Research Article

Anti- neuroinflammatory activity of *Etlingera pavieana* rhizomal extract in LPS-induced microglial cells

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Abstract

Etlingera pavieana (Pierre ex Gagnep) R.M. Sm., a plant in family Zingiberaceae, has long been used as a spice and a traditional medicine in the East of Thailand. Previous studies have shown that *E. pavieana* rhizomes inhibited inflammatory response in mouse macrophage cells and human endothelial cells. However, the anti-inflammatory activity of *E. pavieana* rhizome in microglial cells is yet to be demonstrated. Thus, the aim of this study was to evaluate the anti-inflammatory effect of ethanol extract from *E. pavieana* rhizomes (EPE) in lipopolysaccharide (LPS)-induced BV2 microglial cells. Anti-inflammatory activity was evaluated by measurement of the production of inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandins E_2 (PGE_2). Cell viability was evaluated by MTT assay. The level of inflammation-responsive protein and mRNA was investigated by Western blot analysis and qRT-PCR, respectively. EPE at non-cytotoxic concentrations (25 to 100 µg/mL) reduced NO and PGE_2 production in a concentration-dependent manner. Furthermore, EPE significantly inhibited the expression of iNOS and COX-2 at protein and mRNA levels. In addition, EPE suppressed the phosphorylation of inhibitor of nuclear factor-ΚB (NF-ΚB). Taken together, our results indicate that EPE exhibits the anti-inflammatory effect in microglial cells in part by inhibiting of the production of NO and PGE_2 as well as the expression of iNOS and COX-2 via suppressing nuclear factor-ΚB activation. The ethanol extract from the *E. pavieana* rhizome has the potential to be used as a functional food ingredient and dietary supplements for preventing neuroinflammation-related diseases.

Keywords: Anti-inflammatory activity, *Etlingera pavieana*, Microglial cell, Nitric oxide, Prostaglandins E_2

Introduction

Neuro- inflammation is an immune response of the brain's innate immune cells to harmful stimuli within the central nervous system (CNS) mediated by the production of inflammatory mediator such as nitric oxide (NO) and prostaglandin E_2 (PGE_2). [1,2,3] Microglia, a resident immune cell in CNS, can be activated by several stimuli such as bacterial lipopolysaccharide (LPS), β- peptide and neurotoxin to secrete inflammatory mediators. [4,5] However, the excessive and chronic production of these mediators leads to loss of function and degeneration of neurons resulting in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease. [6] Therefore, the inhibition of these inflammatory mediators may be an alternative approach to prevent neurodegenerative diseases.

NO is a free radical signaling molecule that can be synthesized from L-arginine by nitric oxide synthase (NOS). NOS are classified into three types; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). [7,8] PGE_2 is the most abundant PGs in a body synthesized from arachidonic acid by the action of cyclooxygenase (COX). [9] Two isozymes of COX have been identified; COX-1 is a constitutive form and COX-2 is inducible form can be induced by many stimuli like LPS and pro- inflammatory cytokines. [10] Synthesis of NO and PGE_2 in microglial cells are catalyzed by iNOS and COX-2, respectively. [8,9] The expression of LPS- induced iNOS and COX-2 genes are regulated by nuclear factor-kappa B (NF-κB) transcription factor. [11] The ubiquitous heterodimers of NF-κB compose of p65 (RelA) and p50 subunits which interact with inhibitory protein of NF-κB (IκB). Upon stimulation with stimulant, IκB inhibitor is phosphorylated by IκB-kinase (IKK) on Ser32 and Ser36 leading to ubiquitination and proteasomal degradation. Then liberated NF-κB dimers translocate into the nucleus and bind to a promotor region of target genes and enhance their transcription. [12]

Etlingera pavieana (Pierre ex Gagnep) R. M. Sm., a plant in family Zingiberaceae, distributes in southeastern Thailand. The rhizome is commonly used as food, spice and medicine for treatment of carminative, diuresis, dyspepsia, flatulence and nausea. [13] Recently, anti-inflammatory activity of *E. pavieana* rhizome has been reported in LPS- stimulated RAW 264.7 macrophages through suppression of iNOS expression and NO production. [14] Recently, Srisook et al. (2019) exhibit anti- vascular inflammatory effect of *E. pavieana* rhizome in TNF-α- induced human endothelial cells. [15] The rhizome suppressed the expression of proteins. Moreover, it inhibited intracellular ROS production in endothelial cells. [16] However, inhibitory activity on neuro- inflammation of rhizomal extract from *E. pavieana* remains unknown. Thus, in the present study, we aimed to evaluate the anti- inflammatory activity of ethanol extract from *E. pavieana* rhizome in LPS-induced microglial cells to compile scientific evidence supporting the use of *E. pavieana* plant for preventing neuro-inflammation in neurodegenerative disorders.

Materials and Methods

Materials

Lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4 (Sigma, USA.). Tetrazolium salt 3-(4,5- dimethylthiazol- 2- yl)- 2,5- diphenyltetrazolium bromide (MTT) (Sigma, USA.). Prostaglandins E_2 (PGE_2) Enzyme Immunoassay Kit (Arbor Assay, USA). Antibodies of iNOS and COX- 2 (BD Bioscience, USA). Phosphatase

inhibitor cocktail, protease inhibitor cocktail and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, USA). NucleoSpin RNA (Macherey-nagel. Germany). 2x iTaq™ Universal SYBR Green Supermix and iScript Reverse Transcription Supermix (Bio-Rad, USA). Antibodies for IκBα, p-IκBα (Ser32/36), GAPDH, anti-mouse IgG: HRP-linked antibody and anti-rabbit IgG: HRP-linked antibody (Cell Signaling Technology, USA).

Extract preparation

Ethanol extract of *E. pavieana* rhizomes (EPE) was prepared as described by Srisook et al. [14] It was dissolved in dimethyl sulfoxide (DMSO) and filtrated through a 0.22 μm nylon syringe filter. In our previous study, HPLC profile of EPE was reported and HPLC analysis revealed the presence of *trans*-4-methoxycinnamaldehyde (MCD) and 4-methoxycinnamyl *p*-coumarate (MCC) as one of major component. [15]

Cell culture

BV2, a murine microglia cell line was obtained from Interlab Cell Line Collection (ICLC) (Genova, Italy) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in a humidified air containing 5% of CO_2 at 37°C. Cells were sub-cultured by scraping.

Cell viability assay

The effect of EPE on cell viability was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This method was performed as described by Srisook et al. [17] Briefly, cells were treated with EPE at various concentration for 24 h and fresh DMEM containing MTT (0.1 mg/mL) was added to each well and further incubated for 2 h. After that, the culture media was removed and formazan crystals was dissolved by DMSO. The absorbance of solution in each well was measured at 550 nm using microplate reader.

Determination of NO and PGE_2 production

BV2 cells (1.5×10^5 cells/well) were cultured in 24-well plate for 18 h. Then cells were pre-treated with EPE for 1 h before incubation with LPS (1 μg/mL) for 24 h. Amount of nitrite, a stable oxidation product of NO, in culture media was determined by Griess reagent. The level of PGE_2 in culture media were evaluated using ELISA kit according to the manufacturer's instructions.

Western blot analysis

Cells (1×10^6 cells/plate) were cultured in 60-mm plates for 18 h and pre-treated with DMEM containing EPE for 1 h before stimulation with LPS for 24 h. Whole cell protein was extracted with ice-cold RIPA lysis buffer. The protein concentrations were determined by Bradford protein assay (Bio-Rad, USA). Protein extracts were electrophoresed in 10% SDS-PAGE and separated proteins were transferred to PVDF membranes. The membranes were blocked with 5% skim milk solution, then, incubated with specific primary antibodies followed by a secondary antibody conjugated to horseradish peroxidase. Afterwards, specific protein bands were detected on an X-ray film by chemiluminescence using SuperSignal West Pico chemiluminescence. The band intensities were quantified by Image Studio Lite 5.2 Quick Start Guide program for windows.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated by NucleoSpin RNA according to the manufacturer's instructions

and measured absorbance using Microvolume Spectrophotometer (NanoDrop). Two micrograms of RNA were converted to complementary DNA using iScript Reverse Transcription Supermix. qRT-PCR reaction was conducted on CFX96 Touch Real time PCR (Bio-Rad, USA) using 2x iTaq™ SYBR Green Supermix. The sequences of the PCR primers which used for the iNOS, COX-2 and elongation factor-2 (EF-2) were as follows: iNOS, 5'-GCACAGCACAGGAAATGTTTCAGCAC-3' (F) and 5'-AGCCAGCGTACCGGATGAGC-3' (R); COX-2, 5'-TGATCGAAGACTACGTGCAACACC-3' (F) and 5'-TTCAATGTTGAAGGTGTCGGGCAG-3' (R); EF-2, 5'-CTGAAGCGGCTGGCTAAGTCTGA-3' (F) and 5'-GGGTCAGATTTCTTGATGGGGATG-3' (R). Relative gene expression was normalized to EF-2 as a housekeeping gene and calculated using $2^{-\Delta\Delta_{ct}}$ to compare the mRNA level between the control and the treated cells.

Statistical analysis

Data were analyzed for statistical significance by Minitab 17 for Windows. Statistical significant difference of extract concentration was determined via one-way analysis of variance (ANOVA), followed by Tukey's method for multiple comparison. A value of $p<0.05$ will be considered significant.

Results

Effect of EPE on cell viability in BV2 microglial cells.

Percentage viability of cells treated with EPE at concentration 200 µg/mL was reduced to 31.70±3.32% of unstimulated control cells **(Figure 1)**, while EPE at concentration 6.25-100 µg/mL did not significantly alter cell viability. Thus, further experiments were performed using EPE at concentrations up to 100 µg/mL.

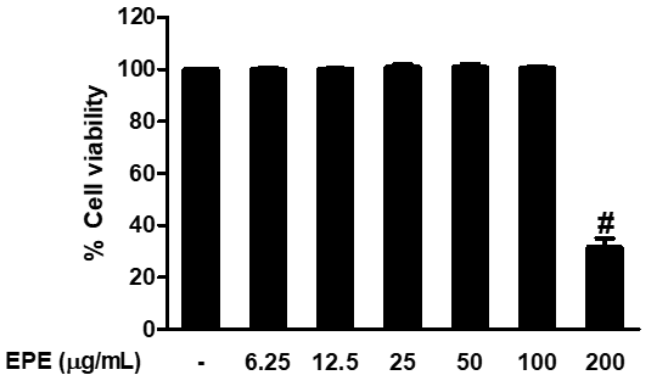

Figure 1: The effect of EPE on the viability of BV2 microglial cells. Cells were treated with EPE at concentration of 6.25-200 µg/mL for 24 h. Each column shows a mean±SD of three independent experiment with triplicated samples. #$p<0.05$ compared with unstimulated control group.

Effect of EPE on NO and PGE_2 production in LPS-induced BV2 microglial cells.

The production of NO and PGE_2 was used as an index of inflammatory response in LPS-induced BV2 microglial cells. The levels of NO and PGE_2 markedly increased in cells-treated with LPS compared with unstimulated control cells. Meanwhile, NO production was significantly reduced by EPE (25- 100 μg/ mL) in a concentration- dependent manner compared with LPS- stimulated cells. Aminoguanidine, an iNOS inhibitor, was used as a positive control and suppressed LPS- induced NO production by 78.59±4.33% **(Figure 2A).** In addition, EPE (25-100 μg/ mL) significantly suppressed PGE_2 production in a concentration- dependent manner. Indomethacin, a COX-2 inhibitor used as a positive control strongly inhibited the production of PGE_2 by 95.13±1.67 % **(Figure 2B).**

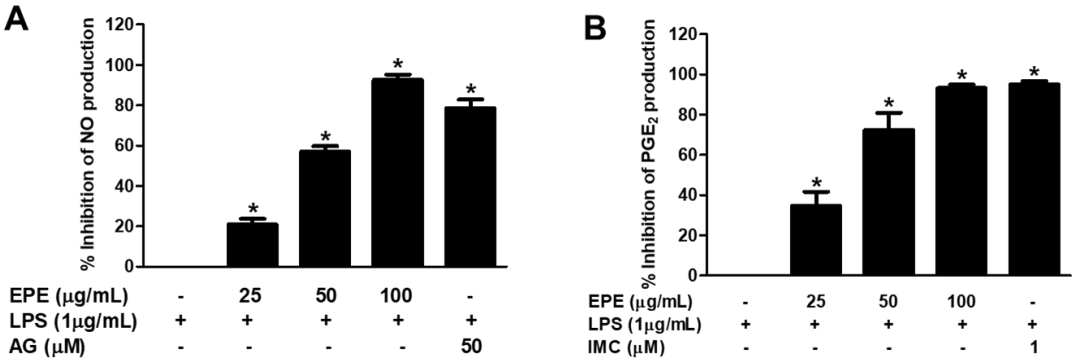

Figure 2: Inhibitory effect of EPE on the production of NO (A) and PGE_2 (B) production in LPS-induced BV2 cells. Microglial cells were pre-treated with EPE for 1 h before stimulation with LPS (1 μg/mL) for 24 h. Each column shows a mean±SD of three independent experiment with triplicated samples. #p<0.05 compared with unstimulated control group, * p<0.05 compared with LPS- stimulated group. AG; aminoguanidine, IMC; indomethacin.

Suppressive effect of EPE on iNOS and COX-2 expression in LPS-induced BV2 microglial cells.

To investigate the mechanism underlying inhibitory activity of EPE on NO and PGE_2 production, Western blot analysis was carried out. The levels of iNOS and COX-2 protein increased in LPS- stimulated cells **(Figure 3A and 3B).** The expression of iNOS protein was remarkably suppressed by EPE in a concentration- dependent manner and EPE at 50- 100 μg/ mL significantly decreased COX-2 protein expression. Furthermore, qRT- PCR analysis data showed the correlation between mRNA levels and the corresponding proteins. EPE significantly concentration-dependently decreased iNOS and COX-2 mRNA expression **(Figure 3C and 3D).**

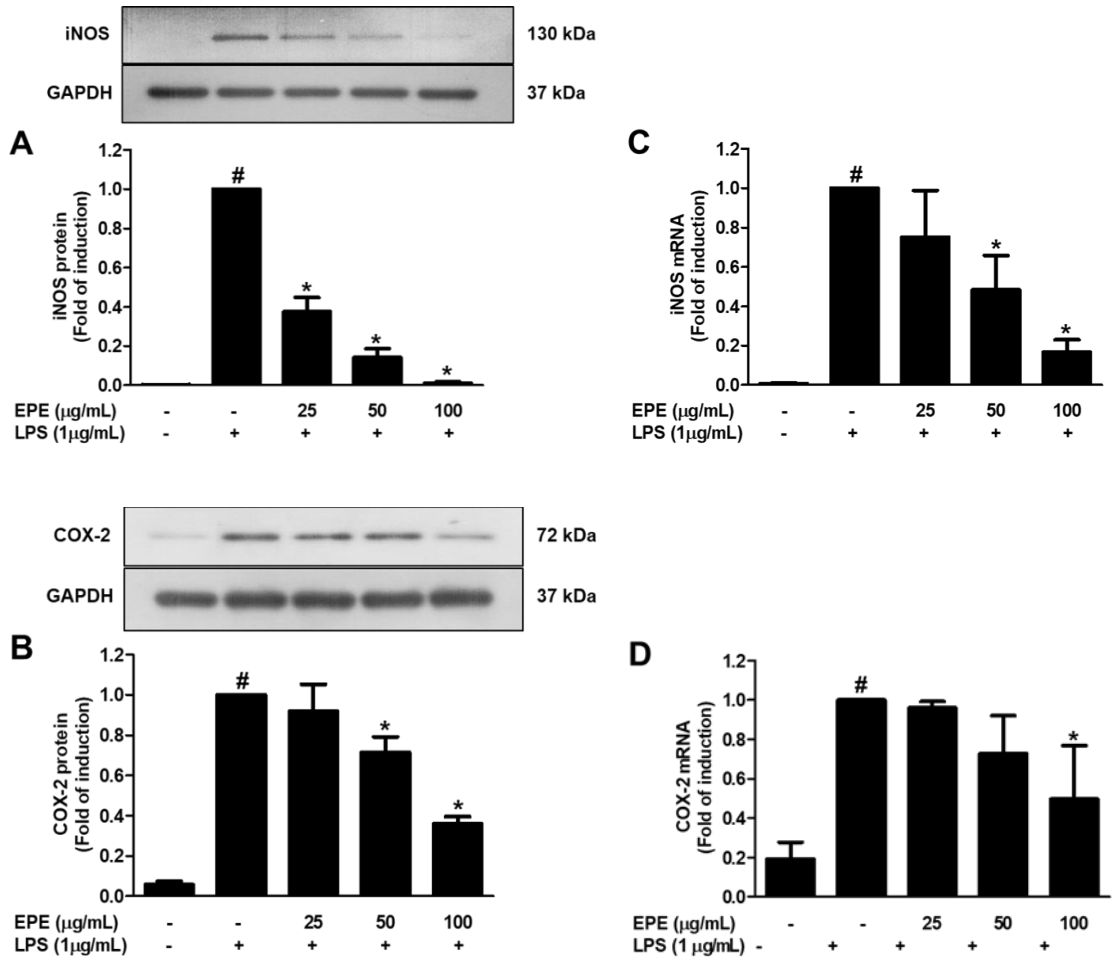

Figure 3: Effect of EPE on iNOS and COX-2 expression in BV2 microglial cells. Cells were pre-treated with EPE for 1 h before stimulation with LPS for 9 h and 24 h for determination of mRNA and protein, respectively. The protein level of iNOS (A) and COX-2 (B) was investigated by Western blot analysis. The mRNA expression of iNOS (C) and COX-2 (D) determined by qRT-PCR. Each column shows a mean±SD of densitometric values. Data are expressed as fold change with respect to cells treated with LPS only. #p<0.05 compared with unstimulated control group, *p<0.05 compared with LPS-stimulated group.

Effect of EPE on NF-κB activation in LPS-induced BV2 microglial cells.

Since EPE exhibited the inhibitory effect on LPS-induced expression of iNOS and COX-2, we determined further such inhibitory effect might be occurring through activation of NF-κB signaling pathway. Western blot analysis showed the level of phosphorylated IκBα was increased while total IκB protein was decreased in cells stimulated with LPS. EPE significantly suppressed LPS-induced phosphorylation of IκBα. Furthermore, EPE did not restore the level of total IκBα protein **(Figure 4).** BAY 11-7082, a NF-κB inhibitor, was used as a positive control.

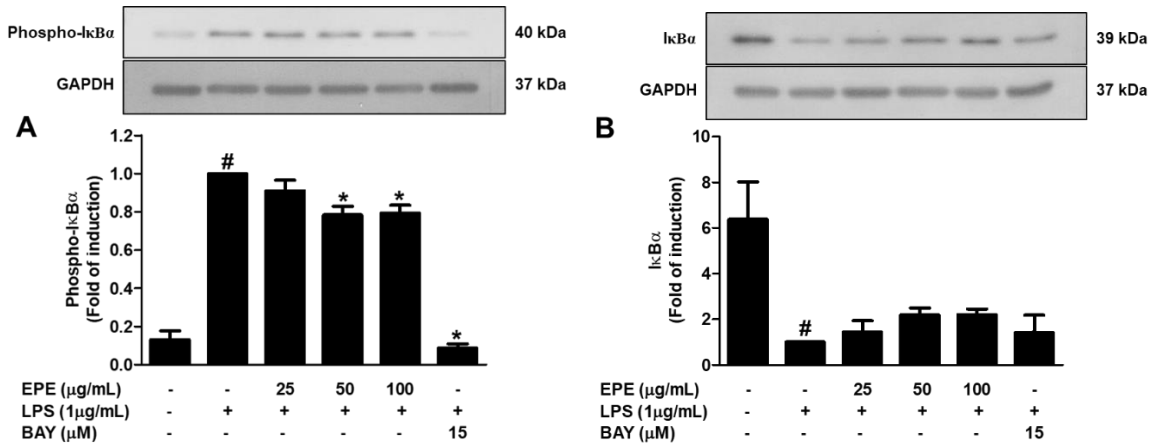

Figure 4: Inhibitory effect of EPE on NF-ΚB activation in BV2 microglia. Cells were pre-treated with EPE for 1 h before exposure to LPS for 30 min. Amounts of p-IΚBα (A) and total IΚBα (B) in whole cell protein extract were determined by Western blot analysis. Each column shows a mean±SD (n = 2) of densitometric values and normalized with GAPDH. #p<0.05 compared with unstimulated control group, *p<0.05 compared with LPS-stimulated group. BAY; BAY-117082.

Discussion and Conclusion

Reducing inflammatory mediator secretion is another way of preventing neuro-inflammation which is one cause of neurodegenerative diseases. Currently, the treatment options for neurodegenerative disease are focused on the alleviation of symptoms and slow the progression of the diseases. In addition, drugs available to treatment can cause in several side-effects. [18,19] Therefore, the discovery of natural agents having a potential to inhibit of inflammatory mediator production has gained an interest from researchers.

In this study, LPS-induced BV2 cell line was used as a study model for assessing inflammatory response of microglia cells by analyzing the amount of inflammatory mediators. The results revealed that EPE at 25-100 μg/mL can reduce the production of NO and PGE_2 without reducing cell viability compared to control cells. It elucidated that the reduction in NO and PGE_2 amount was not attributed to cytotoxicity of EPE. NO and PGE_2 produced in LPS-activated microglial cells are generated by the regulation of iNOS and COX-2 enzymes, respectively. [8,10,20] Thus, EPE was evaluated the effect on iNOS and COX-2 expression in BV2-microglial cells. EPE at test concentrations decreased the expression of iNOS and COX-2 enzymes in both mRNA and protein levels. Our data indicated that EPE inhibit NO and PGE_2 production by suppressing the expression of iNOS and COX-2 mRNA and protein. It should be noted that the degree of PGE_2 suppression by EPE was higher than that COX-2 protein expression. This appears to be due not only to diminished expression of COX-2 but also to inhibited its activity. However, further experiment is needed to clarify this point.

NF-ΚB is a transcription factor that has been known to play a major role in the regulation of genes associated with inflammation in microglia activation such as iNOS and COX-2. [11,21] In the present study, we found that EPE reduced phosphorylation on IΚBα but not restore IΚBα degradation. Thus, the anti-inflammatory mechanism of EPE in mouse microglia BV2 cells

was partially due to inactivation of the NF-κB signal pathway by reducing IκBα phosphorylation resulting in suppressing the transcription process of iNOS and COX-2 genes. The result of the present study is in the line with our previous study reported that *E. pavieana* rhizomes suppressed NF-κB activation in LPS-induced RAW 264.7 macrophages [14] and in TNF-α-induced human endothelial cells. [15]

In conclusion, our results provide scientific evidence that *E. pavieana* rhizomes exhibits an anti-inflammatory effect in LPS-induced BV2 microglial cells in part by inhibition of NO and PGE_2 production. The mechanism underlying this inhibitory effect was mediated through reduction of iNOS and COX-2 expression which might be occurring via suppressing NF-κB activation. We demonstrated that *E. pavieana* plant is a promising one to be developed as plant-based functional ingredient and dietary supplement. However, further studies are required to clarify the effectiveness *E. pavieana* rhizomes in animal model of neuro-inflammation and perform standardization of the plant extract.

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