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Zerumbone ameliorates neuroinflammation in LPS-induced SH-SY5Y cells, an *in vitro* model of neuropathic pain: targeting NO, IL-6, and TNF-α

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Abstract: Neuropathic pain is initiated by lesions or diseases affecting the somatosensory nervous system. The development and persistence of this condition involve complex and interconnected mechanisms, including those related to neuroinflammation and neuronal hyperexcitability. Due to an incomplete understanding of these mechanisms, conventional therapies for neuropathic pain often result in adverse effects. Recent research has proposed that zerumbone, a crystalline sesquiterpene compound extracted from *Zingiber zerumbet*, can attenuate neuropathic pain in animal models. Lipopolysaccharide (LPS)-induced SH-SY5Y cells were employed to allow tight control of the 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 enables the observation of one of the hallmarks of neuropathic pain pathophysiology, which is the expression of pro-inflammatory mediators. This study aims to evaluate the anti-inflammatory effect of zerumbone by measuring its influence on the expression of nitric oxide (NO), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) in LPS-induced SH-SY5Y cells, an *in vitro* model of neuropathic pain. The anti-neuroinflammatory effect of zerumbone was first investigated through the expression level of NO, whereby the inhibitory concentration of zerumbone was determined at 8 μg/ml (p<0.0001 compared to the LPS-only group). Zerumbone treatment significantly reduced the expression of IL-6 (p<0.05 compared to the LPS-only group). Although a reduction in TNF-α levels was observed, it did not reach statistical significance in the enzyme-linked immunoassay (ELISA). Data from each experiment were analysed by using the One-way Analysis of Variance (ANOVA) followed by the *post hoc* Tukey test, p<0.05. Zerumbone demonstrates an anti-neuroinflammatory effect in LPS-stimulated SH-SY5Y cells by suppressing the expression of key inflammatory mediators NO, IL-6, and TNF-α. These findings suggest that zerumbone is a potential therapeutic candidate for managing neuropathic pain associated with neuroinflammation.

Keywords: Zerumbone; Neuropathic pain; Anti-neuroinflammatory; NO; IL-6; TNF-α; LPS-induced SH-SY5Y cells

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1.0 INTRODUCTION

The pathophysiology of neuropathic pain involves the mechanisms of neuronal plasticity and neuroinflammation for the development and maintenance of the condition. The upregulation and activation of pro-inflammatory cytokines will trigger the activation of signalling molecules. This results in the up-regulation and sensitisation of pro-nociceptive downstream receptors and ion channels (Kiguchi et al., 2017; Ma et al., 2024). Cytokines are secreted not only within the central and peripheral nervous systems but also in other peripheral tissues and the bloodstream. In neuropathic pain conditions, nerve injury causes damaged cells to secrete signalling molecules. These molecules act to recruit circulating leukocytes, mainly macrophages, in the peripheral nervous system. In the central nervous system, they recruit microglial cells to the injury site. Once at the injury site, these immune cells produce pro-inflammatory cytokines. Common pro-inflammatory cytokines include interleukin-6 (IL-6), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and oxidative agents such as nitric oxide (NO), which are observed to be upregulated within the dorsal horn in the spinal cord and the dorsal root ganglia (DRG). The inhibition and downregulation of pro-inflammatory cytokines in animal models of neuropathic pain managed to prevent the development and attenuate neuropathic pain (Chen et al., 2018; Gopalsamy et al., 2017; Kiguchi et al., 2017).

The first-line treatments of neuropathic pain utilise gabapentinoids (gabapentin and pregabalin), tricyclic antidepressants (TCAs) (amitriptyline) and selective serotonin-noradrenaline reuptake inhibitors (SNRI) (duloxetine and venlafaxine) (Antoniazzi et al., 2024). The second-line therapy involves the use of opioids and topical lidocaine, while the third-line treatment involves the use of strong opioids and neurotoxin. Even though the first-line treatments and most of the second-line and third-line treatments have been able to attenuate neuropathic pain symptoms, such as allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (increased sensitivity to pain), these treatments result in adverse effects in patients (Cavalli et al., 2019). Lethargy, peripheral swelling, constipation, nausea and vomiting, erythema and seizures are the commonly reported adverse effects of the current treatments (Zhu et al., 2019).

Zerumbone, an active compound from *Zingiber zerumbet*, has been widely studied for its anti-inflammatory properties. Zerumbone has been observed to attenuate neuropathic pain in chronic constriction injury (CCI)-induced mice model through the suppression of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α (Gopalsamy et al., 2017). Hence, this study aims to determine the anti-inflammatory property of zerumbone via its effect on the expression of NO, IL-6 and TNF-α in LPS-induced SH-SY5Y cells. The study of molecular and signal transduction of neuropathic pain in an *in vivo* model faces limitations due to the complex systems and interactions. Hence, having *in vitro* models helps study crosstalk of molecular signalling and reduce animal usage (Hattangady and Rajadhyaksha, 2009; Mohammed Izham et al., 2022). It was hypothesised that treatment of zerumbone would reduce the production of inflammatory cytokines in LPS-induced SH-SY5Y cells.

2.0 MATERIALS AND METHODS

2.1 SH-SY5Y cell culture and differentiation

SH-SY5Y neuroblastoma cell line was purchased from ATCC (ATCC CRL-2266, Passage 15). The cells were plated in 96-well plates at a density of 1 x 10^5 cells/well. The cells were initially cultured in Dulbecco's Modified Essential Medium/Ham's Nutrient Mixture (DMEM:F12) (containing 4.5 g/L glucose with 2 mM of L-glutamine and sodium pyruvate), supplemented with 15% (v/v) FBS, 1% (v/v) of non-essential amino acids (NEAA), 1% (v/v) of Penicillin-Streptomycin mixed solution and incubated at 37°C with 5% carbon dioxide (Chia et al., 2020; Mohammed Izham et al., 2018). After 24 hours, the growth media was replaced with a differentiating media consisting of DMEM:F12 (containing 4.5 g/L glucose with 2 mM of L-glutamine and sodium pyruvate), supplemented with 2.5% (v/v) FBS, 1% (v/v) of NEAA, 1% (v/v) of Penicillin-Streptomycin mixed solution and 10 µM retinoic acid. The cells were incubated at 37°C with 5% carbon dioxide. The differentiating media was replaced daily for five consecutive days.

2.2 Cell viability assay

Cell viability assay of the differentiated SH-SY5Y cells was corroborated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The differentiated cells were treated with 2.0, 4.0, 8.0 and

16.0 μg/ml of zerumbone (Rahman et al., 2014), and incubated at 37°C with 5% carbon dioxide for 24 hours. Then, the culture media was aspirated, and 100 μL of MTT solution (#23547-21, Nacalai Tesque) with a concentration of 5 mg/mL prepared in culture media, was added into each well. The plates were wrapped with aluminium foil and the cells were incubated at 37°C for 3 hours until the formazan crystals were formed. Then, 100 μL of MTT solvent, sterile dimethyl sulfoxide (DMSO), was added to dissolve the crystals and shaken for 15 minutes. The absorbance values were measured at 570 nm (Haque et al., 2018). The percentage viability of the cells was measured by comparing the zerumbone-treated groups with the normal control (non-treated group) by using the following equation:

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100\%$$

2.3 LPS induction and treatment of zerumbone

The extraction and isolation of the target compounds followed the procedures outlined (Chia et al., 2016; 2020). The rhizomes of *Zingiber zerumbet* were purchased from a local market in Jalan Chow Kit, Kuala Lumpur, and identified by a botanist at the Institute of Bioscience (IBS), Universiti Putra Malaysia (specimen reference number: SK 622/07). Zerumbone was extracted using the hydrodistillation method. Fresh rhizomes (approximately 18 kg) were washed, cut into pieces (approximately 0.5 – 1 mm thick), and placed in a round-bottom flask connected to a Dean-Stark trap and condenser. Water was added, and the mixture was boiled for 4 hours until the soluble oils were collected. Hexane was used during boiling to improve oil extraction. The hexane phase was then collected and evaporated to obtain the crude hydrodistillate. This was stored at 4°C overnight, allowing the crystallised solids to separate. The solids were thawed and recrystallised three times to increase purity. The purity of the zerumbone was confirmed using nuclear magnetic resonance spectroscopy (NMR) and high-performance liquid chromatography (HPLC), showing more than 99.0% purity.

The differentiated cells were induced with 1 μg/mL of LPS from *Escherichia coli*, serotype O55:B5 (#03553, Sigma-Aldrich). LPS were added to the culture medium for 12 hours at 37°C with 5% carbon dioxide (Chia et al., 2020; Pandur et al., 2018; Si et al., 2024). The cell culture in the normal control group was not induced with LPS. After 12 hours of LPS induction, the cells were treated with zerumbone at concentrations of 8 μg/mL, 16 μg/mL of amitriptyline was added to the cells for 24 hours in the positive control group; while phosphate-buffered saline (PBS) was added to the cells in the vehicle control group for 24 hours at 37°C with 5% carbon dioxide.

2.4 Measurement of nitric oxide

The production of nitric oxide (NO) after LPS induction was estimated spectrophotometrically via the formation of nitrites through the Griess' assay. A total of 100 μL culture media was harvested and mixed with 100 μL of Griess' reagent (#03553, Sigma-Aldrich) and incubated for 10 minutes at room temperature under dark condition. The Griess' reagent was made up of 1% sulphanilamide in 0.1 mol/L hydrochloric acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride. The absorbance was measured at 540 nm using a microplate reader. The nitrite formed was calculated based on a standard curve constructed with sodium nitrite ($NaNO_2$) (Chae, 2004).

2.5 Enzyme-linked immunosorbent assay (ELISA)

The cells were then harvested for the quantification of IL-6 and TNF-α by using human-specific, enzyme-linked immunosorbent assay (ELISA) kits (#E-EL-H0102, Elabscience). First, the samples were prepared by removing the culture medium and adding ice-cold PBS to rinse the cell culture. Then, 150 μL of RIPA lysis buffer with protease inhibitor was added, and the cells were scraped using the cell scrapper. The sample was then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used for the assay.

100 μL of each sample and different concentrations of standard solutions were added in duplicate wells and incubated at 37°C for 90 minutes. After removing the liquid, 100 μL of biotinylated detection antibody solution was added to each well and incubated for 1 hour at 37°C. Wells were washed three times with 350 μL of wash buffer, 1 minute each time. Then, 100 μL of HRP conjugate solution was added to each well and incubated for 30 minutes at 37°C. The washing steps were repeated, and 90 μL of substrate reagent was added to each well, incubating at 37°C for 15 minutes in the dark. Finally, 50 μL of stop solution was added, and absorbance was measured at 450 nm using a microplate reader.

2.6 Statistical analysis

All experiments were conducted in triplicate with three independent biological replicates (n = 3), each including three technical repetitions. The data presented represent these independent experiments, with statistical analyses performed on the mean values from

these replicates. Cell viability assay data were analysed using the one-way ANOVA followed by *post hoc* Tukey test in the GraphPad Prism v6.0 software. Results are expressed as mean ± standard error of the mean (SEM), with significance determined at $p \leq 0.05$.

3.0 RESULTS

The effect of zerumbone on the viability of differentiated SH-SY5Y cells was tested at several concentrations ranging from 2.0, 4.0, 8.0 and 16.0 µg/mL (**Figure 1**). The results demonstrated that the treatment of zerumbone at the concentrations did not reduce the viability of differentiated SH-SY5Y cells. The cell viability remained above 90%, with a higher viability percentage observed following zerumbone treatment compared to the normal control.

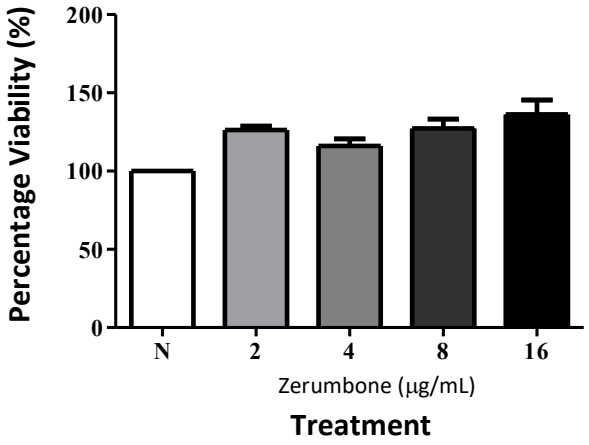

Figure 1: **Effect of zerumbone on the viability of differentiated SH-SY5Y cells.** Differentiated SH-SY5Y cells were treated with zerumbone at concentrations of 2.0, 4.0, 8.0 and 16.0 µg/mL for 24 hours at 37°C with 5% carbon dioxide. The treatment groups were compared with a normal control. The data represent three independent experiments, and the results are expressed in mean ± SEM. The data was analysed via one-way ANOVA. N = normal control.

The anti-neuroinflammatory effects of zerumbone were first studied through the measurement of NO production in LPS-induced SH-SY5Y cells. The cells were treated with four concentrations of zerumbone - 2.0, 4.0, 8.0 and 16.0 µg/mL for 24 hours, followed by the NO quantification by the Griess' assay. In **Figure 2**, it can be observed that all concentrations of zerumbone treated on the LPS-induced cells significantly reduced the production of NO as compared to the LPS-only and vehicle groups. Treatment with zerumbone in LPS-induced cells reduced the NO level to the normal physiological level, compared to the normal control, with no significant differences observed. All concentrations of zerumbone used have shown no significant differences as compared to the positive control group (L-NAME-treated on LPS-induced cells), suggesting zerumbone may have a similar mechanism as L-NAME in reducing NO production.

After observing the potential of zerumbone to reduce NO production, the anti-neuroinflammatory effects of zerumbone were further studied through ELISA assay for the quantification of IL-6 and TNF-α. The half maximal inhibitory concentration value, IC_{50}, was calculated from the NO assay to decide the concentration of zerumbone to be used in ELISA and downstream experiments. The IC_{50} value calculated was 7.902 µg/mL. Hence, 8.0 µg/mL of zerumbone was chosen to be used for ELISA and downstream experiments.

IL-6 and TNF-α are distinct pro-inflammatory mediators highly expressed in neuropathic pain (Kaye et al., 2024; Nashtahosseini et al., 2025). **Figures 3 and 4** showed that the 8.0 µg/mL of zerumbone had reduced the levels of IL-6 and TNF-α in LPS-induced SH-SY5Y cells as compared to the LPS-only and vehicle groups. However, a significant reduction was only observed in the IL-6 level. In contrast, the decrease in TNF-α level by zerumbone was not statistically significant as compared to the normal control, positive control, negative control and vehicle. The reduction of TNF-α level was only significant in the amitriptyline-treated group, as compared to the LPS-only group (**Figure 4**). Zerumbone was observed to significantly reduce the level of IL-6 below the normal physiological level, as compared to the normal control group (**Figure 3)**. **Figure 4** also showed no significant differences between zerumbone and the positive control, amitriptyline-treated group.

4.0 DISCUSSION

Normal physiology observes the role of inflammation in eliminating the noxious stimuli and initiating healing within the injured tissues, involving a wide range of immune cells and cytokines. Firstly, immune responses are triggered upon the activation of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) recognition receptors. These receptors are activated in the presence of infection, tissue injury and stress, which results in the recruitment of local immune cells. These immune cells trigger the release of pro-inflammatory cytokines, chemokines, lipid mediators and purines to recruit other circulating immune cells. Common pro-inflammatory mediators include IL-6, IL-1β, TNF-α, prostaglandins, monocyte chemoattractant protein-1 (MCP-1) and adenosine triphosphate (ATP) (Ellis and Bennett, 2013).

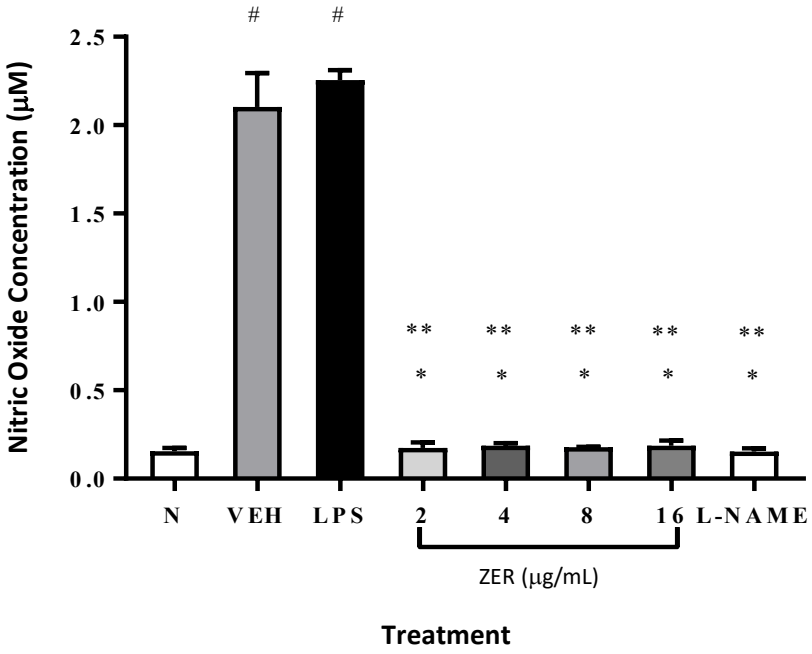

Figure 2: Nitric oxide assay. Differentiated SH-SY5Y cells were induced with 1 µg/mL LPS for 12h, followed by treatment with 2.0, 4.0, 8.0 and 16.0 µg/mL of zerumbone. The treatment groups were compared with a negative control (LPS-only), a normal control (non-treated and non-induced cells), a positive control (L-NAME) and a vehicle group (phosphate-buffered saline). The data represent three independent experiments, and the results are expressed in mean ± SEM. The data was analysed via one-way ANOVA followed by the *post hoc* Tukey test. *p< 0.0001 compared to the LPS-only group, **p<0.0001 compared to VEH; #p<0.0001 compared to N. N = normal control; VEH = vehicle group; LPS = LPS-only group; ZER = Zerumbone group; L-NAME = positive control gorup.

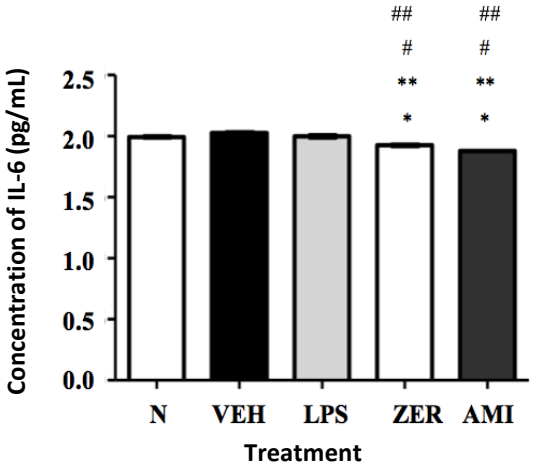

Figure 3: ELISA assay for IL-6. Differentiated SH-SY5Y cells were induced with 1 µg/mL of LPS for 12h, followed by treatment with 8.0 µg/mL of zerumbone. The data represents three independent experiments, and the results are expressed as mean ± SEM. The data was analysed via one-way ANOVA followed by the *post hoc* Tukey test. *p<0.05 compared to LPS; **p<0.05 compared to VEH; #p<0.05 compared to N; ##p<0.05 compared to AMI. N = normal control group; VEH = vehicle group; LPS = LPS-only group; ZER = zerumbone group; AMI = positive control group.

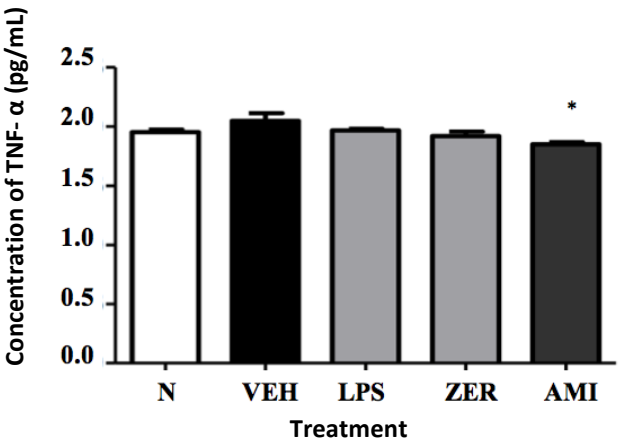

Figure 4: ELISA assay for TNF-α. Differentiated SH-SY5Y cells were induced with 1 µg/mL of LPS for 12h, followed by treatment with 8.0 µg/mL of zerumbone. The data represents three independent experiments, and the results are expressed as mean ± SEM. The data was analysed via one-way ANOVA followed by the *post hoc* Tukey test. *p<0.05 compared to LPS. N = normal control group; VEH = vehicle group; LPS = LPS-only group; ZER = zerumbone group; AMI = positive control group.

Neutrophils, macrophages, helper T cells and microglial cells (in the central nervous system) migrate to the site of injury or stress to enhance the release of pro-inflammatory mediators. The upregulated pro-inflammatory mediators activate the recruited immune cells to perform phagocytosis and degranulation, which are referred to as the cells' effector functions. Following the pro-inflammatory mechanism, there is a switch of mechanism to the resolution of inflammation, in which mediators such as resolvins, lipoxins and protectins are released. During neuroinflammation, degradation of Schwann cells is induced to allow regeneration or healing of nerve cells to take place subsequently. The inflammatory soup results in neuronal sensitisation due to the modulatory effect of the pro-inflammatory mediators on downstream nociceptive receptors and ion channels (Ellis and Bennett, 2013). This phenomenon plays a crucial role in neuropathic pain, a condition characterised by chronic pain due to nerve damage or dysfunction.

Since neuroinflammation plays a prominent role in the pathophysiology of neuropathic pain, LPS was opted for to induce inflammation in the differentiated SH-SY5Y cell culture. LPS is a neurotoxin produced by Gram-negative bacteria, which has been extensively used to induce neuroinflammation in animal models (Kim et al., 2022; Si et al., 2024). Peripheral induction of LPS in *in vivo* models has shown upregulated expression of pro-inflammatory cytokines as well as nerve degeneration due to oxidative stress (Das et al., 2012).

In this experiment, LPS induction prompted a significant increase of nitric oxide in the differentiated SH-SY5Y cells as compared to the non-induced normal control (**Figure 2**). In normal physiology, nitric oxide possesses a wide range of functions, namely regulating vasodilation, energetic mitochondrial respiration, hypoxic nitric oxide signalling, cytoprotection against ischemic stress, host defence and signalling molecules in the nervous system (Kaplish et al., 2024; Lundberg et al., 2008). Nitric oxide is produced by nitric oxide synthase groups, whereas in neuronal cells, NO is produced by inducible and neuronal nitric oxide synthase (Ghimire et al., 2017; Guix et al., 2005; Orfali et al., 2024).

Overproduction of NO gives rise to oxidative stress and pathophysiological effects on the implicated tissues. After nerve injury, NO synthesis is enhanced, shifting its role to a neurodegenerative effect, which results in neuronal death (Freire et al., 2009). The pathophysiological mechanism underlying the upregulation of NO in neuropathic pain was proposed to be due to the activation of the *N*-methyl-*D*-aspartate (NMDA) receptor, which is notable in the development of allodynia and hyperalgesia in neuropathic pain. The activation of NMDA receptors within the post-synaptic neurons results in the influx of calcium ions, which bind to calmodulin. The binding of calcium ions to calmodulin then triggers NO synthase to synthesise NO.

The enhancement of NO production gives rise to the synthesis of neurotransmitters, allowing long-term potentiation to take place (Fronza et al., 2023; Huang, 1997). In addition, NO has also been observed to be able to regulate the synthesis of glutamate in the synapse. In neuronal NO synthase-knockout mouse models, it was reported that the level of *S*-nitrosylation of proteins was significantly reduced. *S*-nitrosylation of proteins is one of the important steps in the cycle of glutamate synthesis, suggesting a retrograde mechanism of NO in regulating glutamate release. Hence, it is important to note that an increase in the synthesis of NO can also increase glutamate release into the synapse (Guix et al., 2005; Oluwole et al., 2022; Raju et al., 2015).

Besides, NO synthesised by inducible nitric oxide synthase (iNOS) is proposed to be modulated through NFκB phosphorylation. The activation of toll-like receptors, such as LPS and pro-inflammatory cytokines, namely IL-1, TNF-α and interferon, triggers a series of signalling cascades that result in the nuclear translocation of NFκB (Guix et al., 2005; Huang, 1997). Abnormal signalling of nitric oxide, synthesised by the upregulated activity of neuronal nitric oxide synthase, results in an immense influx of calcium ions into nerve cells, consequently bringing about excitotoxicity through the increase in glutamate released. This mechanism is coupled with hypoxia, which induces the death of neuronal cells following NO inhibiting cytochrome c oxidase (Förstermann & Sessa, 2012; Lou et al., 2022; Wong-Riley, 2012). Thus, nitric oxide is proposed to be one of the important hallmarks in neuropathic pain pathophysiology.

In **Figure 2**, LPS induction for 12 hours has been observed to significantly increase NO concentration as compared to the non-induced normal control. The mechanism of LPS in inducing NO upregulation was proposed to involve the activation of inducible and neuronal nitric oxide synthase through the mechanisms mentioned above (Anaeigoudari et al., 2016; Yao et al., 2010).

To evaluate zerumbone's inhibitory effect on LPS-induced inflammation, its effect on differentiated SH-

SY5Y cells was first assessed. Since no prior data existed on zerumbone's effect on SH-SY5Y cell viability, reference was made to previous research where 20 µg/mL zerumbone reduced 3T3 cell viability to 20% (Al-Zubairi, 2018). Based on the literature, four concentrations were chosen: 2.0, 4.0, 8.0, and 16.0 µg/mL. **Figure 1** shows that these concentrations did not reduce SH-SY5Y cell viability. The MTT assay further suggested potential modulation of mitochondrial activity by zerumbone, as indicated by a slight increase in SH-SY5Y cell viability (**Figure 1**).

Treatment of zerumbone on LPS-induced SH-SY5Y cells significantly reduced the concentration of NO measured through Griess' assay. The results of the zerumbone treatment group were compared to the positive control L-N^G-Nitro arginine methyl ester (L-NAME), a non-specific nitric oxide synthase antagonist. L-NAME, first developed in the early 90s, was suggested to prevent the conversion of L-arginine to L-citrulline and NO by directly inhibiting the isoforms of nitric oxide synthase (Kopincová et al., 2012; Pfeiffer et al., 1996). In the L-NAME-treated group, there was a similar effect on NO concentration post-LPS induction as compared to the zerumbone-treated group, in which a significant reduction was observed (**Figure 2**). This proposed that the anti-oxidative effect of zerumbone in reducing NO concentration may involve the inhibition of NO synthase. With regards to the interaction of NO synthesis with other pro-nociceptive receptors and ion channels, this could suggest that zerumbone may possess a modulatory effect on the receptors and ion channels through its antagonising effect on nitric oxide synthase. In animal models, inhibiting nitric oxide synthase attenuated hyperalgesia and allodynia (Kaswan et al., 2020; Levy and Zochodne, 2004). Moreover, the treatment of antioxidant compounds in neuropathic pain rat model exhibited a reduction in nitric oxide synthase expression, in addition to the attenuation of thermal hyperalgesia and mechanical allodynia (Jia et al., 2019).

Another prominent hallmark of neuropathic pain, as mentioned above, is the pro-inflammatory cytokines. The coordination and function of pro-inflammatory cytokines are intricated, whereby the cytokines may have a pleiotropic effect, different types of cytokines may produce the same effect or have the same role, and the action of cytokines can be synergistic or antagonistic (Zhang and An, 2007). Pro-inflammatory cytokines are involved both in the early stages of the development of neuropathic pain as well as the maintenance of the condition. Peripheral nerve injury due to diseases or chemotherapy triggers the production of peripheral pro-inflammatory cytokines. Nerve injury also prompts the upregulation of neurotransmitters, proteases, Wnt ligands and chemokines through the activation of immune cells (both CNS and PNS). The cascades of pro-inflammatory mediators signalling ensued, resulting in the abnormal signalling of pro-nociceptive neurotransmitters and the sensitisation of receptors and ion channels. This neuron-glial interaction is not only linked by the pro-inflammatory mediators but also by the modulation of the mitogen-activated protein kinase (MAPK) pathway.

Tumour necrosis factor-α (TNF-α) is widely studied for its role in the pathophysiology of neuropathic pain. In chemotherapy-induced neuropathy, an elevated level of serum TNF-α was identified, while patients receiving TNF-α as anti-cancer treatment also reported neuropathic pain (Leung and Cahill, 2010; Mattar et al., 2024). In the chronic constriction injury rat model, administration of TNF-α to the dorsal root ganglion (DRG) showed retrograde distribution at the dorsal horn on top of the local distribution at DRG. The dispersal of TNF-α following direct administration also precipitates the development of allodynia on the injured and adjacent uninjured nerves in the spinal nerve ligation (SNL)-induced neuropathic pain rat model (Schäfers et al., 2003; Shubayev and Myers, 2002; Tian et al., 2024). In the central nervous system, administration of small inhibitory RNA against TNF mRNA within the hippocampal region resulted in the reduction of thermal hyperalgesia in the CCI-induced neuropathic pain rat model (Gerard et al., 2015). This supported the notion that TNF within the higher-brain centre also plays a role in the modulation of pain, along with the maintenance of neuropathic pain.

Understanding the cytokine network enables the understanding of communication and inter-regulatory modulation of TNF-α with other prominent cytokines in the pathophysiology of neuropathic pain. The upregulated expression of TNF-α following nerve injury enhances the activation of TNF receptors. The amplification of TNF-α expression was due to the production, which is not exclusive to immune cells. TNF-α is also produced by other cells, whereby in the neuropathic pain mechanism, TNF-α are also produced by neuronal cells (Olmos and Lladó, 2014; Tanabe et al., 2010). The binding of TNF-α to the receptor instigates the phosphorylation of I-kappa B kinase (IKK), which brings about the phosphorylation of inhibitor of kappa B alpha (IκBα). This is followed by the degradation of IκBα induced by the proteasome. Degradation of the

IκBα subunit enables the initially bound NFκB subunits, namely RelA and p50, to be translocated into the nucleus and activate NFκB (Liu et al., 2017). Subsequently, the activation of NFκB amplifies the production of more pro-inflammatory cytokines such as IL-6 and IL-1β, chemokines such as MCP-1 and enzymes, namely iNOS (Lin et al., 2014; Liu et al., 2017). In the spinal nerve transection-induced neuropathic pain mouse model, silencing of TNF-α within the dorsal root ganglion region attenuates mechanical allodynia in addition to the suppressed expression of TNF-α in the ipsilateral DRG (Ogawa et al., 2014).

The interlinkage of neuroinflammation and receptors and ion channel sensitisation also mediate the development of neuropathic pain. The enhanced expression of TNF-α can give rise to the activation and/or upregulation of downstream pro-nociceptive receptors and ion channels involved in the development of allodynia and hyperalgesia (Wei et al., 2021). The binding of TNF-α to its receptor causes the activation of the MAPK pathway. Upon activation of the MAPK pathway, a series of signalling cascades takes place, resulting in the activation of pro-nociceptive receptors and ion channels, as well as the enhancement of neurotransmitter synthesis to amplify the pain signal. It was hypothesised that following the activation of the TNF receptor, the MAPK pathway is activated, causing the transient receptor potential subtype V1 (TRPV1) channel to increase in co-trafficking to the membrane and sensitisation of the channel. Consequently, there is calcium ion influx, hence amplifying the release of glutamate into the synapse (Park et al., 2011). Besides, the treatment of TNF-α in hippocampal neurons triggers the exocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and causes the endocytosis of inhibitory γ-aminobutyric acid (GABA) receptors (Heir and Stellwagen, 2020).

To mimic the neuroinflammation in neuropathic pain, LPS was chosen as the inducing agent. Although it is understood that the mechanism of neuroinflammation involves not only neuronal cells but also immune cells, which play a vital role in modulation, this study aims to elucidate the effect of zerumbone on pro-inflammatory mediators produced by neuronal cells through the monoculture of SH-SY5Y cells. The results in **Figures 3** and **4** showed the effect of zerumbone on pro-inflammatory cytokines, which are TNF-α and IL-6. The induction of 1 μg/mL of LPS for 12 hours slightly increased the concentration of both TNF-α and IL-6 as compared to the non-induced normal control (average difference of about 0.09 μM for TNF-α and about 0.03 μM for IL-6). As compared to the NO concentration, slight changes were observed in the LPS-induced culture whereby the increase of inflammatory cytokines was not statistically significant. Understanding the pathophysiology of neuropathic pain and the mechanism of LPS could provide insights to elucidate the underlying mechanism of LPS-induced SH-Y5Y cells. The main player of neuroinflammation in the neuropathic pain mechanism involves the action of immune cells in both the peripheral (macrophage, T cells and neutrophils) and central nervous systems (astrocytes and microglial cells) (Ellis and Bennett, 2013). The major source of pro-inflammatory cytokines is proposed to be higher in these immune cells as compared to neuronal cells, hence explaining the low production and slight difference post-induction of pro-inflammatory cytokines in LPS-induced SH-SY5Y cells. In another study, LPS induction in the monoculture of SH-SY5Y cells showed a significant increase of TNF-α and IL-6 concentration as compared to the non-induced normal control, taking into account that the concentrations of the cytokines were relatively low as compared to the production of cytokines by other immune cells (Pandur et al., 2018). This could be due to the higher concentrations of LPS used in addition to the longer induction period, which was 1 μg/mL of LPS for 24 hours, as compared to our study. Despite the minimal changes post-LPS induction, the effect of zerumbone on the concentration of the pro-inflammatory cytokines was significant in IL-6 as compared to the non-induced normal control and the LPS-only negative control. Whereas the effect of zerumbone on TNF-α concentration slightly decreased with no statistical significance as compared to the non-induced normal control and the LPS-only negative control.

The mechanism of the anti-neuroinflammatory effect of zerumbone could be due to the direct inhibition of zerumbone against TNF-α, which further downregulates the expression of IL-6. As discussed earlier, the inhibition of TNF-α from binding to its receptor prevents the phosphorylation of protein kinases, which in turn inhibits the activation of the NFκB pathway (Li et al., 2020). This inhibition results in the downregulation of other pro-inflammatory mediators such as IL-6. In a molecular docking study, zerumbone was analysed to be able to bind to TNF-α and inhibit the binding to the receptor, as compared to a known crystalised ligand (Fatima et al., 2018). In the CCI-induced neuropathic pain mouse model, administration of zerumbone was observed to suppress the expression of TNF-α and IL-6,

thus attenuating allodynia and hyperalgesia (Gopalsamy et al., 2017).

Another proposed anti-neuroinflammatory mechanism of zerumbone is via the inhibition of toll-like receptors (TLRs). In LPS-induced THP-1 cells, a monocyte-macrophage cell line, the treatment of zerumbone significantly reduced the expression level of TLR2 and TLR4 receptors (Kim and Yun, 2019). In the same experiment, treatment with zerumbone also reduced the mRNA expression level of NF-κB and MyD88, an activator protein in the TLR pathway. This was also supported in a study using a CCI-induced neuropathic pain rat model, whereby the administration of TLR2 and TLR4 antagonists attenuated allodynia and hyperalgesia, suggesting the role of TLR2 and TLR4 receptors in mediating neuropathic pain (Jurga et al., 2016).

To further support the anti-neuroinflammatory properties of zerumbone in LPS-induced SH-SY5Y cells via the previously delineated mechanisms, it is important to understand the mechanism of action of the positive control, amitriptyline. Amitriptyline, a commonly used drug to treat neuropathic pain, was known to possess wide-ranging inhibitory properties in targeting proteins and mediators in the nervous system. Although amitriptyline is a tricyclic antidepressant, it also possesses anti-inflammatory and anti-nociceptive properties (O'Neill et al., 2016). Amitriptyline was proposed to inhibit TNF-α synthesis, but not TNF-α mRNA in rat glial cell culture (Obuchowicz et al., 2006). Besides, amitriptyline was also observed to inhibit TLR4 receptor expression in a dose-dependent manner in a HEK-TLR4 transfected cell line (Hutchinson et al., 2010). In **Figures 3** and **4**, the effect of zerumbone on LPS-induced SH-SY5Y cells was similar to the reduction of TNF-α and IL-6 concentration by amitriptyline. Hence, this observation supports the comparable mechanism of zerumbone to amitriptyline.

While the results suggest a promising potential for zerumbone in modulating inflammatory pathways, it is acknowledged that the inherent limitations of *in vitro* systems may not fully replicate the complexity of biological processes within a living organism. The primary reason for utilising *in vitro* models was to create a controlled environment where we could carefully examine zerumbone's role in modulating neuroinflammation, free from the complexities introduced by systemic factors *in vivo*. By using such a model, it is ensured that the experimental conditions were precisely controlled, allowing the effects of zerumbone on specific neuroinflammatory pathways to be isolated.

The findings from this study, supported by comparisons with existing literature from animal models such as the CCI model, indicate the potential of zerumbone in modulating neuroinflammation. However, while *in vitro* studies have limitations, they remain a critical component of preclinical research. *In vitro* models provide invaluable insights by enabling researchers to examine specific interactions and pathways under well-defined conditions.

While the focus of this study is primarily on the neuronal modulation by zerumbone, it is now recognised that immune cells play a pivotal role in the inflammatory processes underlying neuropathic pain. Therefore, there is a need to explore co-culture models integrating both neuronal and immune cells, to investigate the crosstalk between these cell types and better understand their joint contributions to neuroinflammation and neuropathic pain.

5.0 CONCLUSIONS

The role of reactive oxygen species, such as NO, and pro-inflammatory cytokines, such as TNF-α and IL-6, has been proposed to be involved in the development and maintenance of neuropathic pain. The modulation of nitric oxide, IL-6 and TNF-α has been observed to be able to alleviate neuropathic pain in an *in vivo* model. Similar to the effects of L-NAME, a known nitric oxide synthase inhibitor, zerumbone significantly reduced NO levels in our *in vitro* models. Additionally, like amitriptyline, which inhibits TNF-α synthesis and TLR4 receptor expression, zerumbone demonstrated a comparable reduction in pro-inflammatory cytokines. The anti-neuropathic pain mechanism of zerumbone was proposed to be able to alleviate neuropathic pain through the reduction of nitric oxide, IL-6 and TNF-α expression. Importantly, these *in vitro* results provide preliminary but compelling evidence for the potential of zerumbone as a therapeutic agent for neuropathic pain. By targeting key neuroinflammatory pathways, zerumbone may offer clinical benefits either as an adjunct to existing pharmacological treatments or as a novel natural compound for patients who are unresponsive or intolerant to conventional therapies. Its plant-derived origin also aligns with the increasing demand for safer, naturally derived pain management options with fewer side effects. Nevertheless, given the complex pathophysiology of neuropathic pain, it is essential to recognise that additional cellular players, particularly microglial cells, contribute significantly to

the production of inflammatory mediators. Hence, future studies may need to consider observing the mechanism of action of zerumbone on the mediators synthesised by the immune cells.

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