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Research Article

Oroxylum indicum (L.) Fruits Extract Suppresses BV2 Microglial Activation Through Inhibition of NF-κB and Akt/ERK1/2 Pathway

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Abstract

Background and Objective: *Oroxylum indicum* (L.) Kurz, a plant distributed throughout India and Southeastern Asia. Different parts of the plant applied traditionally for the treatments. The present study attempted to elucidate the effect of OIF extract on NO and ROS production as well as the transcription factor that controls the expression of the pro-inflammatory gene. The molecular mechanisms of OIF on Lipopolysaccharide (LPS)-induced microglial activation were also investigated. **Materials and Methods:** The BV2 microglial cells were incubated with LPS in the OIF extract's absence or presence. The quantity of NO and ROS was determined by using Griess reagent assay and CM-H2DCFDA, respectively. The NF-κB p65 nuclear levels were detected by using an immunofluorescence assay. **Results:** Treatment with OIF extract (25 and 50 μg mL^{-1}) in the BV2 microglial cells were found to be reduced the NO production enhanced with LPS in a concentration-dependently. Moreover, a high ROS level was induced with LPS, which was also significantly reduced after OIF treatment in a concentration-dependent manner. Mechanistically, we found that OIF extract could alleviate inflammatory response by inhibiting NF-κB p65 translocation and activating via the pathway of Akt and extracellular-signal-regulated kinase 1/2 (ERK1/2) in the LPS-stimulated BV2 cells. **Conclusion:** Results concluded that OIF extract exerts anti-oxidative activity by ROS suppression as well as anti-inflammatory activity by NO inhibition in the activated BV2 microglial cells. This could inhibit Akt/ERK1/2-mediated NF-κB pathway. The OIF extracts may act as a promising functional food to allay neuroinflammation in neurodegenerative diseases.

Key words: Akt, microglia, ERK1/2, NF-κB, neuroinflammation, *Oroxylum indicum* (L.) fruits

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neuroinflammation and oxidative stress are pathologic factors that critically contribute to the pathogenesis of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), Multiple Sclerosis (MS) and Parkinson's Disease (PD)[1-4]. Earlier investigations show that microglia, resident immune cells of the Central Nervous System (CNS), also play the same important role[1,3-6]. Although, acute microglial activation contributes to diminishing further injury process and the repair of damaged tissues[7,8], stimulation of chronic microglial is harmful to neuronal survival through the release of pro-inflammatory mediators, such as nitric oxide (NO), proinflammatory cytokines such as Interleukin 1 beta (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α), including the generation of Reactive Oxygen Species (ROS)[9-11]. Thus, to decrease the proinflammatory generation and neurotoxic factors, attenuation of microglial activation is possibly prevented or treated neurodegenerative diseases.

Oroxylum indicum (L.) Kurz, a plant in the Bignoniaceae family, is distributed throughout India and Southeastern Asia. Various parts of the plant have been applied traditionally for treatments in Japan and China[12]. Among *Oroxylum indicum* (L.) Kurz, *Oroxylum indicum* (L.) fruits (OIF) are rich in nutrients[12] and they have been used for thousands of years as part of plant-based diets and herbal medicines in several countries, without any known adverse effects[12-14]. The OIF also contains flavonoids such as oroxylin A, baicalein and chrysin, all of which have been reported to have multiple biological effects. The pharmacological effects of OIF and compounds isolated from the fruit exhibit hepato-protective, anti-diabetic, anti-adipogenesis, anti-oxidant and anti-inflammatory properties[14-17]. A previous study demonstrated that OIF extract could prevent the Aβ25-35-stimulated SH-SY5Y cell injury[18]. Furthermore, OIF extract was found to reduce reactive oxygen species and inflammatory cytokines in activated BV2 cells[19]. Although, several pharmacological beneficial effects of OIF extract have recently been reported, its molecular mechanisms on microglial activation have never been elucidated. Hence, this report investigated the effects of an ethanol extract of OIF on the NO and ROS production as well as the transcription factor that regulates the expression of the gene involved in pro-inflammation with a focus on the underlying molecular mechanisms in BV2 microglial cells stimulated by LPS.

MATERIALS AND METHODS

Study area: The studies were carried out at the Faculty of Medicine, Mahasarakham University, Thailand, from January, 2021 to March, 2022.

Preparation of OIF extract: The OIF was prepared following previous study[18,20].

Cell culture: The murine BV2 microglial cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin-streptomycin in 5% fetal bovine serum (FBS)[19]. All of the cell culture reagents were procured from Hyclone (South Logan, UT).

Evaluation of cell viability: A day after OIF extract treatment at different concentrations of 0-50 μg mL^{-1}, with or without LPS, the incubation medium was discarded. The MTT reagent (0.4 mg mL^{-1}) (Sigma Co St., Louis, MO) in serum-free DAEM was introduced to each well. Then, the MTT medium was discarded after incubation for 2 hrs. The purple formazan crystals formed were solubilized in Dimethyl Sulfoxide (DMSO) and the optical density of each well was then evaluated at 570 nm in a plate reader (Spectramax 340 PC).

Nitric oxide assay: The NO levels in BV2 cells were assessed via a Griess reagent[19].

Intracellular reactive oxygen species assay: After a day of treatment, intracellular ROS's presence was detected via CM-H2DCFDA[19,21].

Western blotting assay: Treated cells were allocated, lysed with RIPA-containing protease and phosphatase inhibitors and then centrifuged at 12,000 g for 5 min at 4 to collect the supernatant. The western blotting assay was explained[19]. All antibodies were received from Santa Cruz, CA, USA. The data were shown as fold changes when compared with the controls.

Immunofluorescence: The BV2 cells were seeded on an adherent 24 well plate in a growth medium. After a day of seeded, cells was treated for 4 hrs, then the NF-κB nuclear translocation was performed by immunofluorescence staining[21].

Statistical analysis: Data are shown as the Mean±SEM from at least three independent experiments in the triplicate.

Multiple comparisons of data were assessed by One-way ANOVA and Bonferroni post-tests. A p-value for statistical significance was considered at a value of less than 0.05.

RESULTS

Effect of OIF extract on BV2 cells viability: To assess the appropriate concentrations of OIF extract treatment, BV2 cell viability was gauged by MTT assay when treated only with OIF and co-treated with LPS. The results exhibited no significant toxicity was detected in BV-2 cells treated either with low or high concentration (50 μg mL^{-1}) of OIF after 24 hrs in Fig. 1. Therefore, the concentration of OIF extract at 25 and 50 μg mL^{-1} was used in the following experiments.

OIF extract suppressed LPS-induced NO production in BV2 microglial cells: To examine the effect of OIF extract on NO level on BV2 microglia stimulated with LPS, the Griess reagent assay was used. Our result demonstrated that NO production of LPS-treated BV2 microglia was significantly increased from the basal level (control 6.23±1.09 μM, LPS treated 35.03±1.90 μM). Co-treatment with OIF extract effectively and concentration-dependently inhibited its induction (23.43±1.31 and 19.29±2.0 μM, respectively) in Fig. 2.

OIF extract reduced LPS-induced ROS production in BV2 microglial cells: To investigate the inhibition of ROS generation in activated BV2 cells by OIF extract, the result showed that LPS could enhance the ROS production (2.65 fold) after 24 hrs of treatment of BV2 cells when compared with the control. On the other hand, high ROS was significantly decreased (2.23 and 2.6 fold), exhibiting a concentration-dependent manner, when OIF was received as compared with the LPS-induced group in Fig. 3.

OIF extract inhibited LPS-induced NF-κB nuclear translocation in BV2 microglial cells: The NF-κB is a key transcriptional factor that controls many of the inflammatory process genes expression. From our immunofluorescence microscopy, it was revealed that the NF-κB p65 subunit was mainly located in the cytoplasm under normal physiology of the untreated group in Fig. 4a and it translocated into the nucleus after stimulation with LPS in Fig. 4b. To study suppression of NF-κB activation after OIF treatment in LPS-mediated BV2 microglia, found that OIF extract could reduce the high nuclear NF-κB p65 translocation in OIF-treated BV2 cells in Fig. 4c and d.

OIF extract inhibited the phosphorylation form of Akt as well as ERK1/2 in LPS-induced microglial activation: To observe the effects of OIF extract on the Akt and ERK1/2 phosphorylation protein, LPS stimulation could significantly induce Akt in Fig. 5a and b and ERK1/2 phosphorylation in Fig. 5c and d. The OIF extract treatment might exponentially diminish the phosphorylation of Akt and ERK1/2 induced by LPS. On the other hand, total protein levels of Akt and ERK1/2 were constant after LPS and OIF extract treatment. After 2 hrs of treatment, total cell lysate was harvested and

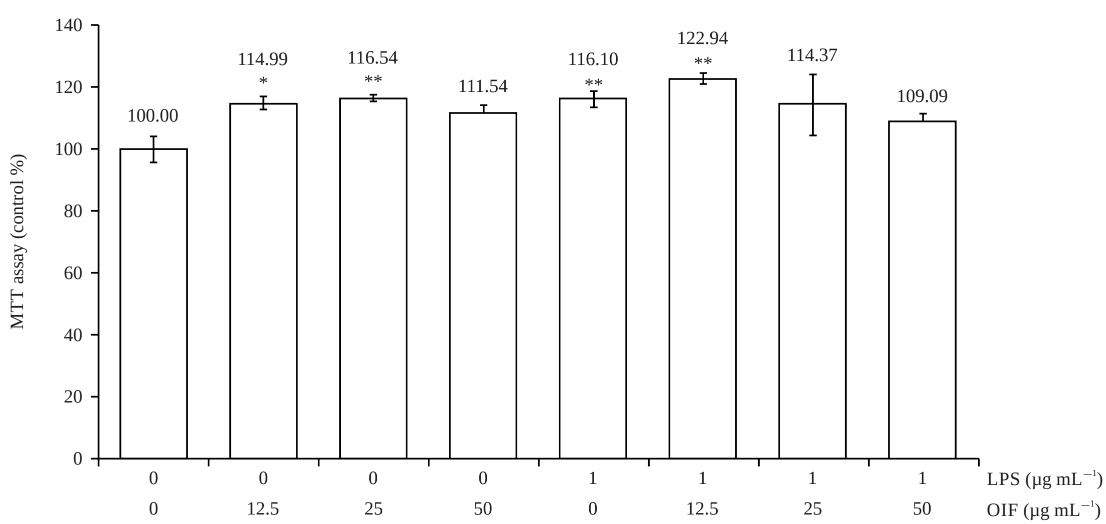

Fig. 1: Effect of OIF extract on BV2 cell viability using MTT assay

BV2 cells were incubated with OIF extract, alone or with 1 μg mL^{-1} LPS. Cell viability was determined after 24 hrs of treatment. Data from three independent experiments are presented as a percentage of control and expressed as Mean±SEM, *Indicated the significant difference ($p<0.05$) relative to the control group and **Indicated the significant difference ($p<0.01$) relative to the control group

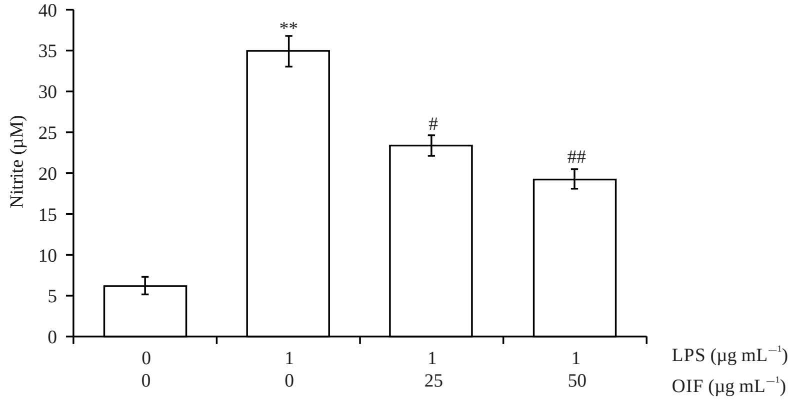

Fig. 2: Effect of OIF extracts on LPS-induced NO production

After 24 hrs of treatment, the culture media were harvested to examine the NO production. Data are presented as Mean±SEM (n = 3), **Indicated the significant difference ($p<0.01$) relative to the control group , #Indicated the significant difference ($p<0.05$) relative to the LPS treated group and ##Indicated the significant difference ($p<0.01$) relative to the LPS-treated group

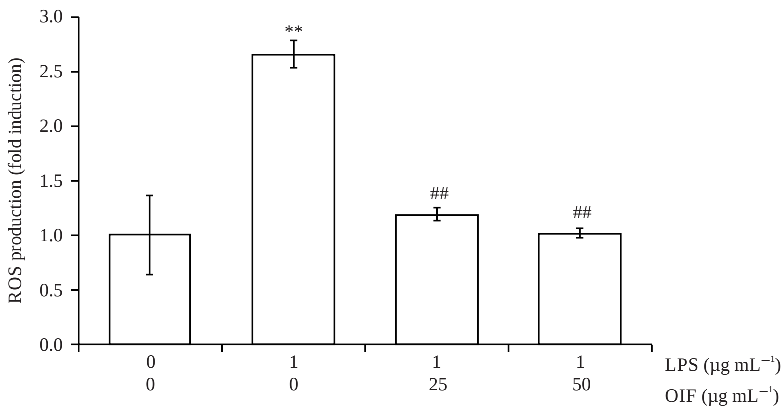

Fig. 3: Effect of OIF extract on the LPS-induced intracellular ROS level

BV2 were treated as indicated in the figure for 24 hrs and accumulation of ROS was measured. Data was represented in Mean±SEM (n = 3), **Indicated the significant difference ($p<0.01$) relative to the control group and ##Indicated the significant difference ($p<0.01$) relative to the LPS treated group

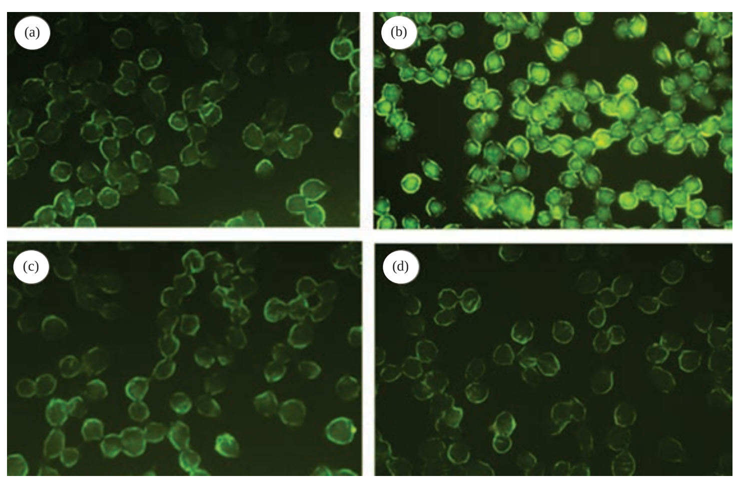

Fig. 4(a-d): Effect of OIF extract on NF-κB nuclear translocation in BV2 microglia stimulated by LPS, (a) Control, (b) LPS, (c) LPS+OIF25 and (d) LPS+OIF50

BV2 cells were induced with LPS in the presence or absence of OIF as indicated and cells after 4 hrs of treatment were examined by immunofluorescence microscopy

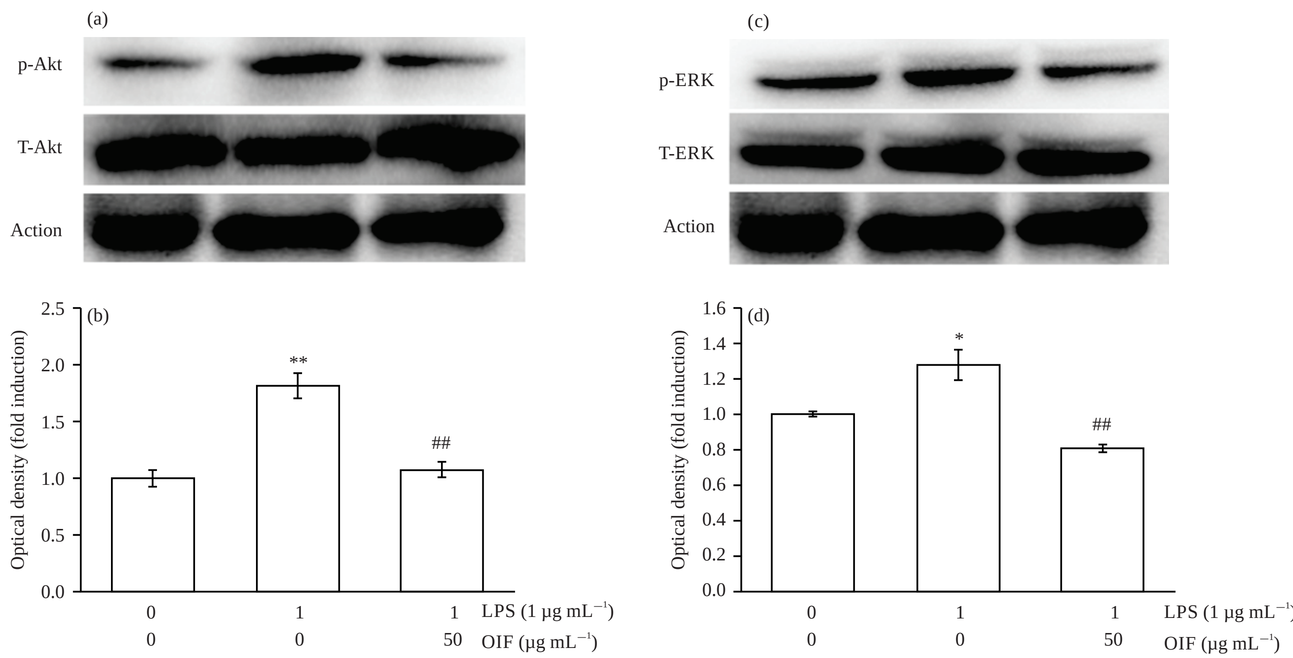

Fig. 5(a-d): Effect of OIF extract on Akt and ERK1/2 phosphorylation induced with LPS, (a) Western blotting of p-Akt, T-Akt and actin, (b) Histograms of p-Akt comparisons with untreated control (represented as fold changes), (c) Western blotting of ERK1/2, T-ERK1/2, actin and (d) Histograms of p-ERK1/2 comparisons with untreated control (represented as fold changes)

All data were represented as Mean±SEM (n = 3), *Indicated the significant difference (p<0.05) relative to the control group, **Indicated the significant difference (p<0.01) relative to the control group and ##Indicated the significant difference (p<0.01) relative to the LPS treated group

expression patterns of anti-p-Akt, T-Akt, ERK1/2, p-ERK1/2 and actin were determined by immuno-western blot assay. Histograms were represented as fold changes of p-Akt and p-ERK1/2 by comparisons with that of untreated control.

DISCUSSION

Chronic neuroinflammation and oxidative stress are two pathologic factors in the development of neurodegenerative diseases[3]. Nowadays, treatments are focused on reducing neuroinflammation caused by microglia to improve neurodegenerative disorders. However, drugs available for treatment can cause several side effects. Therefore, researchers have focused on the potential of natural agents to inhibit the inflammatory process.

Recently, current study demonstrated that OIF extract contained anti-oxidant and anti-inflammatory activity in LPS-stimulated BV2 cells, however, its mechanism is still vague[19]. This study, illustrated that OIF extract at the concentrations of 25 and 50 μg mL^{-1} exhibited no toxicity against BV2 cells, consistent with the previous reports[19]. We also confirmed that at these concentrations, OIF extract treatment with LPS could significantly reduce NO and ROS levels in a concentration dependency scenario. This result agreed with the previous studies[19]. Mechanically, this study demonstrated that OIF extract could attenuate NF-κB activation through the regulation of Akt/ERK signalling pathways in LPS-induced BV2 microglial activation. The NF-κB was found to be a major regulator of inflammatory processes due to its biological abilities, which include the induction of transcription of various inflammatory genes[22]. Under non-stimulated conditions, NF-κB is sequestered in the cytoplasm in which it is bound to its inhibitory subunit, IκB. When reacting to specific stimuli, IκB is phosphorylated, subsequent degradation through ubiquitination and eventually liberated NF-κB[23]. Permitting its translocation to the nucleus promoted the expression of inflammation-associated genes by pro-inflammatory cytokine production[23]. Several investigations reported that signalling pathways activation of NF-κB, PI3K/Akt and ERK caused by LPS resulted in stimulating inflammation[24-27]. The current study demonstrated that OIF extract decreased the NF-κB p65 nuclear level in LPS-treated BV2 cells. The OIF might inhibit the production of NO and ROS by attenuation NF-κB nuclear translocation induced by

LPS. Additionally, this study revealed that the phosphorylation form of Akt in BV2 microglial activation was notably decreased by the OIF extract, reflecting that OIF could inhibit NF-κB activation by suppressing the Akt signalling pathway. The LPS could stimulate the phosphorylation of ERK1/2 and OIF markedly inhibited this effect. This finding suggested that OIF might also attenuate the production of NO and ROS in activated BV2 microglial mediated by LPS via the ERK1/2 signalling pathway too.

CONCLUSION

It is evident that the OIF extract notably attenuated the release of pro-inflammatory cytokines and mediators, besides the production of NO and ROS in microglial cells induced with LPS. Moreover, the inhibition of NF-κB translocation from the cytoplasm to the nucleus was induced by the OIF extract treatment, which is associated with the signalling pathway inactivation of the Akt and ERK1/2. These results could be implicated that the OIF extract might have the potential for the prevention and treatment of inflammatory diseases and several neurodegenerative diseases involved in microglial activation.

SIGNIFICANCE STATEMENT

This study concluded that OIF extract exerts anti-oxidative activity by ROS suppression as well as anti-inflammatory activity by NO inhibition in the activated BV2 microglial cells. Mechanistically, the OIF extract could alleviate inflammatory response by inhibiting NF-κB p65 translocation and activating via the pathway of Akt and extracellular-signal-regulated kinase 1/2 (ERK1/2) in the LPS-stimulated BV2 cells. The OIF extracts may act as a promising functional food to allay neuroinflammation in neurodegenerative diseases.

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