with a microplate reader (BioTek Instruments, USA). Ascorbic acid (10mM) was used as the standard, 90% DMSO was used as blank and mixture without samples were used as control. The assay was carried out in

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triplicates. The radical scavenging activities were expressed in percentage (%) of DPPH quenched as shown below:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\%$$

**3.3.2 Trolox equivalent antioxidant capacity (TEAC)**

The TEAC assay is an electron based assay involving the decolourisation of ABTS oxidant through the reduction of radical cation. This assay was determined using method outlined by Re *et al.*, (1999). The $ABTS\bullet^{+}$ was prepared in a mixture of 5 mL of 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS\bullet^{+}$) solution and 89μL of 140 mM potassium persulfate ($K_2S_2O_8$). The mixture was kept in the dark for 16 h at room temperature prior to use. After 16 h, the absorbance value of the $ABTS\bullet^{+}$ reagent was adjusted with 95% ethanol to obtain 0.70 ± 0.05 at 734 nm. Sample (10 μL) was mixed with 100 μL of $ABTS\bullet^{+}$ reagent and was allowed to stand for 1 min. The absorbances were measured at 734 nm with a microplate reader (BioTek Instruments, USA). Trolox was used as standard and 90% DMSO was used as blank and mixture without sample was used as control. TEAC values are mean values of triplicates assay and were expressed in percentage (%) of $ABTS\bullet^{+}$ scavenged as shown below:

$$ABTS\bullet^{+} \text{ scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\%$$



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### 3.3.3 Ferric reduction antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) of *L. rhinocerotis* and *C. militaris* extracts were measured according to the protocol developed by Benzie and Strain, (1996). FRAP reagent was prepared by mixing 50 mL of 300 mM acetate buffer; 5 mL of 10 mM 2,4,6-tripyridyl-s-triazine solution (TPTZ) in 40 mM of hydrochloric acid (HCl) and 5mL of 20 mM ferric chloride ($FeCl_3{\bullet}6H_2O$) in the ratio of 10:1:1. FRAP reagent (300 µL) was added to 10 µL of mushrooms extracts (5 mg/mL) plated in a 96 well plate and absorbances were measured at 593 nm after 4 min in a microplate reader (BioTek Instruments, USA). The standard used was iron sulfate ($FeSO_4$) and 90% DMSO was used as blank and mixture without sample was used as control. FRAP results are mean values of triplicate assays and were expressed in mM $FeSO_4$ equivalent (FSE) per gram mushroom (mmol FSE/g extract).

## 3.4 *In vitro* BV2 Cell Culture

### 3.4.1 Cell Culture

The BV2 murine microglial cell line was provided by Dr. Sharmili Vidyadaran (Immunology Lab, Universiti Putra Malaysia). *In vitro* BV2 cells were maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 5% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 ml/L gentamicin, 250 µg/mL fungizone (all Invitrogen), 1X nonessential amino acids (Sigma), 2 mg/mL insulin (Sigma) and 1.5 g/L sodium bicarbonate. Cultures were maintained at 37 $^{\circ}$C in 95% humidified air and 5% $CO_2$. Cells were harvested by treating with 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 37 $^{\circ}$C.

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### 3.4.2 Cell Count

The number of glial cells was estimated using the haemocytometer grid system with trypan blue stain (Schrek, 1936). It is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not exclude trypan blue. The viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Trypan blue dye (10µl) was added to 10µl of cell suspension (1:1 dilution). The mixture was then loaded onto a haemocytometer and visually examined under microscope. The numbers of viable cells were determined using the following formula:

Cell concentration (cells / mL) = mean cell count × dilution factor × $10^4$

(Cells / mL) = Mean cell count × 2 × $10^4$

## 3.5 *In vitro* studies of *Lignosus rhinocerotis* and *Cordyceps militaris* extracts on BV2 cells

### 3.5.1 Cytotoxic effects

The cytotoxic effects were determined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. This assay was carried out according to the method by Tan *et al.*, (2011). In a 96 well flat-bottomed microplate, $5{\times}10^4$ cells were seeded per well and incubated at 37°C overnight for attachment. Stock solutions of 10 mg/mL were prepared for each extract/ treatment. The stock solutions were filter-sterilized using 0.2µm pore sized filters. The filter-sterilized stock solution was then diluted appropriately with phenol free media. Different concentrations (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL and 1000 µg/mL) of the



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extract were then added. After 24 hr of incubation, MTS solution (10 µL) was added and further incubated for 2hr at 37°C in a humidified environment of 5% $CO_2$ and 95% air. The absorbance was measured at 490 nm with a microplate reader (Dynex MRX II microplate reader, USA). The complete growth medium was the blank, and cells incubated in medium only without mushroom extracts were denoted as positive control. Each assay was performed in triplicates. The absorbance value of each well with treated cells was deducted from the absorbance of complete growth medium which served as background reading and labelled as Abs $_{sample}$. Cell viability was calculated in percentage (%) as compared to untreated cells as stipulated below:

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

### 3.5.2 Anti-inflammatory activity

Nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. This assay was carried out according to the method by Tan *et al.*, (2011). The BV2 cells were plated in a 96 well plate at a density of $5 \times 10^4$ cells/well and incubated overnight. Stock solutions were prepared and diluted for each extracts/ treatment as previously described in section 3.5.1. Cells were then treated with different concentrations of the extract and incubated with 1µg/mL of *Escherichia coli* (O55:B5) lipopolysaccharide (LPS) (Sigma, US) for 24 h. After 24 h, the culture supernatants were collected for nitrite measurement. Fifty µL of the spent medium were plated in a 96 well plate and 50µL of Griess reagent (0.1% N-1-[naphthyl]ethylenediamine-diHCl, 1% sulfonamide and 2.5% $H_3PO_4$) were added. The plate was incubated for 10 min, and the absorbance was measured at 530 nm using a

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microplate reader (Dynex MRX II microplate reader, USA). The amount of NO was calculated using a sodium nitrite standard curve. Each experiment was conducted in triplicates.

## 3.6 Bioassay guided fractionation of ethanolic extracts of *Lignosus rhinocerotis* and *Cordyceps militaris*

### 3.6.1 Fractionation of ethanol extract

The crude ethanolic extracts were further fractioned with n-hexane (Fisher Scientific, UK) giving a hexane soluble fraction and an insoluble fraction. The insoluble fraction was further partitioned with ethyl acetate (Fisher Scientific, UK): water mixture (1:1) to obtain the ethyl acetate soluble fractions (Kanagasabapathy *et al.*, 2011). Each fraction's supernatant was decanted, filtered and concentrated in a reduced pressure rotary evaporator (Buchi, Switzerland) at 40°C. The fractions were then combined and concentrated. The fractions were weighed and stored in an air tight bottle at 4 ± 2$^{o}$C prior to use. Concentrated extracts of hexane and ethyl acetate were subjected to vacuum liquid chromatography (VLC) to isolate lipid components. Prior to assay, the required fractions were dissolved in 90% dimethyl sulfoxide (DMSO, D5879, Sigma-Aldrich, St.Louis, MO, USA) and filter sterilised in 0.2 µM nylon filter. The filtered extract was further diluted to the required concentrations with DMEM media.

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### 3.6.2 Isolation of Lipid Components.

#### 3.6.2.1 Sample preparation.

The ethyl acetate fractions (5g) were weighed and mixed with silica gel (0.063-0.200nm; mesh 70-230) (Merck, Germany) and a small amount of hexane. The mixtures were then dried in oven at 50°C for 3h to get a powdery consistency.

#### 3.6.2.2 Vacuum Liquid Chromatography (VLC).

The dried, powdered mixture of extracts and silica were subjected to VLC. The column was packed with 300g silica gel as a stationary phase and the ratio of the fraction to silica gel was 1:60. The components in the extract were eluted with 100% n-hexane solvent and subsequently with a mixture of n-hexane: acetone in an increasing percentage of acetone (increasing the polarity). The elutions, which were collected in each vial were monitored using TLC plates. Eluents with similar TLC pattern were combined, concentrated under reduced pressure rotary evaporator and collected in numbered 25 mL volume vials (Malek *et al.*, 2011).

#### 3.6.2.3 Thin-Layer Chromatography (TLC).

Thin layer chromatography was carried out using the precoated silica gel 60 $F_{254}$ (20x20 mm) (Merck, Germany) glass TLC plates. A small drop of the sample was spotted at the base line 1.0 cm from the bottom of the TLC plate using a capillary tube and left to dry for a minute. Spots were labelled at the base line of the plate with a pencil. After the sample has dried, the TLC plate was placed in a TLC tank containing pre mixed organic

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solvent system. Various percentages of hexane and ethyl acetate solvents were used as the developing solvent systems. The TLC plates were removed from the tank when it has fully developed to solvent front. Then the TLC plates were left to dry for 2 minutes. The spots were visualized under the short wavelength (254 nm) and long wavelength (365 nm) ultraviolet (UV) light. The plates were placed into an iodine vapour chamber to stain the spots of separated organic components (Waksmundzka-Hajnos *et al.*, 2008). The subfractions were pooled according to the similarity of the spots on TLC plates. The subfractions with similar spots were combined, reduced with rotary evaporator and stored in 25 mL vials. Components in the isolated subfractions were identified using GC-MS.

### 3.6.3 Identification of major bioactive constituents by gas chromatography-mass spectrum (GC-MS)

Samples were prepared by diluting one (1) mg of each extract in in 1mL chloroform in a 1.5ml vial. The GC-MS analysis was performed on a Agilent Technologies 6890 N (United States) gas chromatograph equipped with a 5979 Mass Selective Detector (70 eV direct inlet) and a HP-5ms (5% phenylmethylpolysiloxane) capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) initially set at 100 °C, then the temperature was increased at a rate of 5 °C per minute to 300 °C using helium as the carrier gas at a flow rate of 1 mL/min. The total ion chromatogram obtained was auto integrated by Chemstation and the constituents were identified by comparison with the accompanying mass spectral database (W9N11. Mass Spectrum Library, USA, 2011).

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## 3.7 Gene expression studies of *Lignosus rhinocerotis* and *Cordyceps militaris* bioactive constituents

BV2 microglia cells were seeded with the density of $5 \times 10^6$ into the 25 $cm^2$ culture flask and were incubated overnight to allow attachment of cells. A stock solution of 10 mg/mL was prepared for each extract/ treatment. The stock solutions were filter-sterilized using 0.2µm pore sized filters. The filter-sterilized stock solution was then diluted appropriately with phenol free media. The cells were treated with extracts (10 µg/mL final concentration) and co-incubated with 1 µg/mL of LPS for 24 hours. Aspirin (0.1 mM final concentration) was used as positive control. Meanwhile untreated cells were used as negative control. The total RNA was isolated from the macrophage cells as recommended by the manufacturer's manual using Ambion-RNAqueous Micro® kit (Applied Biosystems, USA).

Briefly, cells were dislodged from the bottom of the flask using the cell scraper and were transferred into a 15 mL centrifuge tube. Cells were centrifuged at 1800 g for 5 minutes. Then, the cells were lysed with lysis solution that disrupts cell membranes and capable of protecting the RNA from endogenous RNases. Subsequently, the cells were homogenized by pipetting vigorously and vortexing. The homogenate was then mixed with ethanol thoroughly and centrifuged through a microfilter cartridge supplied with a silica-based membrane that selectively binds RNA at 10,000 g for 30 seconds. The impurities were effectively removed by specific washing step. Finally, total RNA was eluted by running the elution solution through an elution cartridge. Homogenates were kept on ice to prevent the RNase activity. Purified RNA was used for reverse transcription. The concentration of the RNA was calculated as follow:

Total RNA (µg RNA/mL) = $A_{260} \times 40$ µg/mL $\times$ dilution factor

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Purified RNA was used to synthesise complementary DNA (cDNA). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) which contains all required reagents needed for reverse transcription (RT) of total RNA to single-stranded cDNA was used in this study. In general, 10 µL of RT buffer, 1µL of Enzyme Mix and 9 µL of RNA sample was added in a microcentrifuge tube. The mixture was mixed thoroughly and was centrifuged to spin down the contents and eliminate air bubbles. The mixture was then loaded into a thermal cycler (Eppendorf, USA) and PCR was carried out according to optimized thermal cycling conditions provided by the manufacturer. The cDNA product was stored under -80°C prior use for gene expression study (Kanagasabapathy *et al.*, 2012).

Reaction setup for all TaqMan® Gene Expression assays was performed according to the reaction setup instructions generated by the StepOne software (Ver 2.0, Applied Biosystems). Briefly, a reaction which consisted of the TaqMan® Gene Expression Master Mix and the assay mix was prepared separately, as each assay mix contained corresponding primers and probe for each gene targeted. The list of genes used in this assay is given in Table 3.1. All reagents were kept on ice, once thawed. Each reaction was assayed in triplicate. The reaction mix was mixed with either sterile ultra-pure water for no template control reactions (NTC) or isolated cDNA. Subsequently, the mixture was transferred into fluorescence compatible MicroAmp™ Fast Reaction Tube Strips and capped with MicroAmp™ Optical Cap Strips. The strips were centrifuged and loaded into the real time PCR thermal cycler (StepOnePlus™ Real Time PCR System).

The relative expression of the investigated genes was normalized with the endogenous control (β actin rRNA). $C_T$ values are the means of triplicate measurements.

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The calculations and formulas involved were as follows.

$C_{T\ Target} - C_{T\ Endogenous\ control} = \Delta C_T$

$\Delta C_{T\ Sample} - \Delta CT_{\ Calibrator} = 2^{-\Delta\Delta Ct}$

Target = gene of interest

Endogenous control = a gene that is present at a stable amount in total RNA despite experimental conditions (β actin rRNA)

Sample = treated sample

Calibrator = untreated sample or control

**Table 3.1.**: List of genes investigated

| No | Gene name | Abbreviation | Assay ID |
|---|---|---|---|
| 1. | Nuclear factor (erythroid-derived 2)-like 2 | NFE2l2/ NRF2 | Mn00477784_m1 |
| 2. | Heme oxygenase-1 | HO-1 | Mm00516005_m1 |
| 3. | NAD(P)H quinone oxidoreductase 1 | NQO-1 | Mm01253561_m1 |
| 4. | Inducible nitric oxide synthase | NOS2 | Mm00440502_m1 |
| 5. | Beta- actin | β-actin | Mm00607939_s1 |
| 6. | Cyclooxigenase-2 | COX-2 | Mm00478374_m1 |
| 7. | Inter leukine 1β | IL-1β | Mm00434228_m1 |
| 8. | Nuclear factor- κB | NF-κB | Mm00479810_g1 |

General abbreviation of genes selected for this study and corresponding assay ID was 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.

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## 3.8 Statistical Analysis.

All data were recorded as means ± standard deviation (SD) and analysed by SPSS for Windows (ver. 18.). One-way analysis of variance (ANOVA); and Dunnett and Duncan comparisons were carried out to test any significant differences. $P < 0.05$ was considered statistically significant.

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# CHAPTER 4: RESULTS

## 4.1. Aqueous and ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies

### 4.1.1. Physical characterisation of aqueous and ethanol extracts

Freeze dried powders of the sclerotium of *Lignosus rhinocerotis* cultivar and the stroma of *Cordyceps militaris* were purchased from Ligno Biotech and Biofact Life Sdn. Bhd. respectively. The freeze dried powder of each mushroom, underwent two different types of extractions; hot aqueous and ethanol extractions. The extraction flow charts of aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris* mushrooms are shown in Figure 4.1 and Figure 4.2.

The hot aqueous extraction of *L. rhinocerotis* yielded 3.12 g (31.2%) of light brown flaky substance from a 10 g of starting material. The *L. rhinocerotis* produced 234.22 g (9.4%) of ethanol extract out of the initial 2.5 kg freeze dried mushroom powder as shown in Figure 4.1. Ethanol extract was prepared from a mixture of ethanol and water in a ratio of (8:2) to enhance the extraction capability of ethanol soluble compounds (secondary metabolites) as water has higher polarity (Figure 4.1). The ethanol extract was a dark brown viscous liquid. The yield of ethanol extract was three fold lower than the aqueous extract as the hot aqueous extract also contains soluble carbohydrates and some amino acids. The ethanol extract contains more secondary metabolites, but less of carbohydrates and probably no amino acids.

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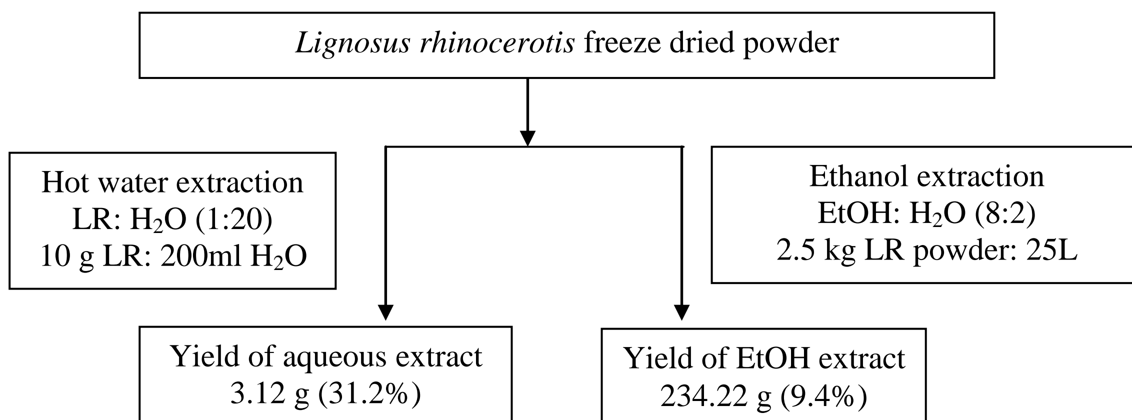


**Figure 4.1:** Flow chart for aqueous and ethanol extracts of *L. rhinocerotis*

Hot aqueous extraction of *C. militaris* yielded 3.14 g (31.4%) of brown granulated powder from 10 g of freeze dried mushroom powder. The ethanol extraction of *C. militaris* produced 233.19 g of extract which amounts to more than 1/4 of the starting material (0.9 kg). A dark brown viscous ethanol extract was formed from the bright orange ethanolic solution of *C. militaris*. According to the results shown in Figure 4.2, water soluble and ethanol soluble constituents present in the mushroom powder are similar because the yield percentage of both extracts amounts to 26-31% each.

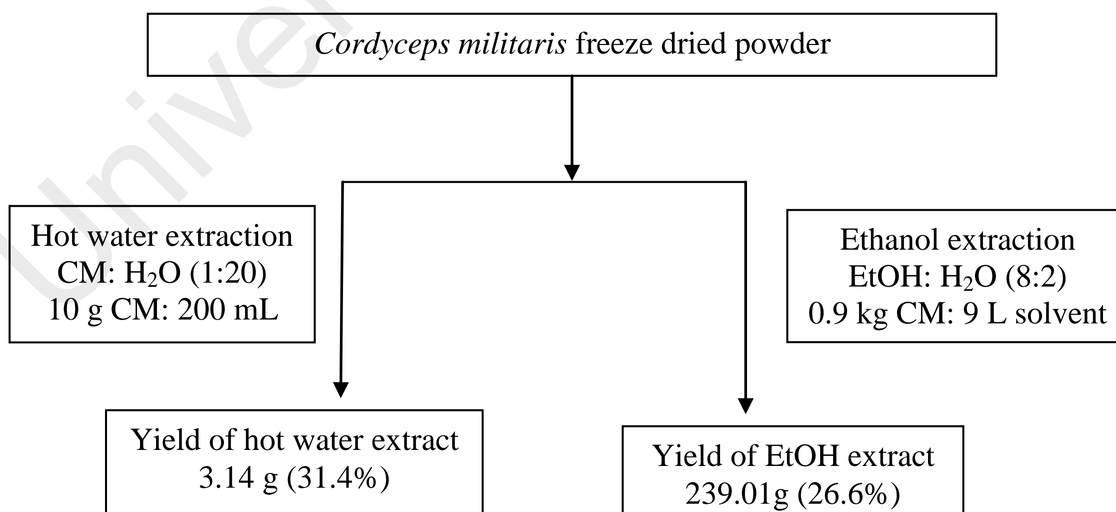


**Figure 4.2:** Flow chart for aqueous and ethanol extracts of *C. militaris*

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The differences between the yield of hot aqueous extracts of *L. rhinocerotis* and *C. militaris* were only 0.2%. However the yield of *C. militaris* ethanol extracts (26.6%) was almost three fold higher than the amount of *L. rhinocerotis* ethanol extract (9.4%). The physical appearance of *L. rhinocerotis* was in the range of brown colour and viscous liquid texture. However the appearance of *C. militaris* extracts was orange in colour.

### 4.1.2 *In vitro* antioxidant activity of aqueous and ethanol extracts

The effects of hot aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris* on DPPH scavenging activity, ABTS scavenging capacity and ferric reduction activity were analysed and tabled in Table 4.1. Cinnamon (*Cinnamomum zeylanicum),* a good antioxidant herb was used as a comparison standard.

The DPPH scavenging activity of *L. rhinocerotis* ethanol extract was 29.35% while the aqueous extract was only 2.29% (Appendix D, Table 1). The ethanol extract showed 12 times higher scavenging activity compared to aqueous extract. However the scenario is reversed in *C. militaris*, whereby the aqueous extract is shown to possess two times higher DPPH scavenging activity compared to the ethanol extract with 67.29% and 31.23% inhibition respectively (Appendix D, Table 1). *C. militaris* aqueous and ethanol extracts had higher inhibition percentage compared to *L. rhinocerotis* extracts. The ethanol extracts of both mushrooms are similar to the range of inhibition between 29-31%. The aqueous extracts on the other hand, have very vast differences between their ability to scavenge the DPPH radicals. *C. militaris* aqueous extract have shown more than 30 times higher activity compared to *L. rhinocerotis* aqueous extract.

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The ABTS radical scavenging capacity of *L. rhinocerotis* ethanol extract was 25.81% inhibition while the aqueous extract had less than 1% inhibition. The *C. militaris* ethanol and aqueous extracts showed 22.91% and 42.17% ABTS radical quenching capacity respectively. The ABTS radical scavenging capacity of the aqueous extract has double the effect compared to ethanol extract. Based on the results, it is shown that the inhibition percentages of both the ethanol extracts are similar with 22-26% (Appendix D, Table 2). The aqueous extract of *C. militaris* projected the highest ABTS radical quenching capacity among all the crude extracts but *L. rhinocerotis* aqueous extract did not possess the scavenging effect.

The FRAP value of *L. rhinocerotis* ethanol and aqueous extracts were 122.58 and 77.1 mg FE/ g extract respectively. Where else the *C. militaris* ethanol and aqueous extracts showed 112.46 and 692.23 mg FE/ g extract FRAP value (Appendix D, Table 3). Similar to ABTS and DPPH scavenging activity, the aqueous of *C. militaris* showed the highest FRAP level. It was 6 fold higher than the aqueous extract. The *L. rhinocerotis* ethanol extract also showed 1.6 fold higher FRAP value compared to the aqueous extract.

**Table 4.1:** Antioxidant activities of hot aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris*

| **Mushroom** | **Extracts** | **DPPH** (%) | **TEAC** (%) | **FRAP** (mg FE/g) |
|---|---|---|---|---|
| *Lignosus rhinocerotis* | Hot aqueous | 2.29 ± 0.35[a] | 0.51 ± 0.36[a] | 77.10 ± 3.66[a] |
| | Ethanol | 29.35 ± 1.67[b] | 25.81 ± 1.26[b] | 122.58 ± 4.79[b] |
| *Cordyceps militaris* | Hot aqueous | 67.29 ± 2.66[c] | 42.17 ± 1.13[c] | 692.23 ± 13.27[c] |
| | Ethanol | 31.23 ± 4.25[b] | 22.91 ± 1.00[d] | 112.46 ± 4.14[d] |
| *Cinnamomum zeylanicum* * | Aqueous | 84.43 ± 3.48 | 64.88 ± 3.74 | 723.75 ± 0.15 |

* Source: Dudonne *et al*., (2009)
Different alphabets in a row means significant differences ($p<0.05$)



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Based on the results in Table 4.1, the aqueous extract of *C. militaris* possess the best anti-oxidant properties among all the extracts. It is also projected that the *C. militaris* extracts have better antioxidant properties compared to *L. rhinocerotis* extracts. In between the ethanol and aqueous extracts of *C. militaris*, the aqueous extract exhibited minimum of two fold higher antioxidant properties compared to the ethanol extract. However, in between *L. rhinocerotis* extracts, the ethanol extract exhibited better antioxidant properties with 10 times more scavenging activity compared to its aqueous extract.

### 4.1.3 *In vitro* cytotoxic effect of aqueous and ethanol extracts

The cytotoxic effect of aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris* was evaluated using the MTS assay. The viability of BV2 microglia cells was assessed after 24 hours incubation with various concentrations of *L. rhinocerotis* and *C. militaris* extracts. The cell viability of untreated cells (BV2) was scored 100%. The cell viability was scored in percentage, as the ratio of treated cells over untreated cells.

Figure 4.3 shows the percentage of cell viability of BV2 cells incubated with various concentrations of *L. rhinocerotis* extracts. There was no significant ($p<0.05$) cytotoxic effect observed in BV2 cells at 0.1 µg/mL-100 µg/mL concentrations (Appendix D, Table 4). A dose-dependent increase of cell viability of BV2 cells treated with the extracts was shown at concentrations ranging from 0.1µg/mL – 10 µg/mL followed by a dose dependent decrease from 10 µg/mL - 1000 µg/mL. An increase of 4-10% in cell viability was seen in BV2 cells treated with 10 µg/mL of each extract tested. However, there was no significant ($p < 0.05$) difference in the cytotoxic effect at concentrations 0.1µg/mL – 100 µg/ml compared to the positive control. At 1000 µg/mL

the extracts were cytotoxic to the BV2 cells. The cell viability of BV2 cells incubated with 1000 µg/mL of aqueous and ethanol extract displayed only 79% and 11% respectively. Thus, in all subsequent assays 1000 µg/mL concentration of the extracts was omitted.

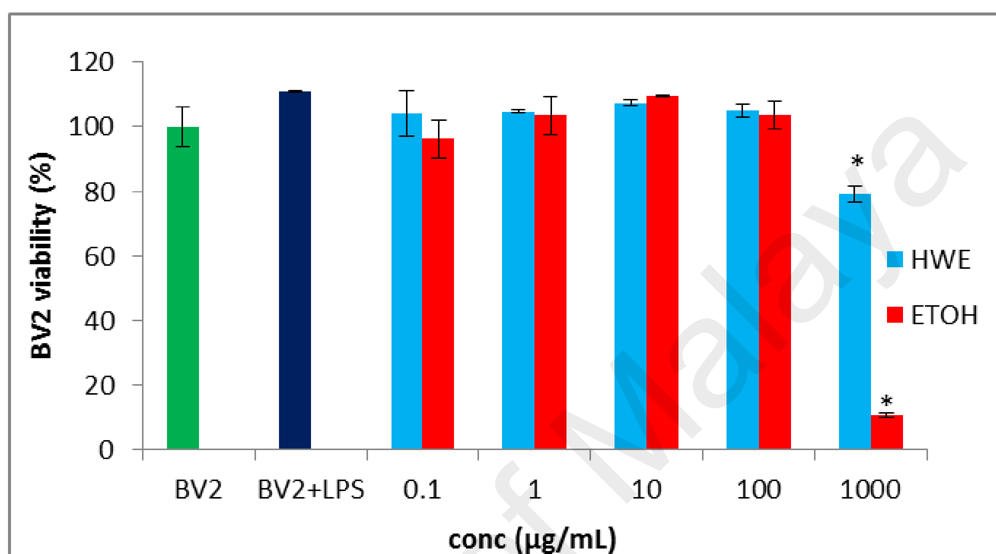


**Figure 4.3: Effects of aqueous and ethanol extracts of *L. rhinocerotis* on the viability of BV2 cells.** The data represent the mean ± SD from a representative of three independent experiments carried out in triplicates. HWE = Hot aqueous extract; ETOH = Ethanol extract. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control. Control (BV2) = 100%

Figure 4.4 showed the percentage of cell viability of BV2 cells incubated with various concentrations of *C. militaris* extracts. The cell viability of BV2 cells incubated with 0.1 µg/mL-100 µg/mL extracts concentrations individually did not display any cytotoxic effect. A dose-dependent increase of cell viability of BV2 cells treated with the extracts was shown at concentrations ranging from 0.1 µg/mL – 10 µg/mL followed by a dose dependent decrease from 10 µg/mL - 1000 µg/mL. At 1000 µg/ml, there was a significant ($p < 0.05$) 16% reduction for aqueous extract and 84% reduction for ethanol extract in cell viability (Appendix D, Table 5). This clearly shows that 1000 µg/mL dose was too toxic and was not further tested in the following experiments.

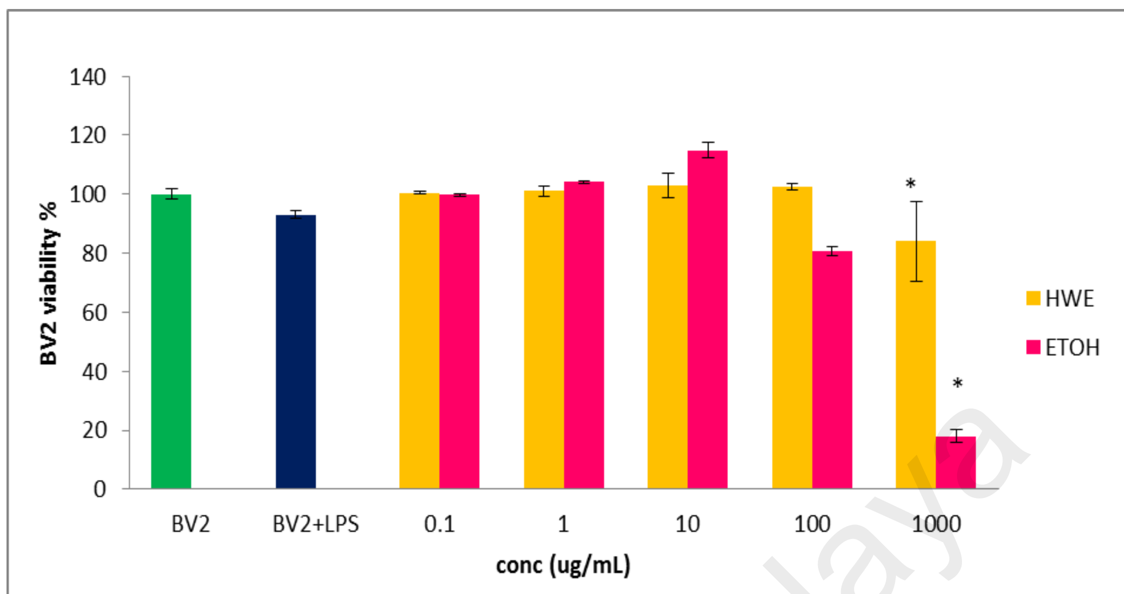


**Figure 4.4: Effects of aqueous and ethanol extracts of *C. militaris* on the viability of BV2 cells.** The data represent the mean ± SD from a representative of three independent experiments carried out in triplicates. HWE = Hot aqueous extract; ETOH = Ethanol extract. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control. Control (BV2) = 100%

### 4.1.4 *In vitro* anti-inflammatory activity of aqueous and ethanol extracts

The anti-inflammatory activities of the aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris* were evaluated using the Griess reagent by determining the nitric oxide production by cells. L-NAME at 200 µM was used as a positive control because it is a commercial nitric oxide suppressant. It was able to suppress 50% of total nitrite production in the LPS induced BV2 cells. In unstimulated cells (BV2) basal level of NO was detected while LPS stimulated cells (BV2+LPS) were the negative control and showed 100% of NO production in cells.

The effect of *L. rhinocerotis* extracts from 0.1 µg/mL to 100 µg/mL concentrations on NO production by LPS stimulated BV2 cells is given in Figure 4.5.



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The *L. rhinocerotis* aqueous extract treated cells did not have significant reduction of nitric oxide at all concentrations tested ranging 0.1 µg/mL to 100 µg/mL. Thus this extract was not further fractionated to test anti-inflammatory activity. There was a gradual decrease of nitric oxide production in the *L. rhinocerotis* ethanol extract treated BV2 cells from 1 µg/mL to 100 µg/mL with a reduction of 14% to 69%. The nitric oxide production was significantly ($p < 0.05$) reduced at concentration 100 µg/mL compared to the untreated cells (negative control) (Appendix D, Table 6). Thus the ethanol extract was selected for further fractionation with hexane and ethyl acetate.

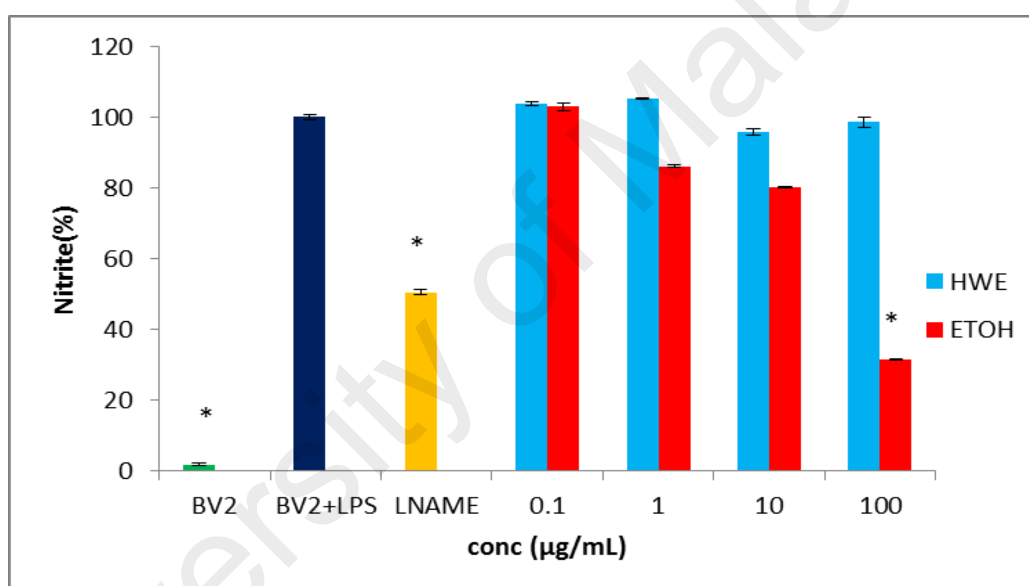


**Figure 4.5: Effects of *L. rhinocerotis* extracts on LPS – induced nitric oxide (NO) production by BV2 microglia cells.** The data represent the mean ± SD and from a representative of three independent experiments carried out in triplicates. HWE = Hot aqueous extract; ETOH = Ethanol extracts. The * symbol denotes a significant difference ($p < 0.05$) when compared to the negative control. Negative control (BV2+LPS) = 100%

Figure 4.6 showed the effect of aqueous and ethanol extracts of *C. militaris* on the LPS induced nitric oxide production by BV2 cells. Aqueous and ethanol extracts of *C. militaris* treated cells showed a dose dependent decrease of nitric oxide production in BV2 cells. The aqueous extract treatment did not show any significant reduction at



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0.1 µg/mL, 1 µg/mL and 10µg/ml but had a drastic reduction of 90% at 100 µg/mL concentration. The treatment with ethanol extract however had a significant ($p < 0.05$) 60% reduction at 10 µg/mL (Appendix D, Table 7). There was almost no nitric oxide production at 100 µg/mL similar to unstimulated cells by 0.23%. The ethanol extract was selected in comparable to the aqueous extract because it managed to reduce nitric oxide at a low concentration treatment. Thus the ethanol extract was used for further fractionation using hexane and ethyl acetate.

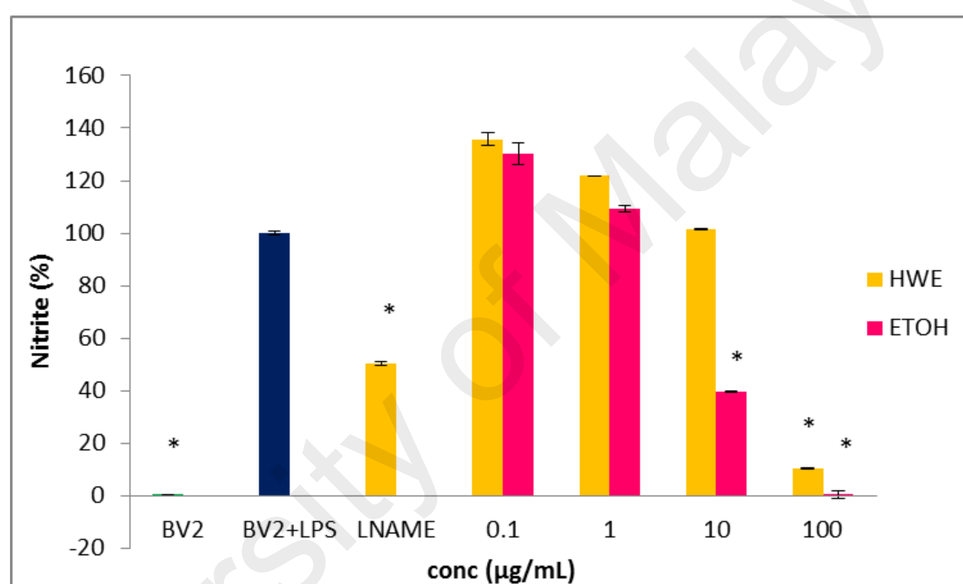


**Figure 4.6: Effects of *C. militaris* extracts on LPS – induced nitric oxide (NO) production by BV2 microglia cells.** The data represent the mean ± SD and from a representative of three independent experiments carried out in triplicates. HWE = Hot aqueous extract; ETOH = Ethanol extracts. The * symbol denotes a significant difference ($p < 0.05$) when compared to the negative control. Negative control (BV2+LPS) = 100%

The ethanol extract of both mushrooms projected a better reduction of nitric oxide by BV2 cells compared to its aqueous extracts. The ethanol extract of *C. militaris* treated cells has significant reductions from 10 µg/mL but *L. rhinocerotis* treated cells only was able to significantly reduce nitric oxide at 100 µg/mL. The ethanol extracts also showed the ability to reduce nitric oxide comparable to the positive control (LNAME).



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At 10 μg/mL, the percentage of nitric oxide reduction with *C. militaris* ethanol extract treatment (60%) was as good as to 100 μg/mL *L. rhinocerotis* ethanol extract treatment (69%). This shows the *C. militaris* ethanol extract is more potent compared to *L. rhinocerotis* ethanol extract. But in this study, we are looking at the components of these medicinal mushrooms that are responsible for the reduction of neuroinflammation rather than which mushroom does work better in curbing neuroinflammation. Thus the ethanol extracts of both mushrooms were further fractionated to narrow down the bioactive components that are able to suppress neuroinflammation.

## 4.2. Bioassay guided fractions from crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies.

### 4.2.1. Physical characterisation of hexane and ethyl acetate fractions

The bioactive ethanol extracts of *L. rhinocerotis* and *C. militaris* were further fractionated with hexane and ethyl acetate solvents. Based on section 4.1, the ethanol extracts of both mushrooms were chosen because they displayed anti-inflammatory activity compared to the aqueous extracts. The process of fractionation is shown in Figure 4.7 and Figure 4.8.

Fractionation of the *L. rhinocerotis* ethanol extract, produced 16.79 g (7.3%) of the hexane fraction as a dark brown viscous liquid while the hexane insoluble fraction was further partitioned with ethyl acetate and water mixture to give the ethyl acetate fraction 9.93 g (4.3%) also a dark brown coloured viscous liquid. Both fractions had very low yields (less than 10%) comparing to the crude ethanol extract as shown in Figure 4.7.

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This shows that most of the constituents in ethanol extract are highly polar and may have large molecular weight.

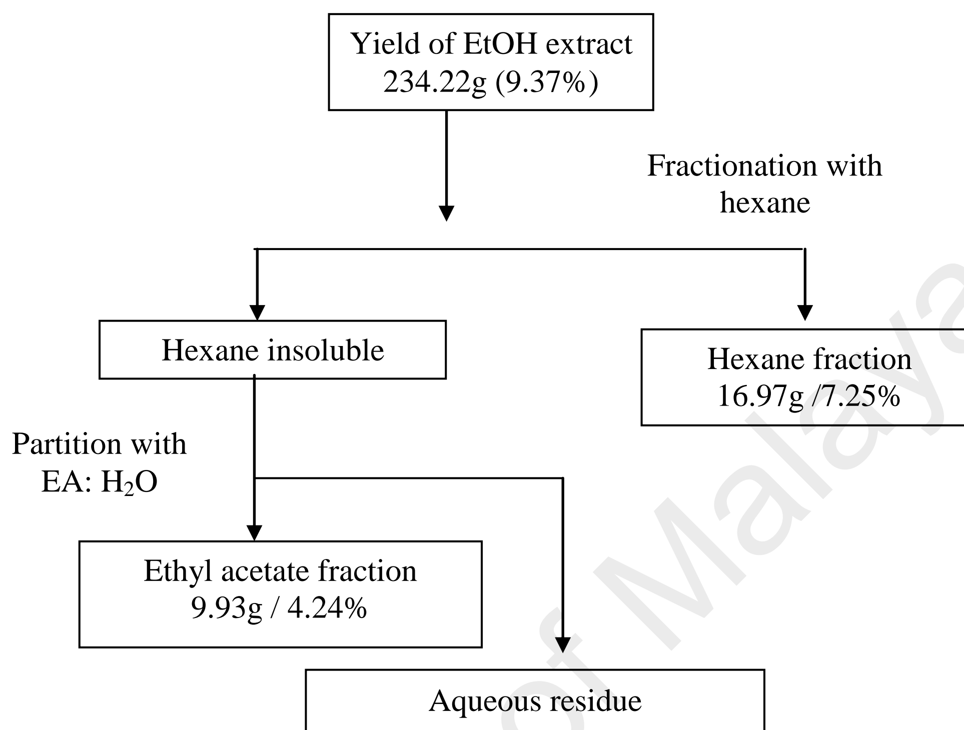


**Figure 4.7:** Flow chart showing fractionation of ethanol extract of *L. rhinocerotis*

Fractionation of the ethanol extract of *C. militaris* using hexane produced a dark brown semi solid extract (20.01g, 8.4%). The ethyl acetate fraction appeared as a bright dark orange semisolid weighing 9.81g (4.1%). As revealed in *L. rhinocerotis* fractions, the *C. militaris* fractions also produced less than 10% yield based on the ethanol extract. The hexane fraction is 50% higher in yield compared to the ethyl acetate fraction.

From both the flow charts, it was presumed that the ethanol extract has less than 20% of low and mid polar constituents and has about 80% high polar constituents. The hexane fractions yielded higher than ethyl acetate fractions of both mushrooms. The fractions obtained from *C. militaris* yielded higher in comparison to the fractions in *L. rhinocerotis*.

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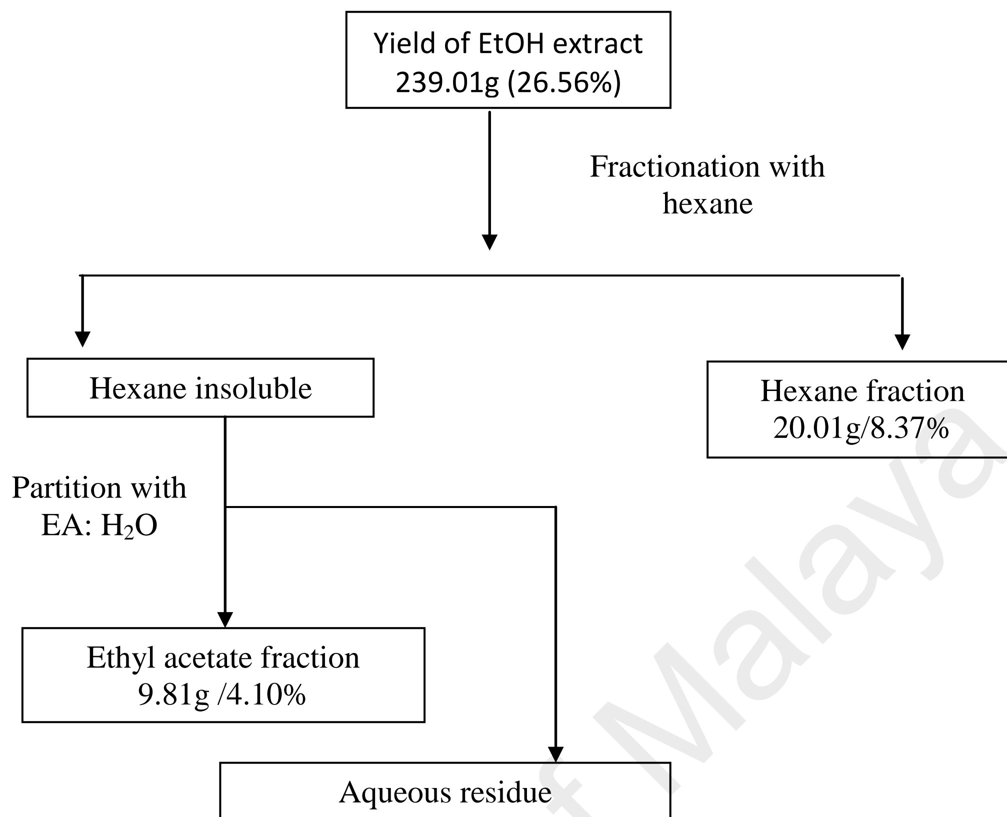


**Figure 4.8:** Flow chart showing fractionation of ethanol extract from *C. militaris*

### 4.2.2 *In vitro* antioxidant activity of the hexane and ethyl acetate fractions

The DPPH scavenging activity, ABTS scavenging capacity and ferric reduction antioxidant power of the hexane and ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* are shown in Table 4.2.

The DPPH radical scavenging activity of *L. rhinocerotis* hexane fraction was 17.21%, while the ethyl acetate fraction was 21.81%. The *C. militaris* hexane and ethyl acetate fractions only showed 6.89% and 9.57% DPPH radical scavenging activity. Both the ethyl acetate fraction had 3-5% more activity compared to the hexane fraction (Appendix D, Table 8). The *C. militaris* fractions showed lower inhibition percentage compared to *L. rhinocerotis* fractions. The hexane fraction of *L. rhinocerotis* showed



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10% higher scavenging activity than the hexane fraction of *C. militaris* whereas the *L. rhinocerotis* ethyl acetate fraction had 12% more scavenging ability compared to *C. militaris* ethyl acetate fraction. The ethyl acetate fraction of *L. rhinocerotis* showed the highest inhibition among the fractions of both mushrooms.

The $ABTS^+$ radical scavenging capacity of *L. rhinocerotis* hexane extract was less than 2%, while the ethyl acetate fraction had 24.57% ABTS radical quenching capacity. The hexane and ethyl acetate fractions of *C. militaris* showed 8.84% and 10.34% ABTS radical quenching capacity respectively. Similar to DPPH scavenging activity, the ethyl acetate *L. rhinocerotis* showed 14% higher ABTS radical scavenging activity compared to ethyl acetate fraction of *C. militaris*. However the *C. militaris* hexane fraction had 7% higher activity compared to *L. rhinocerotis* hexane fraction. In general, the ethyl acetate fraction of both mushroom showed higher ABTS radical scavenging capacity compared to its hexane fraction (Appendix D, Table 9). Amongst all the fractions, *L. rhinocerotis* ethyl acetate fraction had the best ABTS radical scavenging capacity.

Ferric reduction antioxidant power (FRAP) of *L. rhinocerotis* hexane and ethyl acetate fractions were 32.03 mg FE/ g extract and 61.84 mg FE/ g extract respectively. The ethyl acetate fraction had double the activity compared to the hexane fraction. The FRAP value of hexane and ethyl acetate fractions of *C. militaris* were 13.19 mg FE/ g extract and 16.55 mg FE/g extract respectively. Comparing in between the fractions of *C. militaris*, there was only 3% difference in the ferric reduction antioxidant power. The *C. militaris* fractions had 2.5- 3.9 fold lower reducing power compared to *L. rhinocerotis* fractions (Appendix D, Table 10). The *L. rhinocerotis* ethyl acetate fraction showed the best FRAP value.

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Based on Table 4.2, the ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* had better scavenging and reduction activity compared to their hexane fractions. In between the ethyl acetate fractions, the *L. rhinocerotis* ethyl acetate fraction had higher activities. It had two fold higher DPPH scavenging activity, 2.4 fold higher ABTS scavenging capacity and 3.7 fold higher ferric reduction power compared to *C. militaris* ethyl acetate fraction. The ethyl acetate fraction of *L. rhinocerotis* displayed good scavenging and reduction capacity.

**Table 4.2:** Antioxidant activities of hexane and ethyl acetate fractions of *L. rhinocerotis* and *C. militaris*

| **Mushroom** | **Extracts** | **DPPH** (%) | **TEAC** (%) | **FRAP** (mg FE/g) |
|---|---|---|---|---|
| *Lignosus rhinocerotis* | Hexane | 17.21 ± 1.17$^{a}$ | 11.96 ± 1.96$^{a}$ | 32.03 ± 1.33$^{a}$ |
| | Ethyl acetate | 21.81 ± 0.67$^{a}$ | 24.57 ± 1.42$^{ab}$ | 61.84 ± 1.83$^{b}$ |
| *Cordyceps militaris* | Hexane | 6.89 ± 3.14$^{b}$ | 8.84 ± 1.14$^{c}$ | 13.19 ± 0.43$^{b}$ |
| | Ethyl acetate | 9.57 ± 3.06$^{c}$ | 10.34 ± 0.24$^{b}$ | 16.55 ± 2.01$^{c}$ |

Different alphabets in a row means significant differences ($p<0.05$)

### 4.2.3 *In vitro* cytotoxic effect of hexane and ethyl acetate fractions

The hexane and ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* (0.1 µg/mL to 100 µg/mL treatment doses) were tested for their effects on BV2 cell viability as shown in Figure 4.9 and Figure 4.10. Cell viability, (expressed as a percentage) was defined as a ratio of absorbance of treated cells to untreated cells. The cell viability of untreated cells (BV2) was scored as 100%.

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The *L. rhinocerotis* hexane and ethyl acetate fractions showed a dose dependent increase of cell viability with treatment concentration from 0.1 µg/mL to 10 µg/mL and a decrease at 100 µg/mL. The hexane fraction increased from 93% cell viability at 0.1 µg/mL to 110% cell viability at 10µg/ml followed by a decrease of 11% at 100 µg/mL. The ethyl acetate fraction also presented an increase from 98% to 104% in the range of 0.1 µg/mL to 10 µg/mL treatments and declined to 102% at 100 µg/mL. At 0.1 µg/mL concentration of hexane and ethyl acetate treatment individually, the cell viability of BV2 cells was less than 100%. Cells treated with hexane and ethyl acetate fraction at 1 µg/mL and 10 µg/mL showed increase in cell viability percentage (maximum 10%) compared to the untreated cells.

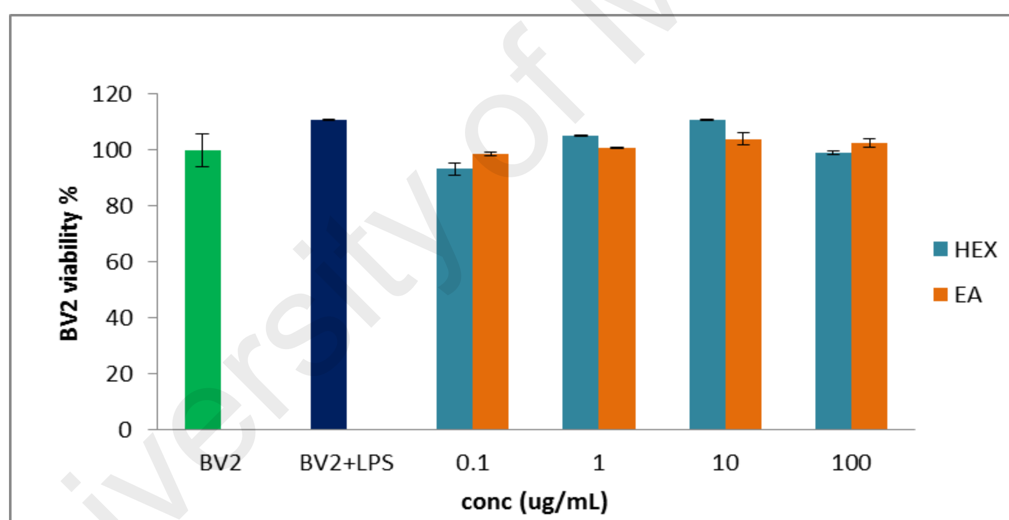


**Figure 4.9: Effects of hexane and ethyl acetate fractions of *L. rhinocerotis* on the viability of BV2 cells.** The data represent the mean ± SD from a representative of three independent experiments carried out in triplicates. HEX = hexane fraction; EA = ethyl acetate fraction. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control. Control (BV2) = 100%

At 100 µg/mL treatment concentration, the hexane fraction treated cells had similar significance in the cell viability as the untreated cells with 99.18% cell viability.



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However the ethyl acetate fraction with the same concentration showed an increase of 2% in cell viability compared to untreated cells. Overall, there were no significant ($p < 0.05$) differences in cell viability when treated with hexane and ethyl acetate fractions at concentrations ranging from 0.1 µg/mL – 100 µg/mL (Appendix D, Table 11). Thus the reduction in cell viability percentage at 0.1 µg/mL concentration can be neglected.

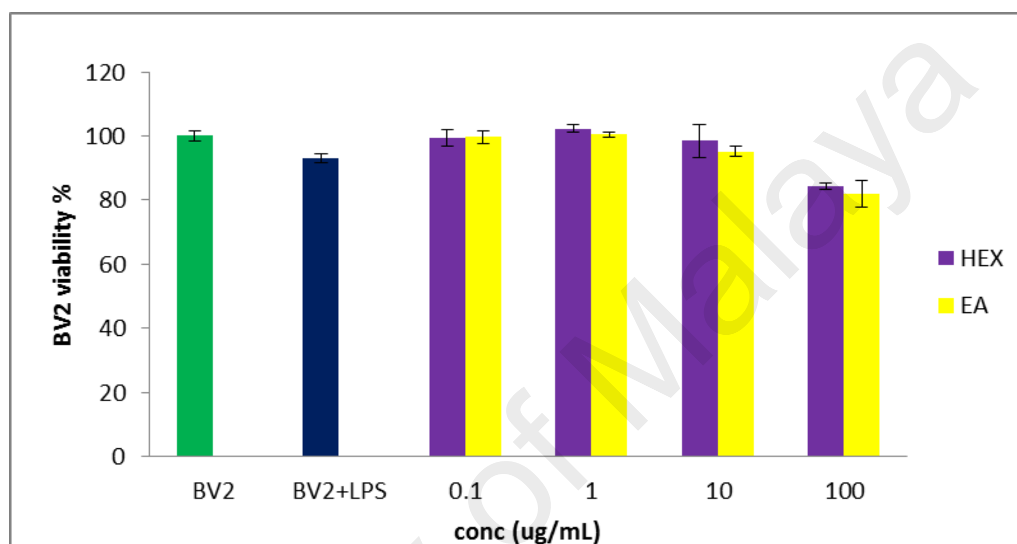


**Figure 4.10: Effects of hexane and ethyl acetate fractions of *C. militaris* on the viability of BV2 cells.** The data represent the mean ± SD from a representative of three independent experiments carried out in triplicates. HEX = hexane fraction; EA = ethyl acetate fraction. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control. Control (BV2) = 100%

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The hexane and ethyl acetate fractions from *C. militaris* ethanol extract were tested at concentrations from 0.1 µg/mL to 100 µg/mL on the BV2 cells to obtain cell viability percentage. The 1000 µg/mL concentration was omitted as it was toxic to the cells. Cells treated with hexane and ethyl acetate fractions showed an increase of cell viability percentage from 0.1 µg/mL to 1 µg/mL concentration followed by reduction at 1 µg/mL to 100 µg/mL concentration. The BV2 cells treated with 0.1 µg/mL hexane and ethyl acetate fraction independently showed only 99% cell viability compared to the

untreated cells. As treatment concentration was increased to 1 µg/mL, the cell viability increased by 1% and 3% by hexane and ethyl acetate respectively.

There was a decline in cell viability percentage with treatment concentration from 1µg/mL to 100 µg/mL. The hexane fraction treated BV2 cells showed reduction up to 16% while the ethyl acetate fraction showed 18% reduction in cell viability percentage compared to the untreated cells. There were no significant ($p < 0.05$) differences in cell viability when treated with hexane and ethyl acetate fractions at concentrations ranging from 0.1µg/mL – 100 µg/mL (Appendix D, Table 12). Thus the reduction in cell viability percentage can be neglected and continued to be tested on the following assays.

### 4.2.4 *In vitro* anti-inflammatory activity of hexane and ethyl acetate fractions

The anti-inflammatory activity of the hexane and ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* were evaluated by determining the nitric oxide production by BV2 cells (Figure 4.11 and Figure.12). The L-NAME at 200 µM was used as a positive control because it is a commercial nitric oxide suppressant. It was able to suppress 50% of total nitrite production in the LPS induced BV2 cells. In unstimulated cell (BV2) basal level of NO was detected. LPS stimulated cells (BV2+LPS) was indicated as the negative control and denoted as 100%.

The hexane and ethyl acetate fraction treated BV2 cells showed a gradual reduction of nitric oxide from 1µg/mL to 100 µg/mL. Hexane fraction treated BV2 cells reduced nitric oxide production from 109% to 71% at concentrations 0.1 µg/mL to 100 µg/mL but the inhibition was not significant ($p < 0.05$). The ethyl acetate fraction treated BV2 cells, however, showed a gradual dose dependent reduction of nitric oxide



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percentage from 104% to 42% in the range of 0.1 µg/mL to 100 µg/mL treatment concentrations. There was significant ($p < 0.05$) nitric oxide reduction of about 37% and 58% at 10 µg/mL and 100 µg/mL ethyl acetate fraction respectively (Appendix D, Table 13). Treatment with the ethyl acetate fraction showed 25% higher anti-inflammatory activity compared to hexane fraction. At same concentrations, the ethyl acetate fraction reduced the nitric oxide significantly while hexane fraction did not. Thus the ethyl acetate fraction of *L. rhinocerotis* was selected to identify the chemical components. The ethyl acetate fraction was also subjected to vacuum liquid chromatography (VLC) to narrow down the bioactive components in the ethyl acetate fraction.

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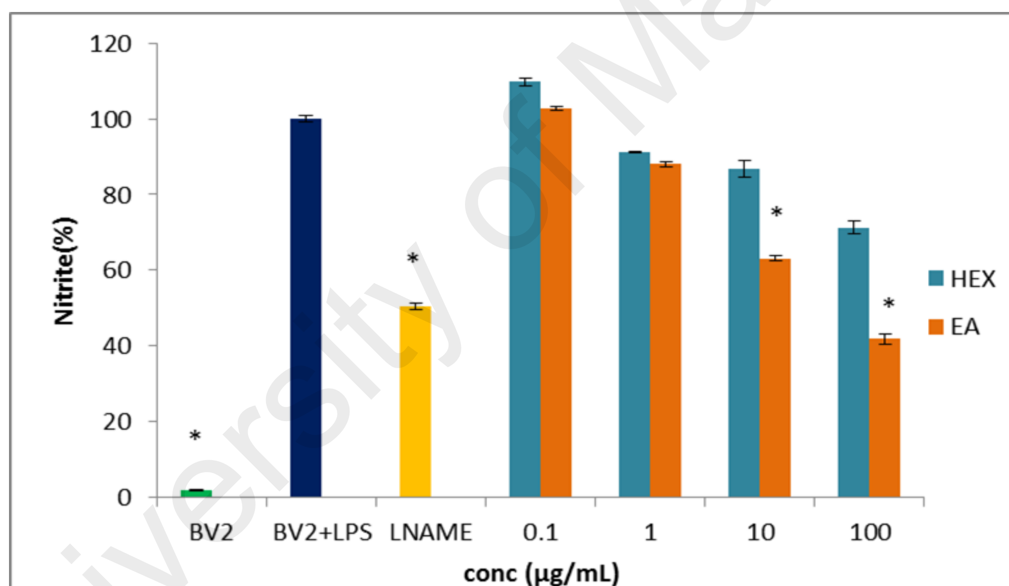


**Figure 4.11: Effects of *L. rhinocerotis* hexane and ethyl acetate fractions on LPS – induced nitric oxide (NO) production by BV2 microglia cells.** The data represent the mean ± SD and from a representative of three independent experiments carried out in triplicates. . HEX = hexane fraction; EA = ethyl acetate fraction. The * symbol denotes a significant difference ($p < 0.05$) when compared to the negative control. Negative control (BV2+LPS) = 100%

The cells treated with hexane and ethyl acetate fractions of *C. militaris* displayed a gradual reduction in nitric oxide from 0.1 µg/mL and 100 µg/mL treatment concentration. Hexane fraction treated cells displayed significant ($p < 0.05$) (Appendix

D, Table 14) reduction of nitric oxide at 10 µg/mL and 100 µg/mL with reduction ranging from 32% and 98% of nitric oxide production respectively. However the ethyl acetate significantly ($p < 0.05$) reduced nitric oxide production by 14%, 48% and 97% at treatment concentrations 1 µg/mL, 10 µg/mL and 100 µg/mL (Figure 4.12) (Appendix D, Table 14). Even though both fractions exhibited good anti-inflammatory activity in BV2 cells, the ethyl acetate fraction was chosen because it was more potent at a lower treatment concentration. Thus the ethyl acetate fraction was proceeded to determine the chemical components via GCMS and further subfractioned using the vacuum liquid chromatography (VLC). The subfractions were also tested for anti-inflammatory activity in the BV2 cells to determine more specific chemical component that are responsible to reduce nitric oxide produced by the cells.

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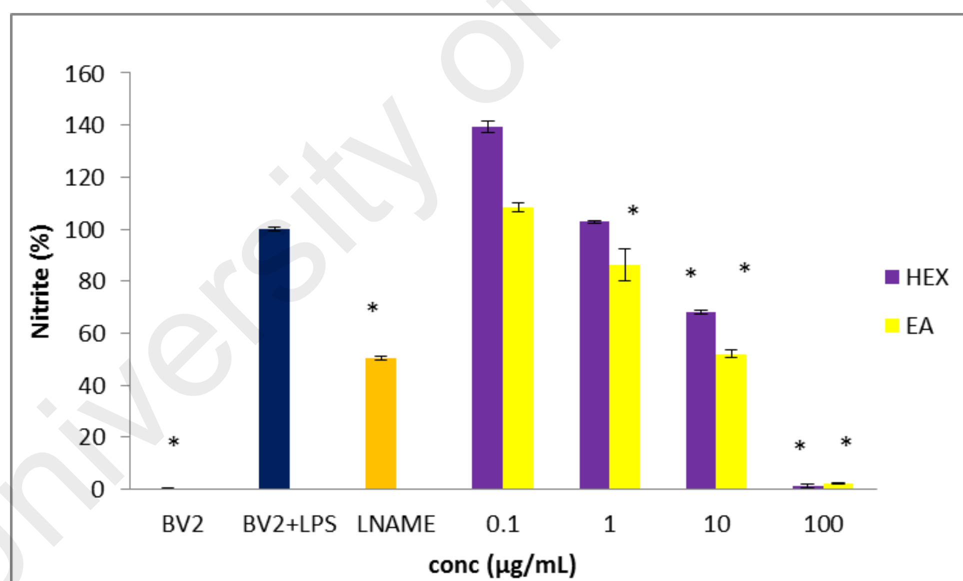


**Figure 4.12: Effects of *C. militaris* hexane and ethyl acetate fractions on LPS – induced nitric oxide (NO) production by BV2 microglia cells.** The data represent the mean ± SD and from a representative of three independent experiments carried out in triplicates. HEX = hexane fraction; EA = ethyl acetate fraction. The *symbol denotes a significant difference ($p < 0.05$) when compared to the negative control. Negative control (BV2+LPS) = 100%.

Overall, a better nitric oxide reduction by BV2 was shown by the treatment of *C. militaris* fractions compared to *L. rhinocerotis* fractions. The *L. rhinocerotis* hexane fraction treated BV2 did not significantly ($p < 0.05$) reduce the nitric oxide production at 0.1 µg/mL to 100 µg/mL treatment concentration. However the hexane fraction of *C. militaris* treatment at 10 µg/mL and 100 µg/mL reduced nitric oxide production significantly. In between the mushrooms, the *C. militaris* ethyl acetate fraction treated BV2 cells showed better reduction of nitric oxide by BV2 at 10 and 100 µg/mL. The *C. militaris* ethyl acetate fraction treated BV2 cells reduced nitric oxide at lower concentration of 1 µg/mL but the *L. rhinocerotis* ethyl acetate fraction treated BV2 cells did not reduce nitric oxide production.

### 4.2.5 GC-MS profiling of bioactive ethyl acetate fraction

The chemical components in the bioactive ethyl acetate fraction of *L. rhinocerotis* and *C. militaris* were identified using gas chromatography - mass spectrometry (GCMS) and tabulated in Table 4.3 and Table 4.4.

The lipid content of ethyl acetate fraction of *L. rhinocerotis* is presented in the Table 4.3. There are various fatty acids, fatty acid esters and sterols present in the ethyl acetate fraction. There is 58.67% of fatty acids present in this fraction and 53.51% of the total fatty acid content are unsaturated fatty acids consisting of linoleic and oleic acids. There is also 16.5% of ethyl ester content in the fraction consisting of ethyl palmitate, ethyl linoleate, ethyl oleate, ethyl stearate, and 2-mono-palmitin. A trace of 5,6-dihydroergosterol (0.81%) was also present in the ethyl acetate fraction. The major components are oleic and linoleic acid amounting to 31.09% and 22.42% respectively of

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the total detected components. A total of 75.98% components were identified in the fraction.

**Table 4.3:** Lipid constituents (%) of ethyl acetate fraction of *L. rhinocerotis*.

| NO | COMPOUND | RT | TM EA |
|---|---|---|---|
| **1.** | 3,5-dimethoxybenzoic acid | 13.145 | 0.92 |
| **2.** | Palmitic acid | 21.409 | 4.24 |
| **3.** | Ethyl palmitate | 21.784 | 2.93 |
| **4.** | Linoleic acid | 24.930 | 31.09 |
| **5.** | Oleic acid | 24.486 | 22.42 |
| **6.** | Ethyl linoleate | 24.836 | 6.76 |
| **7.** | Ethyl oleate | 24.893 | 5.28 |
| **8.** | Ethyl stearate | 25.356 | 0.88 |
| **9.** | 2-mono-palmitin | 30.510 | 0.65 |
| **10.** | 5,6-dihydroergosterol | 40.431 | 0.81 |
| | **TOTAL IDENTIFIED** | | 75.98 |

RT= retention time (min); TMEA = ethyl acetate fraction of *L. rhinocerotis*

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Table 4.4 reveals the chemical components identified in the ethyl acetate fraction of *C. militaris*. A total of 81.28% of its chemical components were identified via GCMS. The fatty acid and ethyl esters encomprising 8.5% was present in the fraction. The fatty acid present was 3.79% namely linoleic acid. There were few ethyl esters identified such as ethyl palmitate (2.83%), ethyl oleate (1.03%) and ethyl stearate (0.85%). The major component in the ethyl acetate fraction of *C. militaris* was sterol (72.78%). Ergosterol contributed to 68.17% of the total sterol identified while dehydroergosterol and dihydroergosterol were quantified as 1.66% and 2.95% respectively.

**Table 4.4:** Lipid constituents (%) of ethyl acetate fraction of *C. militaris*.

| NO | COMPOUND | RT | CD EA |
|---|---|---|---|
| **1.** | Ethyl palmitate | 21.734 | 2.83 |
| **2.** | Linoleic acid | 24.686 | 3.79 |
| **3.** | Ethyl oleate | 24.886 | 1.03 |
| **4.** | Ethyl stearate | 25.374 | 0.85 |
| **5.** | Dehydroergosterol | 39.718 | 1.66 |
| **6.** | Ergosterol | 40.256 | 68.17 |
| **7.** | Dihydroergosterol | 40.356 | 2.95 |
| | **TOTAL IDENTIFIED** | | 81.28 |

RT= retention time (min); TMEA = ethyl acetate fraction of *C. militaris*

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The lipid constituents of *L. rhinocerotis* ethyl acetate fraction consist of fatty acid and fatty acid ester as major components totalling to 75.17%. There was a trace (<1%) of sterol component, 5,6-dihydroergosterol identified in the *L. rhinocerotis* ethyl acetate fraction. However, the components in the lipid constituent of *C. militaris* ethyl acetate fraction were the opposite. This fraction contained 72.78% sterol components and minor components of fatty acid and fatty acid esters of 28.5%. The lipid components of both ethyl acetate fractions contained similar fatty acids, fatty acid esters and sterols but were in different ratios.

## 4.3. Bioactive subfractions from the ethyl acetate fraction and their *in vitro* studies

The ethyl acetate fraction of *L. rhinocerotis* and *C. militaris* mushrooms were further fractionated through vacuum liquid chromatography. The ethyl acetate fraction (5 g) was subjected to vacuum liquid chromatography fractionation method providing seven (7) subfractions for each ethyl acetate fraction. The anti-inflammatory activities of all the subfractions were tested in MTS assay and nitric oxide determination assay. Three

subfractions namely TE3, CE2 and CE3 provided excellent anti-inflammatory activities comparable to L-NAME, a commercial nitric oxide suppressant. Thus the bioactive subfractions are comprehensively discussed in this section.

### 4.3.1 Physical characterization of the bioactive subfractions

The yield and physical characterisations of the three bioactive subfractions are tabulated in Table 4.5. The yield percentage of the bioactive subfraction was calculated based on the starting material (5 g of ethyl acetate fraction). The TE3 subfraction was obtained from ethyl acetate fraction of *L. rhinocerotis*. While CE2 and CE3 subfractions were obtained from the ethyl acetate fraction of *C. militaris*.

The yield obtained for TE3 subfraction was 0.18 g (3.6%). It was golden brown in colour and was in a viscous liquid form. A 0.68 g (13%) of CE2 subfraction was obtained from the fractionation. This subfraction was a bright orange liquid with a viscous texture. However The CE3 subfraction was a yellowish white needle like crystals. They were obtained in the amount of 0.13 g (2.6%).

**Table 4.5:** Yield and physical appearance of bioactive subfractions of *L. rhinocerotis* and *C. militaris*

| Name | Yield (g) | Yield (%) | Colour | Texture |
|---|---|---|---|---|
| TE 3 | 0.18g | 3.6% | Brown | Liquid |
| CE 2 | 0.65g | 13% | Orange | Liquid |
| CE 3 | 0.13g | 2.6% | White | Crystals |



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### 4.3.2 *In vitro* antioxidant activity of bioactive subfractions

The antioxidant properties of the bioactive subfractions were analysed and tabulated in Table 4.6 as shown below. TE3 subfraction was fractioned from *L. rhinocerotis* ethyl acetate fraction; and CE2 and CE3 subfractions were fractioned from *C. militaris* ethyl acetate fraction. The DPPH scavenging activity, ABTS scavenging capacity and ferric reduction antioxidant activity of the three bioactive subfractions were evaluated.

The DPPH scavenging activity of TE3 subfraction was 58.01% while CE2 and CE3 subfractions had 56.20% and 57.59% DPPH inhibition percentage (Appendix D, Table 15). The bioactive subfractions have similar percentage of DPPH quenching activity with 1 % differences in between the subfractions. The ABTS radical scavenging capacity of TE3 subfraction was about 7% while CE2 subfraction was 0.97% and CE3 subfraction was 37%. Among the three subfractions, CE3 subfraction had the highest ABTS radical scavenging ability while TE3 subfraction was moderate and CE2 was very low (Appendix D, Table 16). The ferric reduction power of the bioactive subfraction TE3, CE2 and CE3 were 420.77, 315.76 and 6.69 mg FE/g extract each respectively (Appendix D, Table 17). The highest ferric reduction was given by TE3 subfraction.

**Table 4.6:** Antioxidant activities of bioactive subfractions of *L. rhinocerotis* and *C. militaris*

| **Mushroom** | **Extracts** | **DPPH** (%) | **TEAC** (%) | **FRAP** (mg FE/g) |
|---|---|---|---|---|
| *Lignosus rhinocerotis* | TE 3 | $58.01 \pm 3.24^{a}$ | $6.69 \pm 3.08^{b}$ | $420.77 \pm 8.83^{b}$ |
| *Cordyceps militaris* | CE 2 | $56.20 \pm 4.69^{a}$ | $0.97 \pm 0.35^{a}$ | $315.76 \pm 14.58^{b}$ |
| | CE 3 | $57.59 \pm 4.30^{a}$ | $37.18 \pm 1.80^{c}$ | $6.69 \pm 2.00^{a}$ |

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As investigated, the subfraction did not have very high antioxidant properties. However, amongst the studied subfractions, the CE3 subfraction had high ABTS scavenging and ferric reduction activity. The TE3 had moderate radical scavenging activity but had high FRAP value. The CE2 had low scavenging activity but the FRAP value was comparable to the other subfractions.

### 4.3.3 *In vitro* cytotoxic and neuroprotective effect of bioactive subfractions

The *in vitro* viability of BV2 cells treated with the three bioactive subfractions individually at 0.1 μg/mL to 100 μg/mL treatment doses was tested via MTS assay. The cell viability and neuroprotective effects of the bioactive subfractions are shown in the Figure 4.13. The cytotoxic effect of the subfractions on the viability of BV2 cells is indicated as line labelled 'without LPS'. This shows the cytotoxic effect of the treatments solely towards the BV2 cell viability. The neuroprotective effects of the subfractions on the LPS stimulated BV2 cells are indicated in the line labelled 'with LPS'. This line represents the effect of the treatment on inflamed BV2 cell viability. Cell viability, (expressed as a percentage) was defined as a ratio of absorbance of treated cells to untreated cells. The cell viability of untreated cells (BV2) was scored as 100%.

The TE3 subfraction did not show any significant ($p < 0.05$) cytotoxic effects on the BV2 cell viability (Appendix D, Table 18). The control (BV2) was scored 100% and the negative control (LPS+BV2) was scored 97.37%. There were no significant differences between the positive and negative control for this subfraction. The BV2 cell viability was reducing very slowly with the increase of treatment concentration. At 0.1 μg/mL, the TE3 treated BV2 cells had 97.27% cell viability. It had decreased 10% across the concentrations. The BV2 cell viability at 100 μg/mL treatment concentration was



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87.02%. However, this descent can be disregarded as there is no significant difference between the concentrations. The neuroprotective effects subfractions were done to determine the ability of the bioactive subfractions to protect and prevent cell death of the LPS induced BV2 cells (inflamed microglial). The BV2 positive control was scored 100% and the negative control showed a reduction of 30% in cell viability. The BV2 cell viability treated with TE3 subfraction showed a dose dependent increase from 0.1 µg/mL to 10 µg/mL treatment concentration. At 100 µg/mL treatment concentration, there was a 6% decrease in BV2 cell viability compared to 10 µg/mL treatment concentration. There were no significant ($p < 0.05$) reductions compared to the positive control. The effect of the TE3 subfraction on the LPS induced BV2 cells' viability were relatively lower compared to the effect of the TE3 subfraction on the BV2 cell viability. However, there were no significant differences between the treated and untreated BV2 cells viability. Thus the TE3 subfraction was not cytotoxic towards the BV2 cells and was also able to protect the BV2 cells after induced with LPS.

The CE2 subfraction showed 28% increase of BV2 cell viability with 1 µg/mL treatment concentration compared to 0.1 µg/mL treatment concentrations. A dose dependent decrease of BV2 cell viability was observed from 1 µg/mL to 100 µg/mL treatment concentration. At 1 µg/mL and 10 µg/mL treatment concentrations, there was 24% and 22% proliferation in the BV2 cell viability respectively. Overall the range of cell viability with the various treatment concentrations was 90%-124%. There was also a decrease (23%) of cell viability after BV2 cells were induced with LPS. All the treatment concentration did not impose significant ($p < 0.05$) cytotoxic effect towards the cells (Appendix D, Table 18). The neuroprotective effect of the CE2 subfraction was similar to the cytotoxic effect of the subfraction. There was an increase of 11% in BV2 cell viability treated with 1 µg/mL compared to the 0.1 µg/mL treatment concentration. The

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BV2 cell viability reduced from 104% to 86% with the increase of treatment concentration from 1 µg/mL to 100 µg/mL, the effect of treatment was lower on the BV2 cell viability with LPS compared to cell viability without LPS. Nevertheless the cell viability of LPS induced BV2 cells was similar to the positive control (BV2).

The cytotoxic and neuroprotective effects of the CE3 subfraction treatment on the BV2 cell viability are shown in Figure 4.13. The CE3 subfraction treated BV2 cells showed an increase in cell viability from 0.1 µg/mL to 1 µg/mL treatment concentration followed by a decrease of BV2 cell viability from 1 µg/mL to 100 µg/mL treatment concentrations. The BV2 cells treated with CE3 subfraction proliferated (2% - 23%) at all concentrations except at 100 µg/mL. The effect of CE3 subfraction on the LPS induced BV2 cell viability had similar pattern but relatively lower percentage compared to the effect of the subfraction to non-induced BV2 cells viability. The cell viability range of LPS induced BV2 cells treated with CE3 subfraction was 62%- 105% cell viability. The proliferation and reduction of LPS induced and non- induced BV2 cell viability with CE3 subfraction treatment was not significantly ($p < 0.05$) (Appendix D, Table 18) different from the positive control (BV2).

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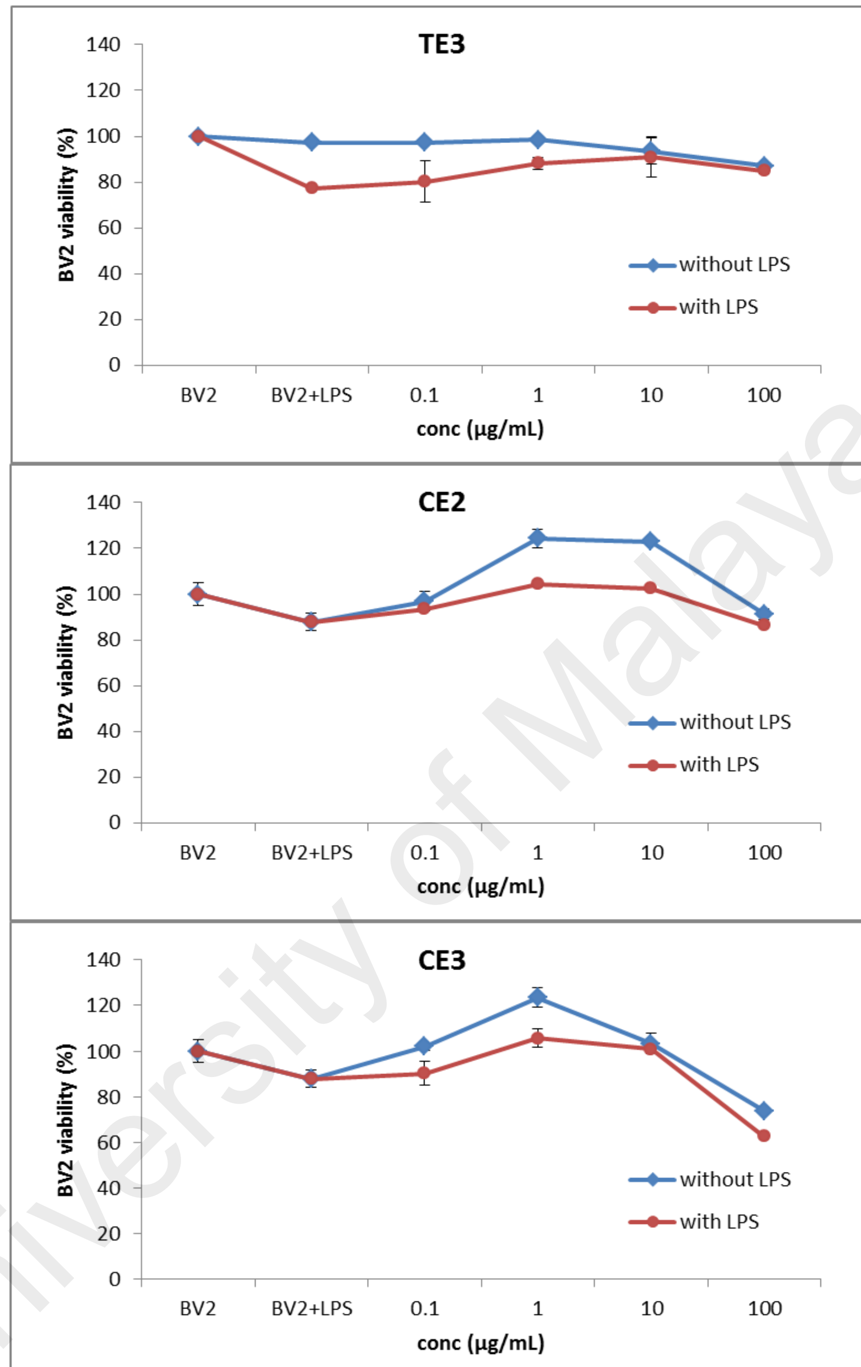


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**Figure 4.13: Cytotoxic and neuroprotective effects of bioactive subfractions on the BV2 cell viability.** The data represent the mean ± SD from a representative of three independent experiments carried out in triplicates. Without LPS = BV2 cell viability without LPS stimulation; with LPS = BV2 cell viability with LPS (1 µg/mL) stimulation. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control. Control (BV2) = 100%

### 4.3.4 *In vitro* anti-inflammatory activity of bioactive subfractions

The anti-inflammatory effects of the TE3, CE2 and CE3 subfractions on LPS induced nitric oxide (NO) production by BV2 microglia cells are displayed in Figure 4.14. In unstimulated cell (BV2) basal level of NO was detected. LPS stimulated cells (BV2+LPS) was indicated as the negative control and denoted as 100%. The L-NAME at 200 μM was used as a positive control because it is a commercial nitric oxide suppressant. It was able to suppress 50% of total nitrite production in the LPS induced BV2 cells without any cytotoxic effect towards the BV2 cell viability.

The TE3 subfraction treated BV2 cells showed significant ($p < 0.05$) nitric oxide reduction at 1 μg/mL, 10 μg/mL, 100 μg/mL treatment concentrations (Appendix D, Table 19). The nitric oxide production was reduced in the range of 44% to 54% at concentrations 1 μg/mL to 100 μg/mL treatment concentration. The reduction is comparable to the positive control, L-NAME. The CE2 subfraction treated BV2 cells also reduced nitric oxide production at 1 μg/mL, 10 μg/mL and 100 μg/mL treatment concentrations, the nitric oxide production was significantly ($p<0.05$) reduced by BV2 cells with 16%, 86% and 92% inhibition (Appendix D, Table 19). This subfraction suppressed inflammation in LPS induced BV2 cell better than the commercial inhibitor, L-NAME. The subfraction brought down the nitric oxide level in the cell almost to the basal level. The CE3 subfraction also showed excellent anti-inflammatory activity on the LPS induced BV2 cells. The CE3 subfraction treated BV2 cells significantly ($p < 0.05$) suppressed nitric oxide production by 45% at 10 μg/mL treatment concentration and 90% at 100 μg/mL treatment concentration (Appendix D, Table 19).

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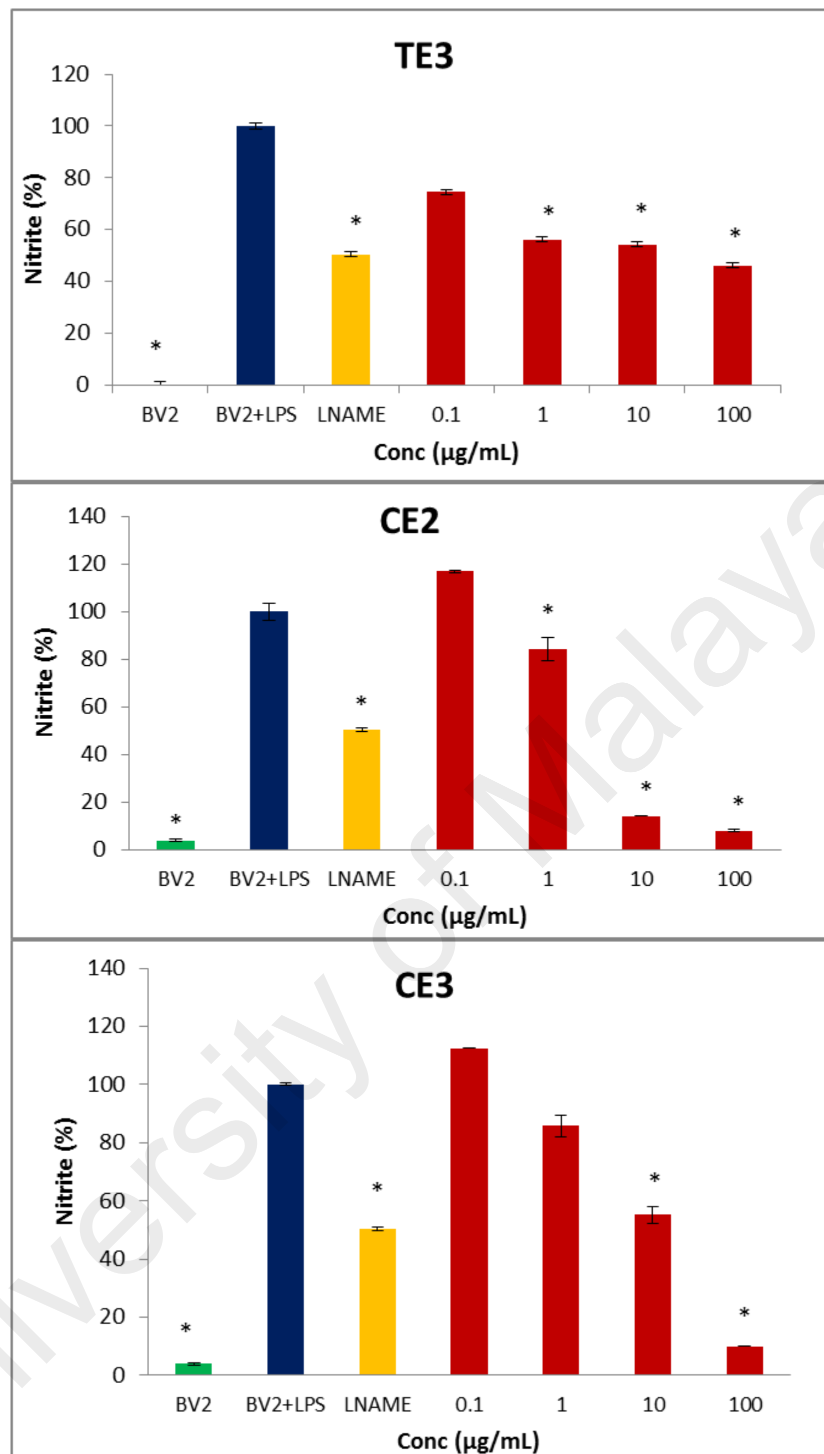


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**Figure 4.14: Effects of bioactive subfraction on LPS – induced nitric oxide (NO) production by BV2 microglia cells.** The data represent the mean ± SD and from a representative of three independent experiments carried out in triplicates. The * symbol denotes a significant difference ($p < 0.05$) when compared to the negative control. Negative control (BV2+LPS) = 100%

### 4.3.5 GC-MS profiling of bioactive subfractions

The chemical components in the bioactive subfractions TE3, CE2 and CE3 were identified using gas chromatography - mass spectrometry (GCMS). The lipid components in the subfractions were profiled and quantified as tabulated in Table 4.7.

The chemical components of subfraction TE3 consists of lipid groups such as fatty acids, fatty acid derivatives and sterols. The major components of TE3 subfractions were fatty acids; palmitic acid (2.37%) and linoleic acid (45.34%). Six other compounds constituting 21.87% of the total are 4-hydroxy benzaldehyde (3.12%); an aldehyde with a benzene ring, vanillin (1.51%); a phenolic aldehyde, palmitamide (3.50%) and oleamide (7.12%); fatty acid amides, 9, 17-octadecadienal (3.36%); an unsaturated aldehyde and dehydroergosterol (2.98%), a sterol. Nevertheless, there were no ethyl esters identified in this subfraction.

The major chemical components identified in CE2 subfraction contained fatty acids; caprylic acid (0.2%), palmitic acid (7.82%) and linoleic acid (43.41%). Five fatty esters comprising of methyl palmitate (0.25%), ethyl palmitate (6.00%), ethyl linoleate (10.08%), ethyl stearate (2.23%) and ethyl lignocerate (0.26%) were identified from the CE2 subfraction. Sterol components such as dehydroergosterol (1.42%), ergone (1.74%) and sitostenone (0.42%) were also present in the subfraction in a minor amount.

The CE3 subfraction consists of three phytosterols namely dehydroergosterol (2.92%), ergosterol (92.98%) and 22, 23-dihydroergosterol (2.12%). The TE3 subfraction of *L. rhinocerotis* and CE2 subfraction of *C. militaris* possess linoleic acid as the major component. Only CE3 subfraction had ergosterol as major component.



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**Table 4.7:** Identified lipid constituents (%) in bioactive subfractions of *L. rhinocerotis* and *C. militaris*

| COMPOUND | TE 3 | CE 2 | CE 3 |
|---|---|---|---|
| **Caprylic acid** | | 0.2 | |
| **4-Hydroxy benzaldehyde** | 3.12 | | |
| **Vanillin** | 1.51 | | |
| **Methyl palmitate** | | 0.25 | |
| **Palmitic acid** | 2.37 | 7.82 | |
| **Ethyl palmitate** | | 6.00 | |
| **Linoleic acid** | 45.34 | 43.41 | |
| **Ethyl linoleate** | | 10.08 | |
| **Ethyl stearate** | | 2.23 | |
| **Palmitamide** | 3.50 | | |
| **Oleamide** | 7.12 | | |
| **9,17-octadecadienal** | 3.36 | | |
| **Ethyl lignocerate** | | 0.26 | |
| **Dehydroergosterol** | 2.96 | 1.42 | 2.92 |
| **Ergosterol** | | | 92.98 |
| **22,23-dihydroergosterol** | | | 2.12 |
| **Ergone** | | 1.74 | |
| **Sitostenone** | | 0.42 | |
| **Total** | **69.28** | **73.83** | **98.02** |

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### 4.3.6. Gene expression study of bioactive constituents.

Quantitative Real time Polymerase chain reaction (qRTPCR) was conducted to determine the effect of the bioactive subfractions on the proinflammatory and anti-inflammatory genes. The expression of the cytokines iNOS, COX-2 and IL-1β induced by LPS in BV2 cells treated with 10µg/mL treatment concentration each is shown in Figure 4.15. Each graph is represented by untreated cells (BV2), stimulated cells without treatment (LPS),

stimulated cells with aspirin 100uM treatment (ASP) and stimulated cells with bioactive subfraction and major compound (10µg/mL treatment concentration) bars. The gene level expressed by the bioactive subfractions is referenced to the LPS bar. The stimulated cells (LPS) have an upregulated expression compared to the untreated cells (BV2). The stimulated cells with aspirin treatment (ASP) as the positive control showed a down regulated expression from the LPS gene expression. Aspirin is a commercial non-steroidal anti-inflammatory drug (NSAID) used to reduce neuroinflammation.

The inducible nitric oxide synthases (iNOS) enzyme catalyzing the production of nitric oxide (NO) from L-arginine was investigated to elucidate the nitric oxide production which is cellular signaling molecule in inflammation. Based on the Figure 4.15, the positive control, aspirin has significantly ($p<0.05$) downregulated the iNOS expression by 1.06 fold compared to the negative control. The expression of iNOS gene was significantly ($p<0.05$) downregulated by bioactive subfractions TE3, CE2 and CE3 with 1.23, 1.14 and 0.547 fold respectively compared to the negative control (Appendix D, Table 20). The subfractions TE3 and CE2 downregulated the gene lower than the commercial NSAID, aspirin.

The inhibition of Cyclooxygenase-2 (COX-2), an enzyme responsible for inflammation and pain was investigated. NSAIDs such as aspirin and ibuprofen exert their effects through inhibition of COX-2. This is shown in Figure 4.15 where the positive control aspirin has shown 1.05 fold downregulation of COX-2 expression compared to the negative control. The bioactive subfractions TE3 and CE 2 had remarkably down regulated the COX-2 expression by 1.20 and 1.50 fold. Where else the bioactive subfraction CE3 had only downregulated the expression by 0.39 fold. All the bioactive subfractions and the positive control reduced COX-2 expression significantly

($p<0.05$) lower than the negative control (Appendix D, Table 21). The subfractions TE3 and CE2 downregulated the COX-2 gene lower than aspirin (positive control).

The expression of Interleukin-1 beta (IL-1β) in the BV2 cells upon treatment with the bioactive subfractions was also investigated. IL-1β is a cytokine protein, which is an important mediator of the inflammatory response. The negative control showed 18 fold upregulation compared to the control. The positive control downregulated the IL-1β gene expression by 15.13 folds compared to the negative control. The TE3, CE2 and CE3 subfractions downregulated the IL-1β gene by 16.70 fold, 17.40 fold and 10.82 fold respectively (Appendix D, Table 22). The TE3 and CE2 subfractions were able to significantly ($p<0.05$) downregulate the expression comparable to and better than the positive control.

The bioactive subfraction CE2 displayed the best reduction of COX-2 and IL-1β gene expression compared to its negative control. However the iNOS gene expression was down regulated the lowest by TE3 subfraction. The CE3 showed moderate but significant down regulation of the cytokine expressions. Thus all three bioactive subfractions were capable of reducing cytokine expression. Since these bioactive subfractions were good at down regulating cytokines (proinflammatory genes), they were further tested for their influence on the expression of anti-inflammatory genes and the transcriptional factors.

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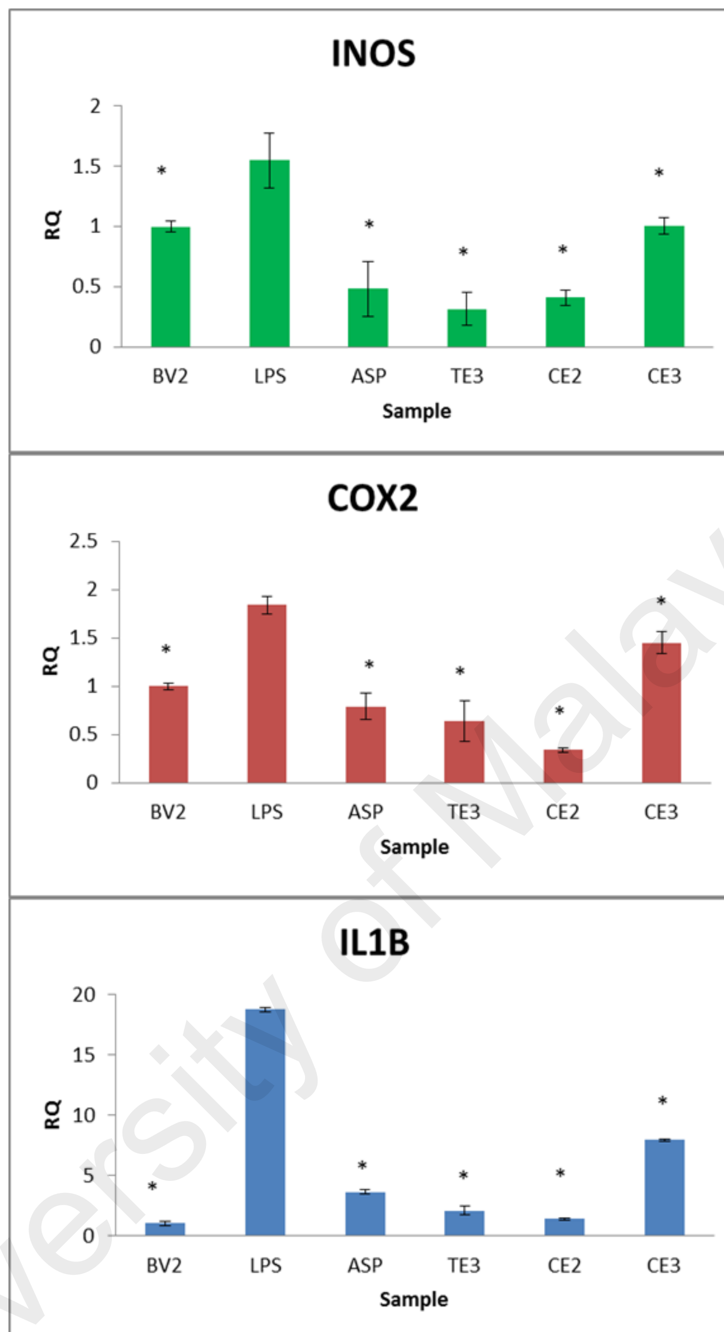


**Figure 4.15: Effect of bioactive subfractions on the proinflammatory gene expression in LPS induced BV2 microglia cells.** Results are expressed as fold of increase compared to LPS treatment as control. The data represent the mean ± SD of experiments carried out in triplicates. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control.

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The effect of the bioactive subfractions on the anti-inflammatory genes namely and HO-1 and NQO-1 was studied on LPS induced BV2 cells to elucidate the signalling pathway of these bioactive subfractions. Each graph in Figure 4.16 is represented by untreated cells (BV2), stimulated cells without treatment (LPS), stimulated cells with aspirin 100uM treatment (ASP) and stimulated cells with bioactive subfraction treatment (10µg/mL treatment concentration) bars. The negative control (LPS) was upregulated but not significant compared to untreated cells (BV2). The positive control (ASP) however was significantly ($p<0.05$) upregulated compared to the negative control (LPS) which indicates the positive control expressed the anti-inflammatory genes.

Heme oxygenase 1 (HO-1) gene is an inducible isoform that is responding to stress such as oxidative stress and cytokines. The effect of the bioactive subfractions on the expression of the gene was investigated. The negative control (LPS) expressed 1.36 folds of HO-1 gene while the untreated cells had 1 fold of HO-1 gene expression. The negative control had an insignificant increase in fold. The positive control (ASP) upregulated the expression of HO-1 gene by 3.32 fold compared to the negative control. The bioactive TE3 and CE2 subfractions showed a significant ($p<0.05$) upregulation in HO-1 gene expression similar to the positive control (Appendix D, Table 23). Both the bioactive subfraction had upregulated the HO-1 gene expression by 3.28 fold and 3.04 fold compared to the negative control. The CE3 subfraction however showed only 0.34 fold of upregulation in HO-1 gene expression and was not significant compared to the negative control.

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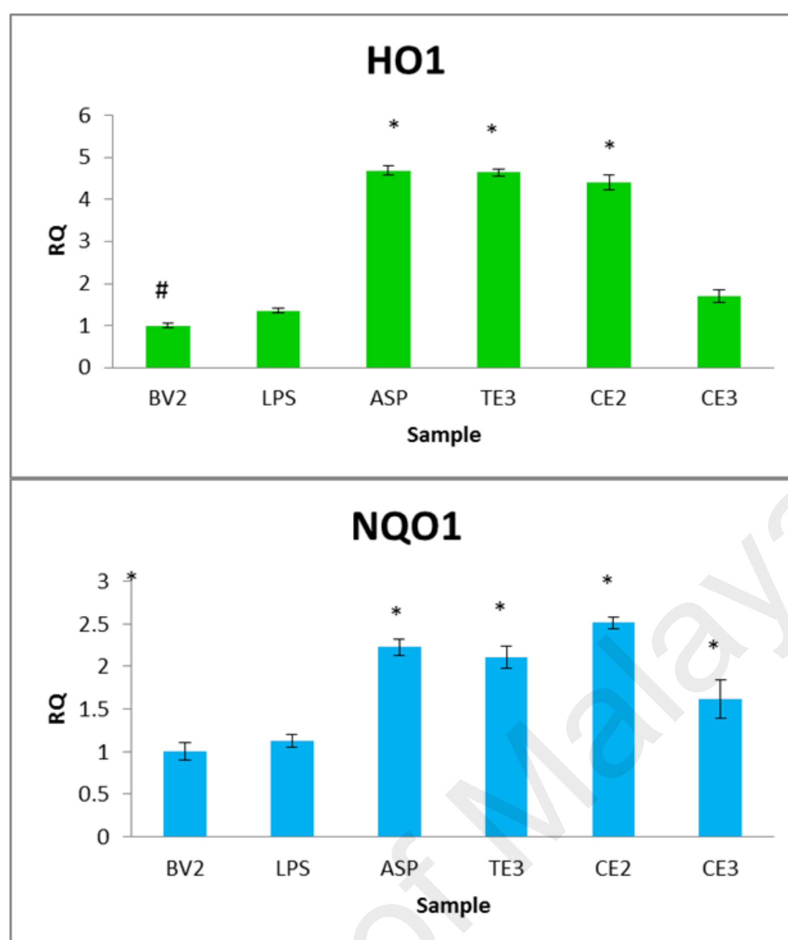


**Figure 4.16: Effect of bioactive subfractions on the anti-inflammatory gene expression in LPS induced BV2 microglia cells.** The data represent the mean ± SD of experiments carried out in triplicates. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control.

The expression of NAD(P)H dehydrogenase [quinone] NQO-1, a FAD-binding protein that reduces quinones to hydroquinones to cytoprotect and inhibit free radical formation was also investigated. The NQO-1 gene expression by the negative control (LPS) was not significantly upregulated compared to the untreated cells (BV2). The NQO-1 gene expression of the positive control (ASP) was 1.11 fold significantly ($p<0.05$) upregulated. The bioactive subfraction TE3 showed an upregulation in NQO-1 gene expression by 1 fold compared to the negative control. The NQO-1 gene expression of subfraction CE2 was upregulated by 1.40 fold compared to the negative control. The CE3 subfraction again had only 0.5 fold increase in the NQO-1 gene expression than the

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negative control. The TE3 subfraction treatment had 0.11 fold lower gene expression than Aspirin treatment. Both the gene HO-1 and NQO-1 were significantly ($p<0.05$) upregulated by two out of three bioactive subfractions (Appendix D, Table 24). Both these genes are the secondary enzyme of NRF2 signalling pathway.

The NRF2 transcriptional gene activates the cytoprotective and antioxidant genes such as HO-1 and NQO-1 while NFκB2 transcriptional gene is activated by the proinflammatory cytokines (iNOS, COX-2 and IL-1β). Effect of bioactive subfractions on the NRF2 and NFκB2 transcriptional factor gene expression in LPS induced BV2 microglia cells were investigated.as shown in Figure 4.18.

A major mechanism in the cellular defense against oxidative or electrophilic stress is activation of the Nrf2-antioxidant response element signalling pathway, which controls the expression of genes that are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity. The effect of nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2, a transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation was investigated with the bioactive subfractions. The negative control (LPS) had 0.2 fold upregulation of NRF2 gene expression compared to the untreated cells. The positive control (ASP) had 1.40 fold upregulation in the NRF2 gene expression. The TE3 and CE2 subfraction treated cells showed a significant ($p<0.05$) upregulation of NRF2 gene expression by 1.49 fold and 1.55 fold respectively (Appendix D, Table 25). Both these subfractions had gene expressions comparable and higher than the positive control. The CE3 subfraction had 0.14 fold insignificant upregulation of the NRF2 gene.

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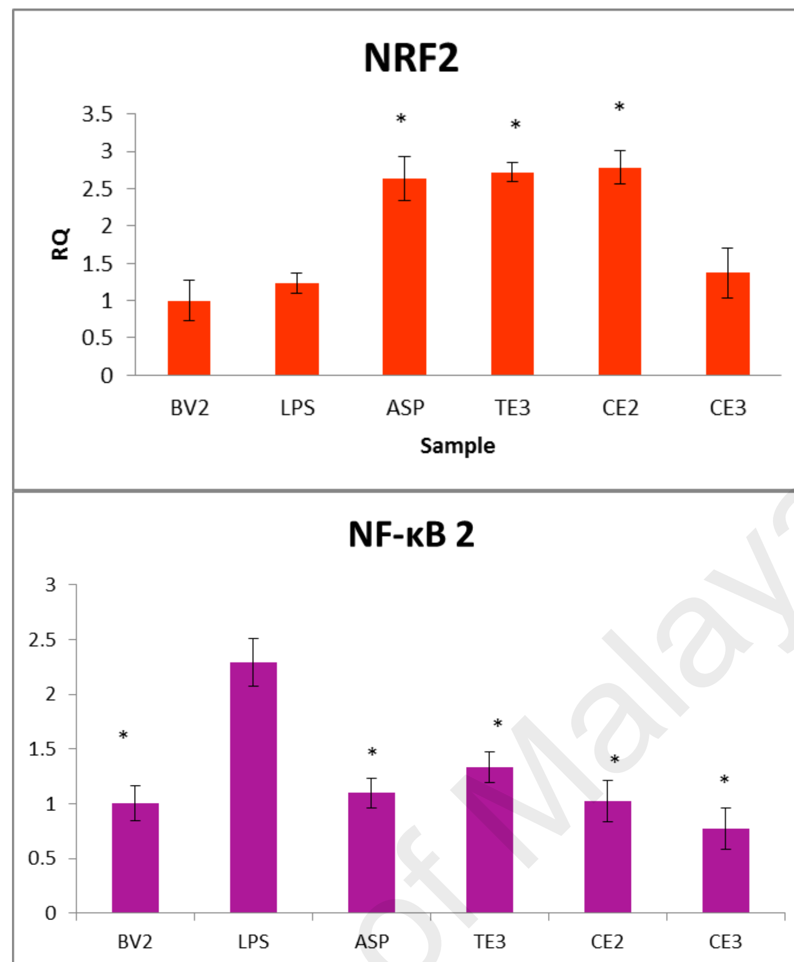


**Figure 4.17: Effect of bioactive subfractions on the NRF2 and NFκB2 transcriptional factor gene expression in LPS induced BV2 microglia cells.** The data represent the mean ± SD of experiments carried out in triplicates. The * symbol denotes a significant difference ($p < 0.05$) when compared to the control.

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NF-kappa B (NF-kB) proteins are eukaryotic transcription factors that are involved in the control of a bulk of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, and apoptosis. In addition, these transcription factors are persistently active in a number of disease states, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases, and heart disease. The NF-kB transcriptional gene was investigated with the bioactive subfraction. The negative control (LPS) had upregulated by 1.29 folds compared to the untreated cells (BV2). Where else the positive control (ASP) down regulated the NF-kB gene by 1.19 folds. It reduced the effect of the negative control by half. All three

subfractions were able to significantly ($p<0.05$) down regulate the NF-kB gene compared to the negative control (Appendix D, Table 26). The TE3 subfraction down regulated the gene by 0.95 fold. The CE 2 and CE 3 subfractions also down regulated by 1.21 and 1.52 folds each respectively.

Based on the gene expression studies of the bioactive subfractions, it is displayed that the TE3 and CE2 subfractions were able to down regulate the proinflammatory cytokines (iNOS, COX2 and IL-1β) and at the same up regulate the antioxidant/anti-inflammatory genes (HO-1 and NQO-1). They also upregulated the NRF2 and down regulated the NFκB2 transcriptional genes. However, the CE3 subfraction was only able to downregulate the proinflammatory genes and NF-kB transcriptional gene; but was not effective in upregulating the NRF2 pathway genes. The TE3 and CE2 subfractions significantly ($p<0.05$) inhibited neuroinflammation via upregulation of genes in NRF2 pathway. All three subfractions, TE3, CE2 and CE3 were also able to suppress neuroinflammation via NFκB2 pathway.

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# CHAPTER 5: DISCUSSION

Mushrooms are consumed globally and are valued not only for the unique taste and flavour but also for their high medicinal and nutritional properties (Manzi *et al.*, 1999). About 100 species of mushrooms are being studied for their health-promoting benefits. Of those hundred, about a half dozen stands out for their ability to deliver a tremendous boost to the immune system. Mushrooms have been intensely used in the traditional Chinese medicine for over 3000 years to impart strength and longevity (Hobbs, 2002). According to the hieroglyphics of 4600 years ago, the ancient Egyptians believed that the mushrooms were plants of immortality and the food of royalty. Other civilisations throughout the world, including Russia, China, Greece, Mexico and Latin America used mushroom in ritual practices (http://mushroominfo.com/history-and-background/#sthash).

Medicinal mushrooms are now gaining wider popularity due to the growing number of scientific studies that confirm the ethnomedicinal knowledge based traditional uses of many fungi; and have found new applications for some fungi. In Malaysia, the Chinese and indigenous communities use many species of mushrooms such as *Amauroderma* sp., *Lignosus rhinocerotis* (Cooke) Ryvarden, *Pycnoporus sanguineus* (L.) Murrill, *Cordyceps militaris* and *Termitomyces clypeatus* (R.) Heim as food and/or medicine (Azliza *et al.*, 2012). Many of these species are used to treat numerous ailments related to inflammation such as fever, cough, cold, epilepsy and asthma.

Uncontrolled production of free radicals may cause oxidative damage and chronic inflammatory related diseases such as atherosclerosis, cancer, rheumatoid arthritis, diabetes, hepatitis, neurodegeneration, and early aging (Uttara *et al.*, 2009). Oxidative

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stress and inflammation are common in a variety of neurodegenerative diseases, including stroke, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Antioxidants are substances that can reduce excess free radicals and prevent oxidative damage in biomolecules, thus preventing the occurrence and reducing the severity of the inflammatory diseases caused by oxidative imbalance (Choi *et al.*, 2006). Inhibition of NO, a free radical produced by activated microglia (Nathan & Xie, 1994) will inevitably inhibit inflammation and inflammation related diseases.

Inflammation can be defined as a general nonspecific but a beneficial response of tissues to injury. It comprises a complex array of adaptive responses to injury which are both local and systemic. The local responses result in recruitment of phagocytic cells and removal of foreign matters. The systemic responses may alter the body functions to allow the processes to occur more efficiently. The cellular processes of inflammations fall into four major groups: changes in blood flow caused by changes in cell function causing vasodilatation, alterations in vascular permeability, migration of phagocytic leukocytes to the inflammation site and phagocytosis (Barbosa-Filho *et al.*, 2006). However, uncontrolled inflammation is undesirable. The reversible features such as pain, redness, heat and swelling are joined by a fifth and less transient feature, loss of function (Wen *et al.*, 2011).

The inflammation in CNS has been appropriately described as a two edged sword. Inflammation in the brain however is characterised by activation of glial cells (mainly microglial and astrocytes) and expression of inflammatory mediators and neurotoxic free radicals; also known as neuroinflammation (Akiyama *et al.*, 2000). Neuroinflammation must be tightly regulated because both deficient and excessive responses will result in pathological disorder. Emerging evidence indicates that neuroinflammation is closely

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associated with neurodegenerative disorders in both acute (stroke, injury) and chronic diseases (multiple sclerosis, Alzheimer's, Parkinson) (McGeer & McGeer, 2004). The usual inflammatory features such as swelling, heat, and pain are not present in the brain. In this state, microglial cells play a crucial role because the uptake of myelin during damage enhances regeneration and repair in CNS. Within CNS, microglial may act as antigen presenting cells and neuron themselves may promote immune activation. Activated microglial were observed in neurodegenerative disorders (Neumann *et al.*, 2009).

*Lignosus rhinocerotis* and *Cordyceps militaris* are both medicinal mushrooms used by indigenous people in Southeast Asia to cure inflammation related ailments for the past few decades. In this study, both these mushrooms were selected based on their folklore medicinal value and to identify the content that is helping to cure inflammation. *Lignosus rhinocerotis* known as tiger milk mushroom is grown in a small tropical region. It is grown in solitary and is an attempt to domesticate the cultivation. *Cordyceps militaris* is known as caterpillar fungus or winter worm summer grass (in Chinese language). This mushroom is native to China and currently is cultivated worldwide for its medicinal properties. Therefore, in this study chemical components of *L. rhinocerotis* and *C. militaris* were identified and evaluated for its biological activity on antioxidant and inhibition of nitric oxide production activities. Identification of active principles that are responsible for the anti-inflammatory properties of these mushrooms were thus attempted. The gene expressions of the potential active principle were tested to determine the mode of action against inflammatory pathways.

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## 5.1. Aqueous and crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies

The aqueous extract of *L. rhinocerotis* and *C. militaris* yielded 31% from the initial freezed dried mushroom powder. While the *L. rhinocerotis* and *C. militaris* ethanol extracts yielded 9% and 26% respectively. Similar results were shown by Lee *et al.* (2014) with *L. rhinocerotis* aqueous extract (37%) and ethanol extract (5%). The yield in our finding is 20% higher compared to *L. rhinocerotis* hot aqueous extract (LR-HA, 11.3%) which were collected in the wild (Lau *et al.*, 2013). The boiling time was 60 mins at 120°C in Lau *et al.* (2013) whereas in this study, the aqueous extraction was done at 100°C but only for 30 mins. The boiling temperature and time may have played a part in the accumulation of the extract yield. The ethanol extract of *C. militaris* displayed a high yield equivalent to that of the aqueous extract. The result in this study was 6 times higher than the yield percentage reported by Won and Park (2005). This is one of the highest extraction efficiency presented for *C. militaris* ethanol extract. The yield differences could have been due to variation in geographic, environmental, nutrients, soil and weather conditions between the cultivated and those collected in the wild.

These extracts were screened for their antioxidant properties via the DPPH radical scavenging activity, trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP). The ethanol extract of *L. rhinocerotis* displayed better antioxidant activity compared to its aqueous extract but the activity was moderate when compared to a reference extract. Li *et al.* (2010) also reported that the ethanolic extract from the *Coprinus comatus* mushroom possessed higher antioxidant activity compared to a hot water extract. Even the results of a study by Elmastas *et al.* (2007) indicated that the methanolic extract of some wild edible mushrooms has significant



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antioxidant activity against various antioxidant systems *in vitro.* A study was done on five *Agaricus* mushroom species; *A. bisporus*, *A. arvensis*, A. romagnesii, *A. sivaticus* and *A. silvicola* on its antioxidant activities. The results indicated the DPPH radical scavenging of these mushroom species had $EC_{50}$ of 5-15mg/ml and reducing power with $EC_{50}$ 2-4 mg/ml (Barros *et al.*, 2008), which is comparable to the results in this study. The various antioxidant mechanisms of the extract may attribute to the scavenging and reducing ability.

On the other hand, the *C. militaris* aqueous extract displayed better antioxidant activity than its ethanol extract. This could be because most of the bioactive compounds which are water soluble compared to the ethanol soluble extract even though both the extract had similar yield. The DPPH activity of *Lentinus edodes* (55.4%) and *Volvariella volvacea* (46%) showed a similar pattern with the current study, the water extract displayed higher activity compared to its methanol extract (Cheung *et al.*, 2003). Between the mushrooms, the *C. militaris* aqueous extract presented the best antioxidant activities. This is consistent with many previous studies done on *in vitro* antioxidant activity of *C. militaris* aqueous extract that has been reported especially the polysaccharide extracts (Chen *et al.*, 2003; Yu *et al.*, 2007; Wang *et al.*, 2012). The *C. militaris* ethanol extract also has 31% DPPH scavenging activity at 5mg/mL. This is higher than the methanolic extract of *C. militaris* $EC_{50}$ value (12.17 mg/mL) stated by Reis *et al.* (2013). Pereira *et al.* (2012) also reported the antioxidant activity of the methanol extract of twenty different Portuguese wild edible mushrooms species revealing $EC_{50}$ values for DPPH radical-scavenging activity ranging from 20.02 to 0.68 mg/ml. Some indigenous edible mushrooms also displayed good antioxidant activity of both water and solvent extract, whereby certain species showed better activity in the solvent extract while others had better activity in the water extracts compared to their



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counterparts (Puttaraju *et al.*, 2006). This justification corresponds to the results shown in this study. These extracts were further tested for its anti-inflammatory effect on the BV2 microglial cells.

Glia is composed of distinctive cell types named microglia, astrocytes and oligodendrocytes (Teismann & Schulz, 2004). Microglia is the resident macrophages of the central nervous system and is associated with neurodegeneration and brain inflammatory diseases. Numerous studies reported that the microglial may have entered the brain during embryonic or early postnatal (Barron, 1995; Ginhoux *et al*., 2010). However, in the matured and aging brain, microglial plays a role of surveillance and host defense while maintaining homeostasis as they are very sensitive to their microenvironment. Microglia promptly reacts to subtle alteration in the microenvironment such as alterations in ion homeostasis and brain insults (ranging from aggregated proteins to pathogen) with cytokines, toll like receptors and chemokines receptors (Kreutzberg, 1996). These cells are generally quiescent in the normal brain, their cell bodies barely visible and few detectable fine ramified processes. However the resting microglia cells can quickly proliferate, become hypertrophic and persistently increase expression of many marker molecules as a result of infection and/ or injuries (Liu & Hong, 2003). Various triggers are involved in microglia activation including immunological trigger, neurotransmitter, endogenous disease protein and neuronal injury. The most accepted and widely used are endotoxins that induce a strong neuroinflammatory response in BV2 cell (Ko *et al.*, 2010). The *in vivo* observations support the neuroprotection and regenerative role of microglial in the CNS (Streit, 2002). But there are also other studies stating that the microglial may also cause further damage to the injured CNS. This is commonly claimed because the overproduction of activated

microglial produces majority of proinflammatory and neurotoxic factors (McGuire *et al.*, 2001).

In this study, the effect of the extracts towards reducing the NO production due to overproduction of microglial namely BV2 cells was studied to curb the inflammatory response towards the cells. Prior to analysing the anti-inflammatory properties of the extracts, the cytotoxic effect of the extracts on the cells was tested to make sure the extract itself do not inflame the cells. The aqueous and ethanol extracts of *L. rhinocerotis* and *C. militaris* had no significant ($p<0.05$) cytotoxic effect on BV2 cells up to 100 μg/mL concentrations except for 1000 μg/mL. This can be due to the oversaturation of compounds that enables the compounds to react with the cells. At 10 μg/mL treatment, 4-10% increase in cell number was observed in BV2 cells for both the mushroom extracts. Lee *et al.* (2012) demonstrated that the *L. rhinocerotis* cold water extract (LR-CW) did not show significant cytotoxic effect on human normal breast and lung cell lines (184B5 and NL 20) at concentration ranging from 15.6 to 1000 μg/mL. However, anti-proliferative activity against both MCF-7 and A549 cancer cell lines was exhibited. Hendra *et al.*, 2011 also reported that the mesocarp and pericarp of *Phaleria macrocarpa* extracted with methanol extract at concentrations up to 200 μM showed more than 90% cell viability of RAW 264.7 cells. At the concentrations 50–200 μg/ml used in an article by Jung *et al.* (2009), the ethanol extract of *Ecklonia cava,* a type of brown algae did not affect cell viability of BV2 cells. The concentrations used in the previous studies were similar to the range of concentration used in the present study. Thus the 0.1-100 μg/mL concentrations were used to determine the anti-inflammatory effects on the BV2 cells.

The aqueous extract of *L. rhinocerotis* and *C. militaris* did not inhibit NO production at concentrations 0.1 μg/mL-100 μg/mL, even though both extracts had better



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antioxidant activity compared to the ethanol extracts and did not exert any significant impairment on the viability of the BV2 cells. This may be because the compounds with antioxidant activity in the aqueous extract were not able to scavenge the NO production in the cells. The compounds in the aqueous extract are highly polar and maybe big in size, thus they are not able to pass through the brain microglial cells in order to inhibit the inflammatory response in the cells. The result obtained in this study is in contrast to numerous studies done with various cell lines in the past (Won & Park., 2005; Jo *et al.*, 2010; Eik *et al.*, 2012; Lee *et al.*, 2014).

There was a gradual decrease in nitric oxide production in the *L. rhinocerotis* ethanol extract treated BV2 cells from 1 μg/mL but at 100 μg/mL dose concentration, the ethanol extract of *L. rhinocerotis* significantly ($p<0.05$) inhibited NO production (>60%) compared to the control. The ethanol extract of *C. militaris* reduced NO production by 60% at 10 μg/mL and there was almost no nitric oxide production at 100 μg/mL similar to unstimulated cells with 0.23%. The ethanol extracts of both *L. rhinocerotis* (100 μg/mL) and *C. militaris* (10 μg/mL, 100 μg/mL) at respective concentrations have shown better NO reduction compared to the commercial L-NAME (200 μM concentration).

About 260 kinds of ethanolic extract of plant samples from Jeju Island were tested for their effects as an alternative agent for the treatment of inflammatory diseases accompanied by overproduction of NO. Among the extract, 122 extracts showed potent inhibitory activity against NO production with minimum 25% inhibition at 100 μg/mL concentration against RAW 264.7 cells (Yang *et al.*, 2009). The methanol extracts of *Antrodia camphorate* and *Cinnamomum osmophleum* at 50 μg/mL and 60 μg/mL inhibited NO by 69.5% and 67.2% respectively in LPS activated mouse peritoneal macrophages (Rao *et al.*, 2007). Both of these species are active oriental herbal medicine

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used in various inflammatory diseases similar to the species used in this study. However the *L. rhinocerotis* and *C. militaris* used in this study showed similar inhibition percentage at 5/6 fold lower concentrations. Thus both these mushrooms are more potent than *A. camphorate* and C. *osmophleum*. Many other reports also suggest that solvent (ethanolic / methanolic) extract of mushrooms and plants have the ability to inhibit NO production (Conforti *et al.*, 2008; Nitha *et al.*, 2010; Oskoueian *et al.*, 2011; Debnath *et al.*, 2013).

In conclusion, the data and the supporting explanation suggest that the ethanolic extracts of these two mushroom species have the ability to suppress NO production in cell lines. These mushroom species deserve to be further investigated in order to isolate bioactive secondary metabolites with anti-inflammatory properties. The ethanol extract of *L. rhinocerotis* and *C. militaris* that demonstrated inhibitory effects against NO production are good precede for activity guided isolation of active fractions to further investigate in order to identify and characterise them. The ethanol extracts of these mushrooms were further fractioned with hexane and ethyl acetate solvents yielding hexane and ethyl acetate fractions, which were used to test the anti-inflammatory activities.

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## 5.2. Bioassay guided fractions from crude ethanol extracts of *Lignosus rhinocerotis* and *Cordyceps militaris* and their *in vitro* studies

The *L. rhinocerotis* ethanol extract produced 16.79 g (7.3%) of hexane fraction and ethyl acetate fraction 9.93 g (4.3%). Fractionation of the ethanol extract of *C. militaris* yielded 20.01g (8.4%) of the hexane fraction and 9.81g (4.1%) of the ethyl acetate fraction. It can be deduced that the ethanol extract has less than 20% of low and mid polar constituents as these are usually present in the hexane and ethyl acetate extracts respectively. Fractionation of ethanol extract of *Sargassum micracanthum* by hexane and ethyl acetate gave 12.1% and 1.0% of hexane and ethyl acetate fractions respectively (Yoon *et al.*, 2009). These results are consistent with the results obtained in the current study whereby the ethyl acetate fraction yielded lower than the hexane fraction. The ethyl acetate fraction of *Lentinus edodes* (shiitake mushroom) and *Volvariella volvacea* (straw mushroom) also yielded similar results to that found in the current study with 6.0% and 1.0% respectively (Cheung & Cheung, 2005).

The ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* had better scavenging and reduction activity compared to their hexane fractions. Between the ethyl acetate fractions, the *L. rhinocerotis* ethyl acetate fraction had higher activities compared to *C. militaris* ethyl acetate fraction. Overall the scavenging ability was about 7-25% and the reduction ability was 13-62 mg FE/g. These activities indicate that both mushroom's hexane and ethyl acetate have relatively low antioxidant activities compared to other extracts such as grape seed extract and coriander extracts which are high in antioxidant properties (Jayaprakasha *et al.*, 2001; Wangensteen *et al.*, 2004). Generally, the crude extract (ethanol / methanol) gives better antioxidant activities compared to its fractions

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such as hexane and ethyl acetate. The observations of our study corroborates well with those reported previously (Shon *et al.*, 2003; Ganesan *et al.*, 2008).

The study conducted on white, red and yellow onion showed 2-3 times higher DPPH radical scavenging activity with 40-50% inhibition at 1mg/mL concentration of ethyl acetate extract. The *L. rhinocerotis* and *C. militaris* ethyl acetate extract were only able to inhibit DPPH radical scavenging activity by 22% and 10% respectively. The reducing power on the other hand showed similar absorbance value (0.6-0.7) at 5mg/ml ethyl acetate extract concentration (Shon *et al.*, 2004). It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada *et al.*, 1992). The reducing power of the ethyl acetate fraction of both mushrooms may have produced high amounts of reductones, which could react with free radicals to stabilize and block radical chain reactions. The radical scavenging activity screening of 12 medicinal and aromatic plants has been reported and 80% of the ethyl acetate fraction of plants tested resulted in 2.5-34% DPPH inhibition (Miliauskas *et al.*, 2004). These results are consistent with the results obtained in the current study. Thus the fractions were further tested for their anti-inflammatory activities.

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Inflammation is a naturally occurring reaction in the body in response to trauma, infection and tissue injury (Choy *et al.*, 2008). Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, overproductions of activated microglial may lead to neuronal death and brain injuries (Fontana *et al.*, 2010). The activated cells also increase the secretion of various pro-inflammatory mediators such as nitric oxide (NO) and cytokines. Nitric oxide plays essential roles in mammalian life. Nitric oxide, a short-lived free radical produced from

L-arginine by nitric oxide synthase (NOS), mediates (Salerno *et al*., 2002) a variety of pathophysiological actions ranging from vasodilatation, neurotransmission, inhibition of platelet adherence and aggregation, as well as the macrophage- and neutrophil-mediated killing of pathogens (Oh *et al*., 2008). Unregulated production of NO can cause nitrosative stress leading to damages of protein/ DNA and cell injury/ death (Murphy, 1999). Excess NO production in the cells may be caused by a higher promoter activity of iNOS and progress to also induce COX2 in various *in vitro* and *in vivo* models causing chronic inflammation (Lu *et al.*, 2006). In the last few decades, evidence suggests that excessive NO production may play a role in neurodegenerative diseases. Therefore, inhibition of proinflammatory mediator(s) is beneficial in attenuating an inflammatory disorder. NO is rapidly oxidised to nitrite or / and nitrate by oxygen. The measurement of nitrite concentration is routinely used as an index of NO production. It is a widely used practice to measure the nitrite concentration by well-known method such as Griess assay (Green *et al.*, 1982).

The hexane and ethyl acetate fractions of *L. rhinocerotis* treated BV2 cells showed a gradual reduction of NO from 1μg/mL to 100 μg/mL but the hexane fraction did not significantly ($p < 0.05$) reduce NO in the cells. The ethyl acetate fraction *L. rhinocerotis* treated BV2 cells, however, showed a gradual significant ($p < 0.05$) dose dependent reduction of nitric oxide percentage of about 37% and 58% at 10 μg/mL and 100 μg/mL concentration of ethyl acetate fraction respectively. The cells treated with hexane and ethyl acetate fractions of *C. militaris* displayed a significant ($p < 0.05$) gradual reduction in nitric oxide from 0.1 μg/mL and 100 μg/mL treatment concentration. Nevertheless the ethyl acetate treated BV2 cells significantly ($p < 0.05$) reduced nitric oxide production by 14%, 48% and 97% at treatment concentrations 1 μg/mL, 10 μg/mL and 100 μg/mL which was better than the hexane fraction treated cells

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which displayed reduction of nitric oxide only at concentration of 10 µg/mL (32%) and 100 µg/mL (98%). At 100 µg/mL, the NO reduction was comparable to that of the ethyl acetate treated cells. The ethyl acetate fraction of *L. rhinoceortis* and *C. militaris* had better NO reduction compared to its hexane fraction. Thus the ethyl acetate fractions were further investigated.

A study done on *Tricholoma matsutake* Sing (pine mushrooms) showed that the ethyl acetate fraction exhibited the highest inhibition (61.6%) of NO by BV2 cells at 2mg/ml concentration (Lim *et al.*, 2007). The findings in this study showed higher NO inhibition at lower concentration compared to the study by Lim *et al.*, (2007). Further, *Houttuynia cordata*, a traditional plant used as folk medicine for treating several ailments including allergic inflammation and anaphylaxis also showed that HC-EA (ethyl acetate fraction) inhibited the LPS-stimulated increase of NO release by BV2 cells in a concentration-dependent manner. The fraction was able to inhibit approximately 66% at 80 µg/mL fraction concentration (Park *et al.*, 2013). The *L. rhinocerotis* ethyl acetate fraction displayed a similar reduction in NO at lower concentrations (58%, 10 µg/mL). The ethyl acetate fraction of *C. militaris* was able to suppress NO production in BV2 cells almost 30% more than HC-EA. Extracts from the flower of *Coreopsis tinctoria* Nutt. has shown to possess anti-neurodegenerative properties in folklore, also known as “snow chrysanthemum” or “snow tea”. The ethyl acetate extract of *C. tinctoria* on NO production by LPS activated N9 microglia were found to be effective because of their significant anti-neuroinflammatory activities with the $IC_{50}$ value at 9.26 ± 1.54 µg/mL (Li *et al.*, 2015), similar to the findings in our study which showed 48% NO reduction at 10 µg/mL concentration of extract. Many more studies done previously have reported the ethyl acetate fraction’s potential to reduce NO production; similar to the ones found in this study (Kim *et al.*, 2013; Cho *et al.*, 2013; Kang, 2014; Kang, 2015).



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A significant number of reports have established that inflammatory mediators, including NO and iNOS are responsible for the symptoms of many neuroinflammatory diseases. It is well documented that microglia produces NO in response to pro-inflammatory stimuli, LPS. The induced NO by the induction of iNOS will lead to increased inflammatory reaction. Therefore, agents that decrease NO production have appreciable therapeutic effect in the treatment of several neuroinflammatory diseases. Our results clearly showed that the ethyl acetate fraction attenuated LPS-induced NO production and hence suppresses inflammation. Thus the ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* were analysed further via GCMS for the chemical constituents of potential bioactive compounds.

Ten chemical constituents were identified in the ethyl acetate fraction of *L. rhinocerotis* comprising 75.98% of the detected constituents. The constituents are mainly made up by fatty acids (58.67%), fatty acid ethyl esters (16.5%) and a trace of sterols (0.81%). The major contents were linoleic acid and oleic acid amounting to 31.09% and 22.42% respectively in the ethyl acetate fraction of *L. rhinocerotis*. These essential unsaturated fatty acids cannot be synthesised in mammalian tissue, therefore, must be obtained from the diet (Burr & Burr, 1930). Linoleic acid (omega-6-fatty acid) promotes cognitive health and supports memory, healthy neurological function and the body's normal inflammatory response (Galli *et al.*, 1994). Oleic acid that occurs naturally in various plant and vegetable fat and oil, is well known to reduce cholesterol and blood sugar as well as to hinder the progression of adrenoleukodystrophy (ALD), a fatal disease that affects the brain and adrenal glands (Rizzo *et al.*, 1986). Beneficial physiological effects of these fatty acids and their derivatives include inhibition of chemically induced carcinogenesis, enhancement in immune response, reduction in catabolic effects of



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immune stimulation, reduction of atherosclerosis, growth enhancement and body fat reduction in many rodents, animals and even in humans (Pariza *et al.*, 2001).

There have been many studies on the biological effects of these fatty acids and there is growing evidence that they modify neuronal membrane structure and functions (Raz & Livine, 1973). Low concentrations of several unsaturated fatty acids such as oleic, linoleic and linolenic acid may cross the blood brain barrier (BBB) (Betz, 1991), as had been explained by Edmond (2001) on the transportation mechanism of certain unsaturated fatty acid, as it progresses from the blood into the brain especially during infancy and in aging. It is also reported that structural changes occur in the BBB complex, in Alzheimer's patients due to fatty acid intake. These components penetrate into the cells to reduce NO production of glial cells by down regulating the expression of iNOS.

Based on the results of several *in vitro*, *in vivo* and *ex vivo* studies, growing evidence revealed that omega-6 fatty acids have anti-inflammatory effects that are likely to be relevant to neurodegenerative diseases. The anti-inflammatory effects of omega-6 PUFAs include competitive inhibition of arachidonic acid, the metabolites of which are involved in promoting inflammation (Callegari & Zurier, 1991; Gil, 2002; Namazi, 2004). In addition, the production of anti-inflammatory prostaglandins $E_1$ and $E_2$, which are derived from the omega-6 PUFA dihomo-$\gamma$-linolenic acid, can inhibit the production of proinflammatory cytokines such as interleukin (IL)-2 and interferon γ (IFN-γ) (Santoli & Zurier, 1989; Mertin *et al.*, 1985). A study demonstrated that linoleic acid and oleic acid suppressed the release of proinflammatory mediators NO and PGE2 as well as the expression of iNOS and COX-2 in LPS-stimulated BV2 microglia (Oh *et al.*, 2009). An isomer of linoleic acid, the conjugated linoleic acid also has resulted in reducing anti-



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inflammation via NF kB (Cheng *et al.*, 2004). Based on previous reports, it is shown that the polyunsaturated fatty acid content in the ethyl acetate fraction of *L. rhinocerotis* evidently reduced the inflammation in LPS stimulated BV2 cells.

A total of 81.28% of chemical components were identified in the ethyl acetate fraction of *C. militaris* via GCMS. The major components in the ethyl acetate fraction of *C. militaris* were sterols amounting to 72.78% and minor components of fatty acid and ethyl esters (8.5%) present. The major constituent is ergosterol, which represents 68.17% of the identified constituents. Ergosterol is a white crystalline organic solid belonging to the steroid family and is commonly found in prokaryotic membranes and lower eukaryotes like fungi (Arora *et al.*, 2004). This compound is structurally similar to cholesterol; present in fungi but absent in animals (Czub & Baginski, 2006). It can be converted by ultraviolet irradiation ultimately into vitamin $D_2$ (Rajakumaran *et al.*, 2007). This happens naturally to a certain extent, and many mushrooms are irradiated after harvest to increase their Vitamin D content. Fungi are also grown industrially so that ergosterol can be extracted and converted to Vitamin D for sale as a dietary supplement and food additive. Ergosterol is an important source of Vitamin D as it is able to activate expression of a number of defense genes and increase the resistance against the pathogens. Therefore, ergosterol-containing foods and supplements provide an important nutrient (vitamin D) (Holick, 2004).

Studies were done to validate the benefits of ergosterol as an anti-inflammatory agent (Hausman & Varanasi, 2012; Kuo *et al.*, 2011; Yazawa *et al.*, 2000; Akihisa *et al.*, 2007; Yasukawa *et al.*, 1994). An article by Ma *et al.* (2013) reported that isolated ergosterol from the ethyl acetate fraction of *Inonotus obliquus* mushroom was found to have significant inhibitory effects on NO production and NF-κB luciferase activity in



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macrophage RAW 264.7 cells. Ergosterol identified in *Agaricus blazei* Murill has also reported antitumour properties by inhibiting the Lewis lung carcinoma tumour-induced neovascularization and Matrigel-induced neovascularization at doses of 5- 20 mg/kg (Takaku *et al.*, 2001). Ergosterol isolated from *S. aspratus* (Berk) S. Ito repressed LPS induced TNF-α by RAW 264.7 cells by more than 50% at 60 μM without decreasing cell viability (Kobori *et al.*, 2007). These details indicate that the major compound ergosterol in the ethyl acetate fraction of *C. militaris* may well be responsible for lowering the nitric oxide production in the treated BV2 cells.

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### 5.3. *In vitro* anti-inflammatory and gene expression studies of *Lignosus rhinocerotis* and *Cordyceps militaris* bioactive subfractions

Based on the anti-inflammatory results of the fractions, the ethyl acetate fraction of *L. rhinocerotis* and *C. militaris* both were individually fractioned with vacuum column chromatography. All the subfractions were screened for their antioxidant, cell viability and anti-inflammatory properties. The best three subfractions out of both the mushrooms were identified and selected to be analysed in detail. The antioxidant and anti-inflammatory properties were further investigated. The effects of the bioactive subfractions on the expressions of genes of inflammatory pathways were also studied.

The antioxidant properties of the bioactive subfractions were good. The DPPH scavenging ability of the subfractions was 56-58%. The subfractions were able to inhibit 50% of the scavenging radicals at 5 mg/mL. The ABTS scavenging ability was low with percentages of inhibition ranging from 1-37%. The CE3 subfraction showed 37% inhibition while the other two subfractions TE3 and CE2 were only 7% and 1%. The ferric reducing power of TE3 and CE2 were moderate with 420 and 315 mg FE/ g extract and the CE3 had low FRAP properties with 6 mg FE/ g extract. The radical scavenging ability of the bioactive subfractions is as good as the crude extracts This shows that the identified components in the bioactive fractions may be also responsible of the antioxidant activity in the crude extract. Some bioactive subfractions in this study presented better radical scavenging and reducing power compared to the crude extract and ethyl acetate fraction of *L. rhinocerotis* and *C. militaris*. This deduction was verified by the result of a previous article by Fernandes de Oliveira *et al.* (2012). The antioxidant activities of crude ethanol extract and fractions of the four species of Malvaceae family: *Sidastrum micranthum, Wissadula periplocifolia, Sida rhombifolia* and *Herissantia*

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*crispa* were presented. These species are utilized in folk medicine for anti-inflammatory, diuretic, arthritis, gastrointestinal disorders and asthma (Franzotti *et al.*, 2000; Yesılada *et al.*, 2003). The scavenging ability of the crude extract of these four species was reported to be in the range of $IC_{50}$ = 114-127 mg/mL where else the ethyl acetate fraction had $IC_{50}$ of 20-148 mg/mL. The $IC_{50}$ values of TEAC of the crude and ethyl acetate fraction were 36-81 mg/mL and 2-83mg/mL. Two bioactive compounds isolated also were tested to compare its effectiveness as antioxidant agent. Based on the results, it is shown that the bioactive compound did not display better DPPH activity compared to the crude and fraction. For TEAC assay, the compounds exhibited a low $IC_{50}$ value (lower than the crude and fraction). The antioxidant activity of the subfractions in the current study also displayed similar trend as the spices extracts.

A previous study carried out by Barros *et al.* (2007) showed antioxidant activity of the fruiting body of the wild mushroom, *Lactarius piperatus* at different maturity stages. It is shown that the mature fruiting bodies (cap opened) with immature spores (Stage II) had high antioxidant properties. The antioxidant activities increased with the concentration of the extract. The $EC_{50}$ of DPPH activity was 5.19 mg/ml more or less the same with the current study, whereby the $EC_{50}$ is below 5mg/ml. The reducing power, however in this study was lower compared to the study done by Barros *et al.* (2007) as their $EC_{50}$ was 2.29 mg/ml. This report supports the antioxidant results presented in the current study. A study by Babbar *et al.* (2011) showed that phenolic compounds alone are not fully responsible for the antioxidant activity. Other constituents such as ascorbates, tocopherols, carotenoids, terpenes, and pigments as well as the synergistic effect among them could possibly contribute to the total antioxidant activity. The synergistic effects among non-phenolic components in the bioactive subfractions of the current study may have contributed to the high antioxidant activity. The follow up MTS

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and NO assays were carried out to determine the anti-inflammatory effect of the bioactive subfractions.

The cytotoxicity effects of the bioactive subfractions on the BV2 cells were evaluated prior to anti-inflammatory activity. The bioactive subfractions tested individually without and with LPS stimulation did not exert any cytotoxic effects towards the cells in MTS assay. The bioactive subfractions were not only non toxic but also were able to protect the BV2 cells from the LPS stimulus. Thus the bioactive subfractions from *L. rhinocerotis* and *C. militaris* have good prospects of being neuroprotective agents that maybe helpful in many inflammation related medical conditions. Bioactive compound such as 1-docosanoyl cafferate isolated from *Rhus verniciflua* was not cytotoxic to BV2 cells in MTT assay up to 50 µM concentration (Lee *et al.*, 2011). While the cytotoxic effects of cilostazol (a drug used in the treatment of ischemic symptoms) in BV2 cells with treatment dosage up to 30 µM didn't affect the cell viability in the absence and presence of LPS stimulant via MTT assay (Jung *et al.*, 2010). Similarly, Tan *et al.* (2011) reported that palm α, γ and δ tocotrienol didn't affect the cell viability of BV2 stimulated with and without LPS up to 50 µM concentration. Besides these reports, synthetic hydroxypropenone showed no cytotoxic effect in RAW 264.7 and U937 cells up 25 µM (Liew *et al.*, 2010). All these bioactive compounds presented high cell viability and were able to inhibit NO production simultaneously by the macrophages and microglia cells. Thus, the bioactive subfractions in this study were further assayed to determine its anti neuroinflammatory effects on the cell line.

The TE3, CE2 and CE3 subfractions exhibited strong anti-inflammatory activities by significantly decreasing NO production. This result corresponds to previous studies done on anti-inflammatory and anti-neuroinflammatory activity of mushrooms and



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plants. The IOE70 extract of *I. obliquus* reduced NO production in RAW264.7 cells with 25 to 200 µg/mL treatment concentrations (Kim *et al.*, 2007). The results of this study has a similar pattern of NO reduction compared with *Ganoderma lucidum* terpene extract (GLT) which significantly ($p<0.05$) reduced NO levels with the $IC_{50}$ being 11.4 µg/mL (Dudhgaonkar *et al.*, 2009). The anthocyanins from black soy bean seed also demonstrated reduction of NO in the RAW 264.7 cells between 50-100 µg/mL (Jeong *et al.*, 2013). At 10 µM concentration, oleamide significantly inhibited NO production in BV2 cells (Oh *et al.*, 2010) while palmitic acid inhibited NO in peritoneal macrophages (Lee *et al.*, 2010).

On the contrary, the TE3 and CE2 subfractions inhibited NO production at a lower concentration (1 µg/mL) and CE3 fraction (10 µg/mL) better than these individual compounds. This suggests that the bioactive subfractions work better in reducing inflammation than its individual compounds. This may be caused by the combination of the components in the bioactive subfractions. These subtractions exhibited significant anti-inflammatory activity in acute and chronic inflammation *in-vitro* / *in-vivo*. This effect is probably mediated through its significant antioxidant activity.

The relative peak area percentage (based on the GCMS chromatogram) of lipid constituents of TE3, CE2 and CE3 subfractions of ethyl acetate extract is presented in Table 4.7. The TE3 subfraction had a total of 69.28% identified lipid constituents comprising eight compounds. Where else the CE2 subfraction had eleven identified compounds (total of 73.83%) and CE3 subfractions had 98.02% identified lipid constituents containing three compounds.

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Two fatty acids were identified in TE3 subfraction: linoleic acid, an unsaturated fatty acid (45.35%) and palmitic acid, a saturated fatty acid (2.37%). Besides fatty acids, two fatty acid amides, oleamide (7.12%) and palmitamide (3.50%) were identified in this fraction. The phenolic components, 4-hydroxy benzaldehyde and its analogue 4-hydroxy-3-methoxybenzaldehyde (vanillin) were 3.12 % and 1.51% respectively. Other than that, 9, 17-octadecadienal (3.36%) and dehydroergosterol (2.96%) were detected in TE3 subfraction.

Out of the eleven compounds identified in the subfraction CE2, three were fatty acids: caprylic acid (0.2%), palmitic acid (7.82%) and linoleic acid (43.41%); Five compounds were fatty esters comprising of methyl palmitate (0.25%), ethyl palmitate (6.00%), ethyl linoleate (10.08%), ethyl stearate (2.23%) and ethyl lignocerate (0.26%) and three sterol components: dehydroergosterol (1.42%), ergone (1.74%) and sitostenone (0.42%) were present.

Three compounds identified in CE3 bioactive subfraction were sterols; dehydroergosterol (2.92%), ergosterol (92.98%) and 22, 23-dihydroergosterol (2.12%). Some of the major compounds identified in these three bioactive subfractions are well known for their anti-inflammatory properties. The combination/ synergistic effect of various compounds with individual anti-inflammatory properties in these bioactive subfractions might be the reason that these subfractions were able to reduce NO production in BV2 cells.

The major fatty acid components linoleic acid and palmitic acid were identified in TE3 (45.34%, 2.37%) and CE2 (43.41%, 7.82%) respectively. Many mushrooms analysed in previous studies have similar content of linoleic and palmitic acids, both



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acids being the most abundant fatty acids (Günç Ergönül *et al.*, 2013). All mushrooms contain large amount of essential fatty acid especially linoleic acid. Essential fatty acids cannot be produced by animals but are required by the body for healing and good health (Wohl & Goodhart, 1968). The results of this study are in agreement with previous reports stating many mushrooms species had high abundance of unsaturated fatty acid especially linoleic acid (40%-70% of total lipid content) (Pedneault *et al.*, 2006; Kalač, 2009). In general, roughly three quarter of fatty acid contents are similar in all mushroom species including this study.

Linoleic acid had shown good anti-inflammatory activity comparable to its derivatives α-linolenic acid and DHA when treated in THP-1 cells (Zhao *et al.*, 2005). As shown in previous reports and mentioned earlier in section 5.2, linoleic acid has the property to be transported across the blood–brain barrier, thus it may be able to exert anti-neuroinflammation in the brain region (Edmond, 2001). Yu *et al.* (2002) showed that linoleic acid reduced the NO production by the LPS activated cells and decreased the IFNγ-dependent expression of inducible NOS (iNOS) and iNOS promoter activity. Linoleic acid was also responsible for the inhibition of COX2 catalysed prostaglandin biosynthesis as shown in *Plantago major* L. (Plantaginaceae) (Ringbom *et al.*, 2001).

Palmitic acid, a saturated long chain fatty acid has also been commonly identified in mushrooms such as *Tripleurospermum* sp., *Gaultheria itoana* and *Sarcopyramis nepalensis* (Chen *et al.*, 2009; Wang *et al.*, 2009; Erdoğan *et al.*, 2015). This fatty acid was previously reported to modulate inflammation response in stimulated peritoneal macrophages by effectively attenuating the production of pro inflammatory cytokines (IL-6 and TNF-α) and suppress the inflammatory mediators NO and $PGE_2$. Hence the

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NO and $PGE_2$ regulator iNOS and COX-2 expressions also reduced in a dose dependant manner (Lee *et al.*, 2010).

Caprylic acid is one of the major fatty acids in the noni fruit (*Morinda citrifolia*). The noni juice has shown good anti-inflammatory activity by rapidly inhibiting the formation of rat paw edema (Chan-Blanco *et al.*, 2006). This fatty acid also showed COX-1 inhibition activity. This may benefit in reducing pain related to inflammation by acting as a strong COX inhibitor (Henry *et al.*, 2002).

Some of the fatty acid esters in the bioactive subfractions had supporting data indicating them as anti-inflammatory agents in various systems. Methyl palmitate inhibits the activation of Kupffer cells (resident macrophages of the liver) rat peritoneal macrophages and RAW 264.7 cells. Treatment with methyl palmitate inhibited phagocytic activity and the NO production in the cells. It also increased the release of IL-10 and IL-6 while reducing TNF-α significantly (Cai *et al.*, 2005; Sarkar *et al.*, 2006; El-Demerdash, 2011). Similarly a study done by Saeed *et al.* (2012) evaluated the effect of methyl palmitate and ethyl palmitate in different experimental rat models. Both the esters exerted anti-inflammatory activity in local, topical and systemic rat models as evidenced by decreasing carrageenan induced rat paw edema, PGE2 content in exudates, plasma TNF-α, IL-6 levels and NF-κB expression in LPS induced endotoxemia rats liver and lung tissues, croton oil ear edema, myeloperoxidase activity, as well as ameliorating inflammatory histopathological changes.

The ethyl linoleate identified in *Amauroderma rugosum* was able to scavenge NO (antioxidant activity) and inhibit NO production in LPS stimulated RAW 264.7 cells (Chan *et al.*, 2013). Ethyl linoleate isolated from garlic (*Allium sativum*) also exhibited



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the ability to inhibit the NF-κB activation and $MAPK_S$ and Akt phosphorylation that mediated the inhibition of LPS induced NO/ iNOS and $PGE_2$/COX-2 expression and pro inflammatory cytokines production (TNF-α, IL-1β, IL-6 and IL-12) while increasing HO-1 that mediates the attenuation of inflammation (Park *et al.*, 2014).

Fatty acid amides, oleamide (7.12%) and palmitamide (3.50%) were also constituents in the bioactive fractions. Oleamide is an amide of oleic acid; found accumulated in the cerebrospinal fluid of sleep-deprived animals. It induces sedation and physiological sleep in animals (Cravatt *et al.*, 1995). This compound has a cannabinoid-like effect by binding G protein-coupled type 2 that is expressed in immune cells including microglia (Fernández-Ruiz *et al.*, 2007). Oh *et al.* (2010) reported that oleamide suppressed LPS induced inflammation by lowering the expression of iNOS and COX-2. Palmitamide, also known as palmitic acid amide, reduced prostate cancer progression (Liu *et al.*, 2011) similar to its derivative palmitoylethanolamide (PEA), that possesses anti-inflammatory and neuroprotective properties (Perlik *et al.*, 1970).

Other than that, the aldehyde components such as 9, 17-octadecadienal, 4-hydroxybenzaldehyde and its analogue 4-hydroxy-3-methoxybenzaldehyde (vanillin) present have been accepted to be medically important by earlier researchers. 9, 17-Octadecadienal is an unsaturated aldehyde, known for its antimicrobial activity (Rajeswari *et al.*, 2013). 4-Hydroxybenzaldehyde and vanillin are major phenolic compounds that are commonly found in *Gastrodia elata* (family of Orchidaceae) and *Vanilla planifolia* (vanilla bean). The 4-hydroxy benzaldehyde showed anti-inflammatory activity by suppressing NO, iNOS and COX2 expressions; and ROS in murine macrophages (Lim *et al.*, 2008). It has also inhibited COX2 expression and silica induced intracellular ROS (Lee *et al.*, 2006). Vanillin is commonly used for its flavour



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and odour in food, beverages and cosmetics. It displays antimicrobial, antioxidant, chemopreventative effects on carcinogenesis models and inhibits mutagenesis and suppresses the progression and migration of cancerous cells (Liang *et al.*, 2009, Akagi *et al.*, 1995, Kumar *et al.*, 2004). Vanillin has also shown anti-inflammatory activity by inhibiting NFκB and COX2 expression (Murakami *et al.*, 2007).

Sterols are important and common constituents of mushrooms. A few sterol identified in the bioactive subtractions have shown potential as anti-inflammatory agents in previously published journals. The identified sterols are dehydroergosterol, ergosterol, 22, 23-dihydroergosterol ergone and β- sitostenone.

Dehydroergosterol is a naturally occurring fluorescent sterol analogue that mimics many of the properties of cholesterol, which was proven to be a major asset for real-time probing/elucidating the sterol environment and intracellular sterol trafficking in living organisms. Recently it has also been identified in Camembert and Gorgonzola cheeses fermented with *Penicillium candidum*. It inhibits cytokines and chemokines in microglial cells and reduced neurotoxicity in Neuro-2A cells (Ano *et al.*, 2015).

Ergosterol is the principal sterol in fungi cell membrane. It usually represents about 70% of the sterols present in fungi and is abundant in edible and medicinal mushrooms, yeast, and wines. Ergosterol is known to be an important pharmaceutical intermediate and a precursor of Vitamin D2 and cortisone. It has been shown that ergosterol isolated from *Chlorella vulgaris*, a type of green algae inhibited the TPA induced inflammation in mice (Yasukawa *et al.*, 1996). Ergosterol isolated from the fruit of *Cantharellus cibarius* showed potent NFκB inhibitory activity in an enzyme based ELISA NFκB assay and inhibition of NFκB translocation from cytoplasm to nuclear in a

University of Malaya

cell based NFκB assay (Kim *et al.*, 2008). Not only ergosterol can inhibit TNF-α and COX-2 expression through the suppression of NFκB signalling, the proteome signature data showed ergosterol induced down regulation of NFκB associated proteins, TANK and Rho GDIS1 indicating it inhibited NFκB activation by regulating the upstream IκB through Rho GTPase modulation of IKK (Kuo *et al.*, 2011). Comparable to ergosterol, the 22,23-dihydroergosterol was also a potent anti-tumour constituent. The compound 22,23-dihydroergosterol is also known as Vitamin $D_4$. Generally Vitamin D plays a role in immunity and blood cell formation besides maintaining blood levels of calcium and phosphorus. Only a few food sources naturally contain Vitamin D such as shiitake mushroom, fish liver oil, fatty fish, egg yolk and butter (Cimpoiu & Hosu, 2007). Ergosterol and 22,23-dihydroergosterol were the major components in the *Flammunila velutipes* sterol mixture. Both sterol components displayed significant anti-tumour activity in glioma cells (U251) (Yi *et al.*, 2013).

Other than these sterols, ergosta-4,6,8 (14), 22-tetraen-3-one or better known as ergone also possesses anti-inflammatory properties based on previous studies conducted. Ergone was first reported / isolated from the fungus *Fomes officinalis*, then from *Ganoderma applanatum, Ganoderma neo- japonicum* and *Zopfiella longicaudata*. This is the first time this compound was identified in the *Cordyceps militaris* fruiting body in the current study. This compound showed suppression of 100% of NO 50 μM and with no cell viability changes up to 100 μM (Ngoc Quang *et al.*, 2008). Other studies have shown ergone exhibiting interesting bioactivities such as antitumour (Lee *et al.*, 2005); and inhibition of COX and antioxidant (Zhang *et al.*, 2002). One of the active compounds, β-sitostenone found in the leaf of *Terminalia phanerophlebian* and the stem of *Leucosidea sericea* has also shown significant anti-inflammatory activity by inhibition of COX-1 and COX-2 enzymes (Nair *et al.*, 2012; Nair *et al.*, 2012).



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Based on the known biological/ medicinal properties of the compounds identified, combinations of these compounds are responsible for the anti-inflammatory activities in these bioactive subfractions. This may be due to the synergistic effect of the combined constituents (Nallathamby *et al.*, 2015). Thus, these bioactive subfractions were further investigated to determine its effect on the cytokines and signalling genes even though many of the compounds identified have shown good results individually.

A physical or pathogenic event in the CNS is expected to elicit activation of glia cells and secretion of ROS and RNS; prostaglandins, chemokines and cytokines. Some of these factors have neuroprotective and trophic activities and aid in the repair process; while others enhance oxidative stress and trigger apoptosis. To resolve the inflammatory responses such as phagocytosis and production of endogenous, mediators with anti-inflammatory properties are needed. These mediators balance or suppress the proinflammatory gene expression in damaged neurons under chronic neuroinflammatory degenerative conditions that are related to the contribution of neuroinflammatory response.

Activation of microglial cells plays an important role in the initiation of inflammatory responses by producing nitric oxide (NO), induced nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), Interleukin-1-beta (IL-1β) and other inflammatory mediators. iNOS is an enzyme catalyzing the production of NO, functions as a neurotransmitter in the immune response while COX2 is responsible for the edema and vasodilation associated with inflammation. The IL-1β cytokine is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. On the contrary, the anti-inflammatory



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HO-1 and NQO-1 genes phase II enzymes cytoprotective proteins decreased in the activated microglia leading to disrupted cellular redox homeostasis against ROS generation and oxidative stress. HO-1 is a cytoprotective, anti-oxidative and anti-inflammatory enzyme by catalysing the degradation of the heme group that produces carbon monoxide, biliverdin, and free iron. NQO1 has multiple roles that includes protection against natural and exogenous quinones by reducing quinones to hydroquinones. Cumulative evidence indicates abnormalities or over-production of these mediators may play an essential role in many chronic inflammatory diseases (De Nardin, 2001). Thus, suppression/ activation of these mediators and their function serve an effective therapeutic strategy for preventing neuroinflammatory reaction that led to neurodegenerative diseases.

In the present study, microglial cells were challenged with LPS (1 ug/ml) in the presence of non-cytotoxic concentrations of the bioactive subfractions and the level of iNOS, COX2 and IL-1β expressions were significantly suppressed compared to non-treated cells (negative control). The subfractions TE3 and CE2 down regulated the expression of the proinflammatory genes even lower than the aspirin treated cells. Aspirin is a non-steroidal anti-inflammatory drug (NSAID) known to exert its effects through inhibition of COX2. The expressions of anti-inflammatory genes; HO-1 and NQO-1 were upregulated by the bioactive subfractions. The upregulation of HO-1 and NQO-1 by the TE3 and CE2 subfractions were comparable but marginally lower than the treatment with aspirin. The CE3 subfraction upregulated the NQO-1 gene but had no effect against HO-1 gene.

Previous studies were reported on the effects of the anti-inflammatory agents against proinflammatory and anti-inflammatory genes. Spermidine, a natural polyamine

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compound, effectively decreased iNOS and COX2 mRNA expression in LPS stimulated BV2 cells (Choi & Park, 2012). The expressions of iNOS and COX2 were down regulated by *A. camphorata* treatment on LPS-stimulated RAW 264.7 cells at >50 µg/mL levels (Hseu *et al.*, 2005). LPS-induced HO-1 and NQO-1 expression in human monocytes (Rushworth *et al.*, 2008) showed an increase of 4-5 folds at 10 µg/mL concentration similar to the results in the current study. The HO-1 protein levels increased 2-3 folds significantly to achieve neuroprotective effect without compromising cell viability following treatment with isothiocynate sulforaphane (Innamorato *et al.*, 2008). In a prior study, xanthohumol attenuated LPS-induced NO release, up regulation of iNOS and NRF2 activation by upregulating phase II enzymes NQO-1 and HO-1 to attenuate inflammation in microglial BV2 cells (Lee *et al.*, 2011).

The study on 3,4,5-trihydroxycinnamic acid demonstrated it could significantly inhibit LPS induced NO production, and proinflammatory cytokines such as TNF-α and IL-1β while upregulating the HO-1 anti-inflammatory gene (Lee *et al.*, 2013). Similarly mollugin a bioactive phytochemical isolated from *Rubia cordifolia* demonstrated anti-inflammatory effects by suppressing pro inflammatory mediators, including pro inflammatory enzymes (iNOS and COX2) and cytokines (TNF-α & IL-6). Furthermore, it also displayed the up regulation of HO-1 expression in HT22 cells and HO activity in BV2 cells were the neuroprotective and anti-inflammatory effects of mollugin (Jeong *et al.*, 2011). A study by Kim *et al.* (2012) also demonstrates that licochalcone E obtained from the roots of *Glycyrrhiza-inflata* attenuates LPS-induced inflammatory responses in microglial BV2 cells by inhibiting iNOS and COX-2 enzymes; and inflammatory cytokines such as IL-1β and TNF-α. It also has neuroprotective effects against MPTP-induced nigrostriatal DArgic neurodegeneration in mice and activates the Nrf2 pathway

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by up-regulating downstream antioxidant enzyme expressions (HO-1 and NQO-1) both *in vitro* and *in vivo*.

Numerous other studies in many cell types showed similar anti-inflammatory responses by suppressing the iNOS and COX2 enzymes; and pro inflammatory cytokines while up regulating the antioxidant/ anti-inflammatory/ neuroprotective phase II enzymes. The results reported in previous studies, indicated the suppression of proinflammatory mediators and upregulation of neuroprotective enzymes lead to the activation of upstream molecules controlling inflammatory NRF2 and NFκB cascades (Bisht *et al.*, 2010; Park *et al.*, 2011; Hwang *et al.*, 2011; Lee *et al.*, 2013). In congruence with these results, it is found that the bioactive subfractions down regulate proinflammatory and upregulate anti-inflammatory genes in LPS stimulated BV2 cells followed by the activation of NRF2 and NFκB transcriptional genes. Thus, these transcriptional genes were further investigated.

The NRF2 gene is the transcription factor that activates the cytoprotective and antioxidant genes such as HO-1 and NQO-1. The transcription factor NRF2 is the controller of brain redox homeostasis and regulates inflammatory conditions. It controls both inducible and constitutive gene expression mediated by the antioxidant response element (ARE). Under oxidative stress conditions, NRF2 is liberated from Keap1 protein repression, translocated to the nucleus, forms a heterodimer with small Maf proteins, identifies and binds ARE and assembles the whole transcription machinery such as RNA polymerase II, to transcribe its target genes (Motohashi & Yamamoto, 2004). Aged mice are highly sensitive to oxidative stress in NRF2 null mice.

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The NF-κB transcription factor plays important roles in the immune system by regulating the expression of cytokines; iNOS, COX-2, growth factors and inhibitors of apoptosis. Additionally, NF-κB plays a role in the development and activity of a number of tissues including the central nervous system (Mémet, 2006). Moreover, pathological dysregulation of NF-κB is linked to inflammation and autoimmune diseases as well as neurodegenerative diseases. Investigation on the effect of the bioactive subfractions on the transcriptional genes showed TE3 and CE2 subfractions were able to upregulate NRF2 and downregulate NFκB2 transcriptional genes while CE3 subfraction only inhibited NFκB2 activation.

NRF2 knockout mice were shown to be more susceptible to the inflammatory response in the brain (Innamorato *et al.*, 2008). In addition, aged mice were getting highly sensitive to oxidative stress as a result of loss of NRF2 activity, and NRF2-null mice are similar to aged animals (Duan *et al.*, 2009). A derivative of cinnamic acid, 3,4,5-trihydroxycinnamic acid has also demonstrated significant inhibition of LPS-induced responses through the activation of NRF2 transcription factor in the BV2 cell line (Lee *et al*., 2014). Quercetin a compound found in many fruits, vegetables, and red wine showed suppression of LPS-induced iNOS expression and NO generation in BV2 microglial cells *via* regulation of NF-κB activity and Nrf2-mediated HO-1 expression. Therefore, it is indicated that quercetin could suppress abnormal nitrosative stress-induced neuroinflammation in the brain (Kang *et al.*, 2013). A compound currently in clinical testing as an oral formulation, BG12, has shown activation of NRF2-ARE pathway and inhibition expression of cytokines, demonstrating potential anti-inflammatory properties (Wierinckx *et al.*, 2005). Several reports also describe NF-κB activation in various cell lines related to neuroinflammation. Inhibition of NF-κB can

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reduce the ameloid beta that is deposited in plaques of Alzheimer patients (Kaltschmidt *et al.*, 1997).

NRF2 has a well established role as the regulator of key endogenous defence against oxidative stress in the body. Now emerging evidence suggests that NRF2 pathway may also play an important role in regulating inflammation in the brain. The upregulation of NRF2 has been associated with the reduction of inflammation and dysregulation of vulnerable brain regions and further declining the effects of neurodegenerative diseases. The NFκB has unique roles to the CNS in such processes as neuronal plasticity, neurodegeneration and neuronal developments. The constitutive role of NFκB that occurs in neurons *in vivo* has important neuroprotective roles. Many phytochemicals with anti-inflammatory activities inhibit NFκB activation and in parallel scavenge ROS and induce NRF2 driven defence. This close association between anti-inflammatory and antioxidant properties mediated by the same phytochemical shows a cross talk between NRF2 and NFκB signalling (Surh & Na, 2008). Therefore, NRF2 and NFκB transcriptions could be effective signalling pathway acting independently or simultaneously in reducing neurodegenerative diseases associated with neuroinflammation.

The bioactive subfractions attenuate neuroinflammation by reducing NO, the proinflammatory and anti-inflammatory cytokines via NRF2 and NFκB mediated pathway. The overexpression of HO-1 and NQO-1 inhibited iNOS, COX2 and IL-1β expression in BV2 cells via NRF2 and NFκB pathways; modulating their inflammatory responsiveness, providing a key line of attack in treatment of neurodegenerative diseases. The decreased expression of iNOS and COX2 and the increased expression of HO-1 and NQO-1 by the bioactive subfractions directly affect the NRF2 and NFκB pathway



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activation. The data obtained in various animal studies underlines the possibility that NRF2 mediated anti-neuroinflammatory effects may be achieved by activation of antioxidant machinery as well as suppression of pro inflammatory pathways mediated by NFκB signaling. However there are no definite evidence shows that NRF2 can directly inhibit NFκB signaling and vice versa at transcriptional level.

The pathological mechanisms in neurodegenerative disorders have gained enormous attention not only because these disorders are growing cause of disabilities in the aging communities. Nevertheless the immune activation within the CNS is a classic feature of neurodegenerative diseases among others that contributes to neuronal damage. Microglia exerts dual roles in neurodegeneration both as instigators of damage and as guardians of brain homeostasis. In contrast to peripheral macrophages that are highly effective in inciting pro inflammatory responses, microglial take an opposing role of limiting inflammation. Despite the notion that the CNS is an immune privileged site, innate and adaptive immune responses regularly take place; and are crucial for eliminating infectious agents and stimulate tissue repairs. Yet chronic activation of immune responses may lead to many problems such as neurodegenerative diseases (Amor *et al.*, 2010).

Collateral neuronal damage is clearly inherent to primary neuroinflammatory diseases; as neuroinflammation is likely the consequence of primary neurodegeneration. Studies support the notion that neuroinflammation promotes the neurodegenerative effects; early intervention with anti-inflammatory therapies may represent a unique opportunity to reduce the progression of neurodegenerative diseases. The formation of intracellular and extracellular protein led to the formation of activated microglia in CNS and promotes chronic neuroinflammation leading to chronic neurodegenerative



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conditions. Most neurodegenerative diseases triggered by neuroinflammation could be prevented through modification of lifestyle and dietary habits as well.

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# CHAPTER 6: CONCLUSION

In conclusion, this study has demonstrated that the extracts of *L. rhinocerotis* and *C. militaris* exhibited considerable antioxidant activity. The extracts were not cytotoxic to BV2 cells, and they also possess anti-neuroinflammatory effects as demonstrated by the ability to reduce NO production. The extracts also down regulated the proinflammatory genes (iNOS, COX2 and IL-1β) expression and upregulated anti-inflammatory cytokines (HO-1 and NQO-1) via NRF2 and NFκB mediated pathway. The anti-neuroinflammatory activity of *L. rhinocerotis* and *C. militaris* are demonstrated for the first time.

The crude extracts of *L. rhinocerotis* and *C. militaris* extracts exhibited no cytotoxic effect on BV2 cells up to 100ug/ml treatment concentration. The antioxidant activity ethanol extract of *L. rhinocerotis* was 12 fold (DPPH), 50 fold (TEAC) and 1.6 fold (FRAP) higher compared to its aqueous extract. On the other hand, the aqueous extract of *C. militaris* showed higher antioxidant activity of 2 fold (DPPH), 2 fold (TEAC), 3 fold (FRAP) compared to the ethanol extract. The ethanol extract of both *L. rhinocerotis* and *C. militaris* showed 40% and 60% higher anti neuroinflammatory activity respectively compared to its hot aqueous extracts. The crude ethanol extract was further fractioned to hexane and ethyl acetate fractions to determine the anti-neuroinflammatory activity of non-polar and medium polar components on BV2 cell line. Hexane and ethyl acetate fractions of both *L. rhinocerotis* and *C. militaris* mushrooms showed no cytotoxic effects on the cells up to 100ug/ml treatment concentration. The ethyl acetate fraction of *L. rhinocerotis* significantly ($p<0.05$) reduced nitric oxide production 37% and 98% at 10µg/ml and 100µg/ml respectively. Whereas, the ethyl acetate fraction of *C. militaris* significantly ($p < 0.05$) reduced nitric oxide production by 14%, 48% and 97% at treatment concentrations 1µg/ml, 10µg/ml and 100µg/ml.

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The ethyl acetate fraction of *L. rhinocerotis* and *C. militaris* each had 16% higher anti-neuroinflammatory activity compared to the hexane fractions. Thus, the ethyl acetate fractions of *L. rhinocerotis* and *C. militaris* were subjected to vacuum liquid chromatography respectively, yielding three bioactive subfractions TE3, CE2 and CE3. These bioactive subfractions were tested for their antioxidant properties, cell viability and anti neuroinflammation properties by BV2 cells; and their effect on anti-inflammatory pathways' gene expressions. The bioactive subfractions showed a relatively low antioxidant activity compared to known standards (*Cinnamomum zeylanicum*). They also did not exert any cytotoxic effects on the BV2 cells individually with and without LPS stimulation at 0.1μg/ml to 100μg/ml treatment doses via MTS assay. The bioactive subfractions did not affect the cells and at the same time were able to protect the cells post inflammation. The three subfractions downregulated the proinflammatory genes (iNOS, COX-2 and IL-1β). The TE3 and CE2 subfractions upregulated the antioxidant- anti-inflammatory genes (HO-1 and NQO-1) by 1.5 folds. TE3 and CE2 subfractions were able to upregulate NRF2 and downregulate NFκB2 transcriptional genes while CE3 subfraction only inhibited NFκB2 activation. The transcription factor NRF2 expression upregulated by 1.5 folds by TE3 and CE2 subfractions while NFκB activation was inhibited by 0.5-1.5 folds by all subfractions.

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## 6.1 Future investigations

This study has exhibited interesting and valuable outcomes. Additional research may be conducted in the future to resolve contradictions or explain exceptions related to this study as follows. Firstly, the identified chemical constituents of the bioactive fractions should be isolated, purified and confirmed using rpHPLC and NMR; and then tested for anti-neuroinflammatory activity *via in-vitro* and *in- vivo*. Protein expression study such as western blot or Elisa kit should be conducted to determine the effects of the bioactive subfractions/isolated chemical constituents on enzymes related to various neuroinflammatory pathways. Next, i*n vivo* studies should be done to confirm the anti-neuroinflammatory activity of whole mushrooms and/ or the bioactive subfractions for alternative drug discovery. Other than that, the bioactive constituents may be used to synthesise nanoparticles which will increase drug delivery to target organs.

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# LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

## Publication

1. Nallathamby, N., Lee G.S., Vidyadaran, S. Malek, S.N.A., Raman, J., Sabaratnam, V. Ergosterol of *Cordyceps militaris* attenuates neuro-inflammation in BV2 cells. *Natural Product Communication 10*(6), 885-886.

2. Nallathamby, N., Guan-Serm, L., Raman, J., Malek, S. N. A., Vidyadaran, S., Naidu, M., Kuppusamy, U. R. & Sabaratnam, V. (2016). Identification and in vitro evaluation of lipids from sclerotia of *Lignosus rhinocerotis* for antioxidant and anti-neuroinflammatory activities. Natural Product Communications (Accepted).

## Proceedings

1. Nallathamby, N., Malek, S.N.A., Naidu, M., Wong K.H., David, P., Sabaratnam, V. (2013, August 26-29). Cytotoxicity effect of selected indigenous mushrooms on BV2 microglial cells. The 7th International Medicinal Mushroom Conference, Beijing, China. (Oral presentation)

2. Nallathamby, N., Malek, S.N.A., Kuppusamy, U.R., Sabaratnam, V. (2014, Jan, 6-8). Antioxidant properties of ethanol extract and its fractions of Lignosus rhinocerotis (Cooke) Ryvarden. 18th Biological Sciences Graduate Congress, Kuala Lumpur, Malaysia. (Oral presentation)

3. Baskaran, A., Nallathamby, N., Chua, K.H., Sabaratnam, V., Kuppusamy, U.R. (2012, July 11). The Effect of Extracts of Selected Medicinal Mushrooms on RAW 264.7 macrophage. National Postgraduate Seminar 2012, University Malaya, Malaysia. (Poster presentation)

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