# *METABOLIC VARIATIONS IN BIOLOGICAL EVALUATION OF Clinacanthus nutans (Burm. f.) Lindau LEAF EXTRACTS ON LIPOPOLYSACCHARIDES-INDUCED NEUROINFLAMMATION IN RATS*

**AMALINA BINTI AHMAD AZAM**

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**By**

**AMALINA BINTI AHMAD AZAM**


**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

**February 2020**

**DEDICATION**

*This thesis is dedicated to my lovely parents, family and friends*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**METABOLIC VARIATIONS IN BIOLOGICAL EVALUATION OF *Clinacanthus nutans* (Burm. f.) Lindau LEAF EXTRACTS ON LIPOPOLYSACCHARIDES INDUCED NEUROINFLAMMATION IN RATS**

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Neuroinflammation is a complex response of injury on any part of the brain resulted in the activation of glial cells, release of inflammatory mediators like cytokines and chemokines, reactive oxygen and nitrogen species, which is a pathological hallmark of many neurological disorders. Therefore, effective control of neuroinflammation is crucial to prevent the related diseases. In this study, the matured leaves of 9-week old *Clinacanthus nutans* (Burm. f.) Lindau (CN) extracted with water and two ethanolic (50% and 100%) phytochemical constituents were profiled by using proton Nuclear Magnetic Resonance ($^1$H NMR) metabolomics approach. The highest nitric oxide (NO) inhibitory activity, in the *in vitro* neuroinflammation model using the lipopolysaccharides (LPS)-induced BV2 cell line, was observed for aqueous extract with an $IC_{50}$ value of 336.2 ± 4.7 µg/ml. Correlation between NO inhibitory activity and CN constituents by partial least square (PLS) analysis resulted in the identification of most potential metabolites responsible for the activity being schaftoside, acetate, propionate, alanine, and Clinacoside C.

The *in vivo* model of neuroinflammed Sprague Dawley rats induced with LPS was also done via the metabolomics approach. The findings from multivariate data analysis (MVDA) highlighted several similarities and dissimilarities in metabolites concentration in LPS-induced rats (LPS+water) and LPS-induced treated with CN extracts. Although CN doses treated group did not alleviate to ascertain level of cure, continuous 14 days oral administration of aqueous CN extract at 500 (CN500) and 1000mg/kg BW (CN1000) was able to moderately ameliorated the neuroinflammation activity in a similar manner as the positive drug, dextromethorphan hydrobromide (DXM) at 5mg/kg BW. A consistent result has been observed for serum by both analytical platforms of liquid chromatography-mass (LC-MS) and NMR, the physiological sickness behavior and $^1$H NMR brain tissue of the neuroinflammed male rats.

The alteration of lipid metabolism; (lysophosphatidic acid (LPA) and 5-diphosphomevalonic acid) in sera of multiplatform model, and the changes of metabolites in the brain tissue namely, lactate, pyruvate, phosphorylcholine, glutamine, and $\alpha$-ketoglutarate in CN500 and DXM exhibited an ameliorating effect when compared to the controlled neuroinflammed rats. CN treatments also significantly reduced IL-1$\beta$, a pro-inflammatory cytokine better than DXM as proven in the quantification of cytokinesby microarray analysis. The physiological sickness behavior such as anxiety, exploration and reduction of locomotion also improved by CN treatments as visualized in the principal component analysis (PCA) model. Hence, herein a comprehensive view of the CN effects in neuroinflammation caused by LPS was successfully profiled, correlated and deciphered between central neuroinflammation, systemic metabolic and physiological disturbance which has potential for future ethnopharmacological and/or nutraceutical studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**VARIASI METABOLIK MELALUI EVALUASI BIOLOGI EKSTRAK DAUN *Clinacanthus nutans* (Burm. f.) Lindau PADA TIKUS NEUROINFLAMASI TERARUH LIPOPOLISAKARIDA**

Oleh

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Neuroinflamasi adalah tindak balas yang kompleks pada mana-mana bahagian otak yang mengalami kecederaan, serta boleh menyebabkan pengaktifan sel glial, pelepasan pengantara keradangan seperti sitokin dan kimokin, serta reaktifikasi oksigen dan nitrogen spesis, yang merupakan salah satu ciri patologi bagi gangguan neurologi. Oleh itu, kawalan yang berkesan mencegah keradangan saraf adalah penting untuk mencegah penyakit berkaitan. Dalam kajian ini, daun matang *Clinacanthus nutans* (Burm. f.) Lindau (CN) yang berumur 9 minggu telah diekstrak dengan air dan dua ekstrak ethanolik (50% dan 100%).untuk mengkaji profil juzuk fitokimia mereka menggunakan kaedah resonans magnetik ($^1$H NMR) yang berasaskan kaedah metabolomik. Nilai tertinggi penghalangan nitrik oksida (NO), pada model neuroinflamasi menggunakan asosiasi titisan (*in vitro*) sel BV2 yang diaruh dengan lipopolisakarida (LPS), telah diperhatikan pada ekstrak akueus dengan nilai $IC_{50}$ 336.2 ± 4.7 µg/ml. Korelasi antara aktiviti penghalangan NO dengan konstituen CN menggunakan analisa separa persegi (PLS) telah mengakibatkan pengenalpastian metabolit-metabolit yang berkemungkinan bertanggungjawab bagi aktiviti tersebut adalah schaftosida, asetat, propionat, alanin dan Clinacosida C.

Kajian model *in vivo* neuroinflamasi menggunakan tikus Sprague Dawley yang diaruh daripada LPS juga telah dilakukan melalui pendekatan metabolomik. Dapatan multivariat data analisis (MVDA) telah berjaya menyerlahkan beberapa persamaan dan kelainan pada kepekatan metabolit-metabolit tikus yang di aruh LPS (LPS+air) dan tikus LPS yang diaruh serta diberikan CN ekstrak. Walaupun hasil rawatan dengan dos-dos CN tidak memadai sehingga ke tahap penyembuhan, pengambilan secara oral ekstrak akues CN secara berterusan selama 14 hari pada dos 500 (CN500) dan 1000mg/kg berat badan tikus (BB) (CN1000) didapati dapat memperbaiki aktiviti anti-keradangan neuro secara sederhana seperti yang diamati dalam cara ubat kawalan, dextromethorphan hydrobromide pada dos 5mg/kg BB. Hasil keputusan yang konsisten juga telah

diperhatikan dalam serum pada kedua-dua platform analitikal; cecair kromatografi jisim (LC-MS) dan NMR, fisiologi tingkah laku berpenyakit dan $^{1}$H NMR neuroinflammasi tisu otak tikus jantan.

Perubahan metabolism lemak; (lisofosphatidik asid (LPA) dan 5-difoshomevalonik asid) dalam model multiplatform serum, dan perubahan metabolit tisu otak seperti laktik, pyruvida, phosphorylkolin, glutamin, dan alpha-ketoglutarat dalam CN500 dan DXM mempamerkan penambahbaikan apabila dibandingkan dengan tikus rawatan kawalan neuroinflamasi aruhan LPS. Rawatan CN juga, secara ketara telah berjaya mengurangkan ekspresi IL-1*ß*, iaitu pro-keradangan sitokin secara lebih baik daripada DXM seperti yang dibuktikan dalam kajian analisa tatasusun kuantitijasad inflamasi tikus. Fisiologi penyakit berkaitan radang otak seperti kebimbangan, penerokaan, serta pengurangan aktiviti lokomotor daripada rawatan CN juga bertambah baik seperti dalam gambaran model analisis komponen utama (PCA). Oleh itu, satu pemerhatian komprehensif bagi kajian kesan CN dalam mengatasi keradangan neuro yang disebabkan oleh LPS telah berjaya diprofil, dikorelasi serta dihuraikan antara radang saraf pusat, gangguan metabolik pada fisiologi, serta keseluruhan sistemik saraf yang mana hasilan ini berpotensi dirujuk bagi kajian etnofarmasi dan/atau pengajian nutraseutikal.

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*Who remember Allah while standing, sitting, and lying down on their sides, and contemplate the creation of the heavens and the earth (with the thought) "Our Lord! Not for nothing have You created (all) this. Glory to You! Give us salvation from the suffering of the Fire*.

(The Quran, 3:191)

In the name of Allah, the Most Merciful and Beneficent. First and Foremost, praise is to Allah (SWT), the Almighty. Devotion to Him the Most Gracious and Merciful, for giving me the opportunity, determination, strength, and patience to complete this thesis. His continuous grace and mercy are with me during the tenure of research and hopefully throughout my life.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 9 April 2020

**Declaration by graduate student**

I hereby confirm that:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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| | |
|---|---|
| % | Percentage |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| δ | Delta for chemical shift in ppm |
| $^{o}$C | Degree centrigrade |
| µg | Micro gram |
| µL | Micro litre |
| µm | Micro meter |
| $^{13}$C | Carbon-13 |
| $^{1}$H | Proton |
| ACUC | Animal Care and Use Committee |
| ANOVA | Analysis of variance |
| BBB | Blood-brain barrier |
| BSA | Bovine serum albumin |
| BW | Body weight of rat |
| CN | *Clinacanthus nutans* |
| CPMG | Carr-Purcell-Meiboom-Gill |
| CV | Coefficient of varience |
| *d* | Doublet |
| *dd* | Doublet of doublets |
| DXM | Dextromethorphan hydrobromide |
| ESI- | Electrospray ionization for negative ion |
| ESI+ | Electrospray ionization for positive ion |
| FDR | False discovery rate |
| GABA | Gamma-aminobutyric acid |
| HCA | Hierarchical cluster analysis |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HSQC | Heteronuclear Single-Quantum Coherence |
| ICV | Intracerebroventicular |
| IFN | Interferon |
| IL | Interleukin |
| IMPaLa | Integrated Molecular Pathway Level Analysis |
| *J* | Coupling Constant in Hz |
| LCMS | Liquid chromatography Mass Spectrometry |
| LPS | Lipopolysaccharides |
| *m* | Multiplet |
| MCP | Monocyte chemoattractant protein |
| MetPA | Metabolic pathways analysis |
| MHz | Megahertz |
| NKEA | National key Economic Area |
| NMR | Nuclear magnetic resonance |
| NO | Nitrite oxide |
| OPLS | Orthogonal partial least square |
| OPLS-DA | Orthogonal partial least square discriminant analysis |
| PC | Principal components |
| PCA | Principal component analysis |

| | |
|---|---|
| PLS | Partial least square |
| PLS-DA | Partial least square- discriminant analysis |
| ROS | Reactive oxygen species |
| *s* | Singlet |
| SD | Sprague dawley |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SIMCA | Soft independent modeling of class analogy |
| *t* | Triplet |
| TCA | Tricarboxylic acid |
| Th | T-helper |
| TLR | Toll-like receptors |
| TNF | Tumor necrosis factor |
| TSP | Trimethylsilanepropionic Acid Sodium Salt |
| VIP | Variable importance of projection |

# CHAPTER 1

# INTRODUCTION

## 1.1 Research background

Neuroinflammation is a key player for severe neurodegenerative diseases. An elevation of neurodegenerative disease that effect mental health brings hindrance to life quality (Beard et al., 2015). A recent report in Malaysian Burden of Disease and Injury Study: 2009 -2014 by the Ministry of Health (MOH), neurological diseases are among the top 12 lead diseases causing morbidity and mortality in Malaysia (MOH, 2017). Hence, there is an alarming interest in the exploration of neuroprotection through the pharmacological application of anti-inflammatory agents either of the synthetic drugs or natural products since both are well documented (Shal et al., 2018).

Herbal medicines or natural products for healthcare have gained popularity due to their safety based on their prolonged usage since the beginning of human civilization. Unfortunately, many of the herbal products for these past decades have been reported with significant side effects. The intentional adulteration and accidental contamination were reported as the primary reasons (Mosihuzzaman, 2012), hence highlights the importance of quality, safety, efficacy, consistency and availability (QSECA) of the claimed herbal medicines.

The assessment of the overall procedures by a conventional method of isolation guided is difficult due to the complexity to ensure the QSECA for the natural products from the original crude extract (WHO, 2008). The quality and safety of an herbal product are practically the outcomes of the standardization of the agronomic steps and material processes (Tripathi et al., 2014). The efficacy of a plant often resulted from the cumulative interaction of a large number of phytoconstituents (Tripathi et al., 2014). Although there were extensive studies on this plant anti-inflammatory properties, to understand the responsible compounds belong to a complex matrix of the plant is not easy.

The metabolomics approach can overcome this problem since it is based on a holistic measurement which possible to relate a collective compound in the whole sample system of a complex unfractionated matrix of crude extract (Verpoorte, 2009) to an activity such as anti-inflammation. The advancement in science and technology of a holistic approach by using modern tools in profiling and fingerprinting the chemical profile of systemic biology of plants and animals has catalyzed and eased the deciphering of the bioactive complex compounds matrix. Metabolomics approach offers a platform to realistically correlate the responsible metabolites to the various components of the biological response, which might be directly linked to the pathological events in the biological system. Hence, a holistic approach enables the identification of multiple compounds from a single extract and their interaction either synergistically or antagonistically in a biological *in vitro*

or *in vivo* system. Also, this approach has the ability to predict the bioactivity of a new set of extracts based on a developed, validated regression model (VRM) of bioactivity-compounds data, which is vital for quality control of a herb preparation. Consequently, the VRM can save time and budget by skipping the assay step of a new set of an extract (Yuliana et al., 2011).

Inflammation is a medical phrase that corresponds to the protective host feedback against infectious or noxious stimuli, where it involves host cells, blood vessel, and protein (Markiewski and Lambris, 2007). One of the primary aims is for defense via the activation of the immune cells to the area by destroying the pathogens and trigger the repair process. Neuroinflammation is a combination of two words, "neuro," which represents brain cells and "inflammation"'. There are a variety of cues that induce neuroinflammation, such as infection, traumatic injury, toxic metabolites, and/or autoimmunity (Gendelman, 2002). Usually, inflammation is under controlled and self-limited. However, an acute brain injury or infection plunges to be a complex inflammatory response when it involves the activation of microglia, which are the main resident innate immune cells in the central nervous system (CNS). In response to infection, microglia activate the production of cytokines, chemokines, and antibodies, which causes stimulation of other cell types such as astrocytes and T-lymphocytes (Dendrou et al., 2016). Since microglia is a critical player in the immune response of the central nervous system (CNS), it is a suitable choice to be examined for neuroinflammation. Prolong activation of all these are sources of multiple neurotoxic factors, including tumor necrosis factor-$\alpha$, nitric oxide (NO), interleukin-1$\beta$, and reactive oxygen species (ROS), which leads to progressive neuronal death or chronic neuroinflammation (Lull and Block, 2010). The imbalance of free radicals of ROS and nitrogen species, which is known as oxidative stress, leads to an outset of anti-inflammation and antioxidant exploration to control neuron activation as a promising therapeutic target to combat neuron-based diseases (Arulselvan et al., 2016).

The therapeutic potential of *Clinacanthus nutans* (Burm. f.) Lindau (CN), locally known as *Belalai Gajah*, is well documented. The most used part of the plant was the leaf, and it was reported that the leaf samples were found to possess more phytochemicals that exhibited antioxidant and $\alpha$-glucosidase activities when compared to the stem (Khoo et al., 2015). Various other reports credited the CN leaf extracts to have antioxidant, anti-viral, anti-inflammatory and anti-cancer activities (Yong et al., 2013; Alam et al., 2016). To date, the standardization of CN leaf based on the quantification of certain marker compounds such as orientin, isoorientin, vitexin, isovitexin or shaftoside (Sarega et al., 2016) and comprehensive phytochemical analysis and the biological activity of inflammation have been recently conducted (Khoo et al., 2018a). Scientific reports on the pharmacological activities of the selected markers have also been well studied (Khoo et al., 2018d; Le et al., 2017). However, to our best knowledge, no report has highlighted the neuroprotective or anti-neuroinflammatory effects of CN leaf extract. Due to CN potentials in triggering the neuromodulation in the liver, heart, and kidney (Lau et al., 2014), its anti-neuroinflammatory activities are worthwhile to be investigated.

A holistic evaluation of rational, evidence-based CN herbal treatment enables a deeper understanding of the association of metabolic signature with its biological events. It is hypothesized that CN extract will show anti-neuroinflammatory effects in the neuron-BV2 cultured neuroinflammation in *in vitro* model, which in turn will be related to the *in vivo* rat model. This will lead to the discovery and development of effective phytomedical intervention and responses to the immunologic events, hence ensuring better constituents' knowledge and efficacy of herbs used in ethno-studies, nutraceutical, and functional food industries.

## 1.2 Aims and objectives

This study aimed to identify the possible neuroinflammation and neuroprotective biomarkers in the rat model and associate them with the possible immunologic events via NMR and LCM-MS. The metabolomic tools associated with multivariate analysis were employed to detect and discriminate the modulatory effects of CN on LPS neurotoxicity.

The aims were achieved through the following set of objectives:

1. To establish the metabolic fingerprint of aqueous, 50% ethanol and 100% ethanol of dried CN leaves extracts and to correlate their NO inhibition activity with the bioactive compounds via BV2 cells and rat model.
2. To identify the neuroinflammatory possible biomarker and the potential of underlying metabolic pathways involved in LPS-induced neuroinflammatory rats.
3. To elucidate the overall mechanism between the periphery and central nervous system, while integrating them with physical behavior and cytokine immunological responses.

## 1.3 Outline of the thesis

This thesis is presented in seven chapters:
Chapter 1 provides a general insight into the research background and aims of the study. The definition of a holistic approach, metabolomic, neuroinflammation, and CN brief background.

Chapter 2 focusses on the comprehensive literature review of the reported data related to the research.

Chapter 3 has been published in the *Records of Natural Products* under the title of "$^{1}$H NMR-based metabolomics of *Clinacanthus nutans* leaves extracts in correlation with their anti-neuroinflammation towards LPS-induced BV2 cells". This chapter discusses on the comparative metabolites evaluation of CN extracts from different solvent extractions by application of nuclear magnetic resonance (NMR) spectroscopy and chemometric methods related to bioassay-guided of BV2 cell toxicity and NO inhibition assays.

Chapter 4 has been published in the *Avicenna Journal of Phytomedicine* under the title of "Effects of *Clinacanthus nutans* leaf extract on lipopolysaccharide-induced neuroinflammation in rats: A behavioral and $^{1}$H NMR-based metabolomics study". This chapter emphasizes on the metabolic profile of sera with LPS and CN intervention and their impact on the behavioral studies of physiological interruption of locomotor, anxiety and exploration.

In Chapter 5, evidence of the $^{1}$H NMR brain tissue in the LPS-induced neuroinflammatory rat model showed that metabolomic analysis able to elucidate both metabolites and the genetic perturbation response via cytokines expression integrated OPLS model.

Chapter 6 presents the diverse biomarkers of the LPS-induced neuroinflammatory rat model with CN intervention using the integration application of LC-MS, a more sensitive analytical platform with NMR data. The results highlighted the identified markers from both platforms.

Finally, Chapter 7 summaries the thesis by connecting a network between metabolic profiles, physical behavior, and cytokines expression activation, where it recapitulates general discussion and ends in future applications and recommendations of the results presented.

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