NEUROMODULATORY EFFECT OF ZERUMBONE ON ALPHA-$_{2A}$ ADRENOCEPTOR, NMDA N2B RECEPTOR AND TRPV1 CHANNEL IN LPS-INDUCED SH-SY5Y CELLS, A NEUROPATHIC PAIN-LIKE *IN VITRO* MODEL

By

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

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

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Neuropathic pain has been denoted as chronic pain that was prompted by lesion or disease of the somatosensory nervous system. The development and persistence of neuropathic pain involve complex mechanisms intertwined, such as the involvement of neuro-inflammation and neuronal hyperexcitability. The conventional therapies to alleviate neuropathic pain triggered adverse effects as a result of indistinct understanding of the mechanisms. Recent research has proposed that zerumbone, a crystalline sesquiterpene compound derived from *Zingiber zerumbet*, was able to attenuate neuropathic pain symptoms in *in vivo* models. Therefore, the present study was carried out to investigate the neuromodulatory properties of zerumbone via the modulation of alpha-$_{2A}$ adrenoceptor, *N*-methyl-*D*-aspartate (NMDA) subtype N2B receptor and transient receptor potential vanilloid subtype 1 (TRPV1) channel in LPS-induced SH-SY5Y cells, a neuropathic pain-like *in vitro* model. LPS-induced SH-SY5Y cells were employed to allow tight control of physiological environment which could not be established in *in vivo* models, in addition to, reducing the use of animals in the study of neuropathic pain. LPS induction in SH-SY5Y cells enable the observation of the hallmark of neuropathic pain pathophysiology which are the expression of pro-inflammatory mediators and the alteration of receptors and ion channels associated with neuronal hyperexcitability. The optimisation of culture media for SH-SY5Y cells were conducted by observing the effect of growth rate of the cells in different medium and foetal bovine serum (FBS) concentration; Dulbecco's modified Eagle's Medium (DMEM) with 15% FBS, DMEM: Ham's F12 mix (DMEM:F12) with 10% FBS and DMEM:F12 supplemented with 15% FBS. SH-SY5Y cells cultured in DMEM:F12 supplemented with 15% FBS had shown the highest growth rate following 24 hours, hence, the culture medium was used throughout the experiments. SH-SY5Y cells were subjected to neuronal differentiation via 10μM of all *trans* retinoic acid induction and serum deprivation, followed by characterization through immunocytochemistry. Then, 3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted and confirmed the viability of SH-SY5Y cell to be above 90% following the treatment of 2, 4, 8 and 16 μg/ml of zerumbone for 24 hours. The neuromodulatory effect of zerumbone

was first investigated through the expression level of nitric oxide through Griess' assay, whereby the inhibitory concentration of zerumbone was determined at 8μg/ml. Enzyme-linked immunoassay (ELISA) was then conducted to observe the expression of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF- α), by which only slight downregulation was observed after the treatment of zerumbone. Next, to understand the anti-neuropathic pain effect of zerumbone, molecular docking and Western blot analysis were performed. The inhibitory properties of zerumbone on pain signal transmission was observed to be involved in the modulation of α_{-2A} adrenoceptor, NMDA N2B receptor and TRPV1 channel. Molecular docking analysis revealed that the -CDOCKER binding and interaction energy of zerumbone to the respective proteins were higher as compared to the native crystallized ligands. Nevertheless, the amino acid interaction of zerumbone with the proteins indicated its possible anti-neuropathic pain effect by which similar interactions were perceived on NECA (α_{-2A} adrenoceptor agonist), ifenprodil (NMDA N2B receptor antagonist) and capsazepine (TRPV1 channel antagonist) with the proteins. Meanwhile Western blot analysis showed that zerumbone increased the expression of α_{-2A} adrenoceptor, proposing the inhibitory mechanism of zerumbone through descending modulation, antagonistic towards the down-regulation of the receptor after LPS induction. Treatment of zerumbone down-regulated the expression of NMDA N2B receptor and TRPV1 channel, as opposed to the upregulation of these proteins after LPS induction. Data from each experiments were analysed by using One-way Analysis of Variance (ANOVA) followed by *post hoc* Tukey test, $p<0.05$. To conclude, the current study proved the neuromodulatory effect of zerumbone on α_{-2A} adrenoceptor, NMDA N2B receptor and TRPV1 channel in LPS-induced SH-SY5Y cells, a neuropathic pain-like *in vitro* model.

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

KESAN NEUROMODULASI OLEH ZERUMBONE TERHADAP ADRENOSEPTOR ALFA$_{-2A}$, RESEPTOR NMDA N2B DAN SALURAN ION TRPV1 DALAM SH-SY5Y SEL TERARUH LIPOPOLISAKARIDA, SEBUAH MODEL *IN VITRO* BAK SAKIT NEUROPATIK

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Sakit neuropatik ditakrif sebagai sakit kronik yang disebabkan lesion atau penyakit sistem saraf somatosensori. Perkembangan dan perlanjutan sakit neuropatik melibatkan mekanisme kompleks yang saling berkaitan, seperti inflamasi saraf dan keterujaan saraf. Terapi kovensional untuk mengurangkan sakit neuropatik mengakibatkan kesan buruk yang berpunca daripada pemahaman yang tidak jelas berkenaan mekanisme keadaan tersebut. Penyelidikan terkini menunjukkan bahawa zerumbone, sebatian seskuiterpena bak hablur yang berasal daripada *Zingiber zerumbet*, dapat mengatenuasi simptom sakit neuropatik dalam model *in vivo*. Justeru, kajian ini dijalankan untuk menyiasat sifat neuromodulasi zerumbone melalui modulasi adrenoseptor alfa$_{-2a}$, reseptor, NMDA N2B dan saluran ion TRPV1 dalam sel SH-SY5Y teraruh lipopolisakarida (LPS), sebuah model *in vitro* bak sakit neuropatik. Sel SH-SY5Y teraruh lipopolisakarida digunakan untuk membolehkan pengawalan ketat terhadap persekitaran fisiologi yang tidak dapat dicapai dalam model *in vivo* serta untuk mengurangkan penggunaan haiwan dalam kajian sakit neuropatik. Aruhan LPS dalam sel SH-SY5Y membolehkan pemerhatian ciri khas patofisiologi sakit neuropatik iaitu ekspresi pengantara pro-inflamasi dan perubahan reseptor dan saluran ion yang berkaitan dengan keterujaan saraf. Pengoptimuman media kultur sel SH-SY5Y dilaksanakan dengan mengkaji kesan kadar pertumbuhan sel dalam setiap jenis kultur media dan serum anak lembu (FBS) dengan kepekatan yang berbeza; Dulbecco's modified Eagle's Medium (DMEM) ditambah dengan 15% FBS, campuran DMEM: Ham's F12 (DMEM:F12) ditambah dengan 10% FBS and DMEM:F12 ditambah dengan 15% FBS. Sel SH-SY5Y dikultur dalam DMEM: F12 ditambah dengan 15% FBS telah menunjukkan kadar pertumbuhan tertinggi berikutan 24 jam, oleh itu, media kultur tersebut digunakan sepanjang eksperimen. Sel SH-SY5Y mengalami pembezaan sel saraf setelah diaruh oleh 10 μM asid all *trans* retinoik dan deprivasi serum, diikuti dengan pencirian melalui immunositokimia. Kemudian, asai 3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dilakukan untuk mengesahkan kebolehhidupan sel melebihi 90% selepas rawatan 2, 4 , 8 dan 16 μg/ml zerumbone selepas 24 jam. Kesan neuromodulasi zerumbone diselediki melalui asai Griess' dengan mengkaji tahap ekspresi

nitrik oksida, yang dengannya kepekatan rencatan zerumbone ditentukan pada 8 μg/ml. Seterusnya, asai imunojerapan berpaut enzim (ELISA) dilakukan untuk mengkaji ekspresi interleukin-6 (IL-6) dan faktor nekrosis tumor alfa (TNF-α), dengan pengurangan ekspresi yang kecil setelah rawatan zerumbone. Bagi memahami kesan sakit neuropatik oleh zerumbone, kajian dok molekul dan pedapan Western dilakukan. Berdasarkan pemerhatian terhadap dua analisis tersebut, zerumbone mempunyai sifat rencatan sakit neuropatik yang dimodulasikan melalui adrenoseptor alfa$_{-2a}$, reseptor NMDA N2B dan saluran ion TRPV1. Analisis dok molekul menunjukkan tenaga pengikatan dan tenaga saling tindak - CDOCKER zerumbone terhadap protein tersebut, masing-masing lebih tinggi berbanding ligan kristal asli. Namun, interaksi asid amino antara zerumbone dengan protein tersebut menunjukkan persamaan dengan NECA (agonis adrenoseptor alfa$_{-2a}$), ifenprodil (antagonis reseptor NMDA N2B) dan capsazepine (antagonis saluran ion TRPV1), yang membuktikan kemungkinan kesan anti-sakit neuropatik oleh zerumbone. Sementara itu, analisis pedapan Western menunjukkan bahawa zerumbone meningkatkan ungkapan adrenoseptor alfa$_{-2a}$, menyarankan mekanisme rencatan zermbone melalui modulasi laluan menurun, berantagonis dengan kesan aruhan LPS yang mengurangkan ungkapan reseptor tersebut. Rawatan zerumbone telah mengurangkan ungkapan reseptor NMDA N2B dan saluran ion TRPV1, berlawanan dengan peningkatan ungkapan protein tersebut setelah aruhan LPS. Data daripada setiap kajian dianalisis menggunakan One-Way Analysis of Variance (ANOVA) diikuti dengan ujian *post hoc*, $p<0.05$. Kesimpulannya, kajian ini membuktikan kesan neuromodulasi oleh zerumbone terhadap adrenoseptor alfa$_{-2a}$, reseptor NMDA N2B dan saluran ion TRPV1 dalam SH-SY5Y sel teraruh lipopolisakarida, sebuah model *in vitro* bak sakit neuropatik.

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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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3D	Three-dimensional
5-HT	Serotonin
AEA	Arachidonoyl ethanolamine
AMI	Amitriptyline
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	One-way Analysis of Variance
ATP	Adenosine-5-triphosphate
Ca^{2+}	Calcium ion
CAMKIII	Calcium ion calmodulin-dependent protein kinase III
cAMP	Cyclic adenosine monophosphate
CCI	Chronic-constriction injury
COX2	Cyclo-oxygenase-2 enzyme
CPM	Conditioned pain modulation
DAMP	Danger-associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
DMEM:F12	DMEM: Ham's F12 mix
DMSO	Dimethyl sulfoxide
DNIC	Diffuse noxious inhibitory control
DRG	Dorsal root ganglia
ELISA	Enzyme-linked immunoassay
EPSC	Excitatory post synaptic current
EPSP	Excitatory postsynaptic potentials
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum

hESCs	Human embryonic stem cells
hiFBS	Heat-inactivated foetal bovine serum
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
hPSCs	Human pluripotent stem cells
IASP	International Association for the Study of Pain
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IPSP	Inhibitory postsynaptic potentials
JNK	c-Jun N-terminal kinase
K^+	Potassium ion
L-DOPA	L-3, 4-dihydroxyphenylalanine
LPS	Lipopolysaccharides
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MTT	(3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normal control group
Na^+	Sodium ion
NEAA	Non-essential amino acids
NECA	5′-N-ethylcarboxamidoadenosine
NMDA NR2B	*N*-methyl-*D*-aspartate subtype N2B
NO	Nitric oxide

PAG	Periaqueductal gray
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PDB	Protein Data Bank
PDMS	Polydimethylsiloxane
PI-3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RAR	Retinoic acid receptor
RVM	Rostroventral medulla
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean
TBI	Traumatic brain injury
TNF- α	Tumour necrosis factor-α
TRP	Transient receptor potential
TRPV1	Transient receptor potential cation channel subfamily V member 1
VEH	Vehicle group
ZER	Zerumbone

CHAPTER 1

INTRODUCTION

1.1 Introduction

Neuropathic pain is a condition that could results in the loss of function and reduce the quality of one's life. The International Association for the Study of Pain (IASP) has interpreted neuropathic pain as a pain that is caused by a lesion or disease on the somatosensory system. The prevalence of neuropathic pain has not been ascertained in most countries in Asia including Malaysia, but, worldwide prevalence has been reported to be 7%, with up to 4- to 6-fold of estimated variation among countries (Zghoul *et al.*, 2017; Ye *et al.*, 2018).

The main mechanism that prompt the development and continuance of neuropathic pain involve peripheral and central sensitization (Cohen and Mao, 2014). The neuronal sensitization could be on account of direct lesion due to injury, viral infections, adverse effects of drugs and other metabolic diseases (Khelemsky, Malhotra and Gritsenko, 2019). In spite of the multifariousness of aetiology and vague underlying mechanism, patients with neuropathic pain share common symptoms which are allodynia, pain due to a stimulus that does not normally provoke pain and hyperalgesia, exaggerated response from a normally painful stimulus respectively (He and Kim, 2020). Other symptoms are burning, numbness, tingling, fatigue and sleep disturbance (Goh *et al.*, 2017).

Current treatments of neuropathic pain involve the management of symptoms, with few approach targeting the mechanism of neuropathic pain. The first-line therapy involve the use of tricyclic antidepressants, such as amitriptyline; calcium ion channel $\alpha 2\delta$ ligands (anticonvulsants), namely gabapentin; and serotonin-noradrenaline reuptake inhibitor (SNRI) namely duloxetine (Fornasari, 2017; Obata, 2017). Earlier studies have exhibited the mechanism of antidepressants in modulating the descending inhibitory pathway, whereby antidepressants managed to inhibit the reuptake of serotonin and noradrenaline at inhibitory neuron synapse (Fornasari, 2017). Moreover, antidepressants have been observed to activate interneurons within the descending pathways which resulted in the release of inhibitory neurotransmitter namely gamma-aminobutyric acid (GABA) (Fornasari, 2017). Besides, treatment of commonly used antidepressants, amitriptyline, was also proposed to modulate NMDA receptor via the calcium ion-dependent desensitization, resulting in the reduced NMDA-activated currents (Stepanenko *et al.*, 2019). Second-line therapy includes the use of lidocaine which block voltage-gated sodium channels; capsaicin 8% patches; and opioids which is also use as third-line therapy (Finnerup *et al.*, 2015; Fornasari, 2017). The established mechanism of lidocaine involved the blockage of voltage-gated sodium channels in peripheral nerves (Kurabe, Furue and Kohno, 2016). Moreover, lidocaine has also been investigated to be able to desensitize transient receptor potential (TRP) channels, thus ameliorate neuropathic pain (Hermanns *et al.*, 2019).

However, due to the complex pathophysiology of neuropathic pain , these treatments were reported to insufficiently provide relief of pain to the patients. Plus, neuropathic pain could also arise from other metabolic diseases, hence making it challenging to decide the accurate treatment. Furthermore, these treatments have been known to have pleiotropic affects which also results in adverse reactions, namely anticholinergic effects, hypertension, seizures, prolongation arrhythmia and nausea (Cavalli *et al.*, 2019).

The foremost mechanism of neuropathic pain are the peripheral and central sensitization which occur because of the alteration of expression of ion channels as well as cellular and molecular changes following the nerve injury and immune response (Khelemsky, Malhotra and Gritsenko, 2019). Monoaminergic pathway, consisting of noradrenergic and serotonergic pathway, could be a prospective target in alleviating neuropathic pain for its role in inhibiting nociceptive signal transmission (Li *et al.*, 2019). α_{-2A} adrenoceptor, one of the key player in modulating the noradrenergic pathway, had been the pharmacological target to alleviate neuropathic pain (Bravo *et al.*, 2019). α_{-2A} adrenoceptor had been reported to be able to modulate other receptor and mediators activation and expression associated to neuropathic pain pathophysiology. As for instances, α_{-2A} adrenoceptor was observed to affect the regulation of glutamate receptors, ion channels and pro-inflammatory mediators, which could synergistically inhibit the nociceptive signal transmission in neuropathic pain (Woo *et al.*, 1997; Liu *et al.*, 2006; Matsushita *et al.*, 2018).

Research have shown that glutamate receptors, specifically *N*-methyl-*D*-aspartate (NDMA) subtype N2B receptors exhibited prominent role in underlying the maintenance of neuropathic pain. In animal models of neuropathic pain, the inhibition or reduction of NMDA N2B receptor expression has been able to attenuate mechanical allodynia and thermal hyperalgesia, the symptoms hallmark of neuropathic pain (Wu *et al.*, 2014; Chia *et al.*, 2020). Alongside NMDA N2B receptor, transient receptor potential subfamily V member 1 (TRPV1) channel has also been proposed to modulate allodynia and hyperalgesia. Similarly to NMDA N2B receptor, the inhibition of expression of TRPV1 channel and the inactivation of the ion channel caused the attenuation of mechanical and thermal allodynia and hyperalgesia (Green *et al.*, 2016; Guo *et al.*, 2019).

The pathophysiology of neuropathic pain also comprises of the role of inflammatory cytokines and chemokines. It is important to take into account that neuronal sensitization and neuroinflammation are not mutually exclusive (Ellis and D.L.H. Bennett, 2013). Commonly identified pro-inflammatory mediators that are suggested to prompt neuropathic pain mechanism are interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and nitric oxide (NO) (Perimal *et al.*, 2011a; Colloca, Ludman, Bouhassira, Baron, Anthony H. Dickenson, *et al.*, 2017; Gopalsamy *et al.*, 2017). Upon nerve injury, these inflammatory mediators have been identified as the early markers of neuropathic pain instigation (Ji, Xu and Gao, 2014).

In order to address the current treatment circumstances, research has been exploring the potentials of natural products with pharmacological effectiveness (Quintans *et al.*, 2014).

Herbal medicines have been widely used as ancient therapies to treat various illnesses such as inflammation and pain. One of the moderately large genus of herbs is genus *Zingiber*, with estimated 141 species found mostly within South East Asia and Pacific Islands (Yob *et al.*, 2011). Within the genus *Zingiber*, *Zingiber zerumbet* has been gaining researchers' interest due to its anti-inflammatory and anticancer properties (Gopalsamy *et al.*, 2017; Girisa *et al.*, 2019a). The plant has been traditionally used in the to treat stomach ache, indigestion, fever and other ailments (Fatima *et al.*, 2015). The bioactive sesquiterpene from *Zingiber zerumbet* that is extensively studied is zerumbone. Highly concentrated within the rhizomes of *Zingiber zerumbet*, zerumbone has been observed to possess potentials as anti-proliferative for cancer treatment, antioxidant, antinociceptive and anti-inflammatory effects (Perimal *et al.*, 2011b; Yob *et al.*, 2011; Gopalsamy *et al.*, 2017; Yan *et al.*, 2017).

Although research has provided some information on the anti-allodynic and anti-hyperalgesic effects of zerumbone in *in vivo* models, the mechanism of action of zerumbone in alleviating neuropathic pain is yet to be understood. Plus, animals usage in conducting pain research has been getting ethical concerns, hence there is the need to develop an *in vitro* model to mimic the pathophysiology of neuropathic pain to further evaluate the therapeutic effects of zerumbone.

Several cell lines have been commonly used in neuroscience research, by which in this study, SH-SY5Y cells have been determined to be develop as an *in vitro* model to mimic the pathophysiology of neuropathic pain. This is because SH-SY5Y cells are human neuroblastoma cell line, which eliminates the species variation difference observed in animal cell lines (Namita G Hattangady and Rajadhyaksha, 2009; Kovalevich and Langford, 2013). Furthermore, SH-SY5Y cells were able to be induced to the differentiated state which express important receptors and ion channels involved in nociceptive signal transmission (Encinas *et al.*, 2000; Shipley, Mangold and Szpara, 2017). In order to trigger the development of neuroinflammation and change the expression of receptors and ion channels incorporated in neuropathic pain mechanism, lipopolysaccharides (LPS) induction was opted for to induce the condition in SH-SY5Y cell culture. LPS-induced SH-SY5Y cells have been established to enable the study of oxidative stress, inflammatory mediators, such as interleukins, and proteins associated with pain signal transmission, namely TRP channels and glutamate receptors, attributable to the activation of toll-like receptors (TLR) by LPS (Meseguer *et al.*, 2014; Pandur *et al.*, 2018).

It was hypothesized that the anti-neuropathic pain properties of zerumbone involved the modulation of α_{-2A} adrenoceptor, TRPV1 channel, NMDA N2B receptors and pro-inflammatory mediators namely nitric oxide, IL-6 and TNF-α. The findings of this research will help to understand the modulation of the receptors in the pathophysiology of neuropathic pain in *in vitro* model and develop zerumbone as promising therapeutics to attenuate neuropathic pain.

1.2 General Objectives

To study the effect of zerumbone on α_{-2A} adrenoceptor, NMDA N2B receptor and TRPV1 channel expression and the pro-inflammatory mediators on *in vitro* model of neuropathic pain-like lipopolysaccharide (LPS)-induced SH-SY5Y cells.

1.3 Specific Objectives

- To determine the effect of different culture medium on the growth of SH-SY5Y cells, to induce differentiation of SH-SY5Y cells and to characterize the differentiated cells via immunostaining of differentiated neuronal markers.
- To determine the effect of zerumbone on the expression of nitric oxide, interleukin-6 and tumour necrosis factor-α in LPS-induced SH-SY5Y cells.
- To study the anti-neuropathic pain effect of zerumbone in LPS-induced SH-SY5Y cells via the modulation of α_{-2A} adrenoceptor through molecular docking and Western blot analysis.
- To determine the anti-neuropathic pain effect of zerumbone in LPS-induced SH-SY5Y cells via the modulation of NMDA N2B receptor through molecular docking and Western blot analysis.
- To investigate the anti-neuropathic pain effect of zerumbone in LPS-induced SH-SY5Y cells via the modulation of TRPV1 channel through molecular docking and Western blot analysis.

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