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Original Research Article

Oroxylum indicum Kurz (L) leaf extract exerted antioxidant and anti-inflammatory effects on LPS-stimulated BV2 microglial cells

Nootchanat Mairuae[1]*, Benjaporn Buranrat[1], Supataechasit Yannasithinon[1], Poonlarp Cheepsunthorn[2]

[1]Faculty of Medicine, Mahasarakham University, Maha Sarakham 44000, [2]Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

For correspondence: **Email: nootchanat.m@msu.ac.th, mairuae.n@gmail.com; **Tel:** +66-43-021021-992; **Fax:** +66-43-021021*

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Abstract

Purpose: *To investigate the antioxidant and anti-inflammatory effects of Oroxylum indicum (L.) Kurz leaf extract on lipopolysaccharide (LPS)-activated BV2 microglia.*
Methods: *BV2 cells were treated with LPS for 24 h in the presence or absence of Oroxylum indicum (O. indicum) leaf extract. The levels of reactive oxygen species (ROS), nitric oxide (NO), and interleukin 6 (IL-6) were quantified with CM-H2DCFDA, Griess reagent assay, and enzyme-linked immunosorbent assay (ELISA) test, respectively. The antioxidant activity of O. indicum leaf extract was assessed in vitro using DPPH, ABTS and FRAP assays.*
Results: *Treatment with LPS resulted in significant increases in the levels of ROS, NO, and IL-6, when compared to untreated cells. However, co-treatment with O. indicum leaf extract significantly suppressed the production of these inflammatory markers ($p < 0.05$). In addition, the leaf extract of O. indicum exhibited antioxidant activity in the DPPH, ABTS, and FRAP assays. The contents of flavonoids and phenolics in O. indicum leaf extract were 13.25 ± 0.163 mg QE/g of dried extract and 82.58 ± 1.772 mg QE/g of dried extract, respectively ($p < 0.05$).*
Conclusion: *The leaf extract of O. indicum exhibits anti-inflammatory and antioxidant properties in LPS-activated BV2 cells. This finding indicates that the leaf extract of O. indicum has promising potentials as a new source of herbal medication with anti-inflammatory properties. However, there is a need for more studies to unravel the fundamental mechanisms of their actions.*

Keywords: *Neuro-inflammation, Oxidative stress, Microglia, Oroxylum indicum leaf*

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INTRODUCTION

Neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), are characterized by gradual loss of neurons and synapses, resulting in cognitive and motor dysfunctions [1]. An increasing amount of evidence suggests that microglia activation, neuroinflammation and oxidative stress play key roles in the etiology of many neurodegenerative diseases [2-4]. Microglia, the resident immune

cells in the central nervous system (CNS), are critical for maintaining homeostasis and responding to damage or disease [5]. When activated, microglia undergo morphological and functional changes, thereby generating a variety of pro-inflammatory cytokines, chemokines, and reactive oxygen species [6,7]. Although initially protective, prolonged microglia activation may cause long-term neuroinflammation, resulting in neuronal damage and death [6,7]. Oxidative stress which results from an imbalance between reactive oxygen species (ROS) production and antioxidant defense, significantly influences the development of neurodegenerative diseases [8,9]. Overexposure to ROS damages lipids, proteins and DNA in cells, leading to apoptosis and dysfunction [9]. Therefore, a thorough understanding of the relationship amongst microglial activation, neuroinflammation, and oxidative stress is critical for designing treatment strategies aimed at slowing down the progression of neurodegenerative disorders. The targeting of these pathways has the potential to reduce neuronal loss and improve clinical outcomes for the affected patients.

The Bignoniaceae member *Oroxylum indicum* (L.) Kurz is native to India, Thailand, and Southeast Asia [10]. *Oroxylum indicum (O. Indicum)* is considered a versatile plant in that it is consumed as food and also used as a traditional medicinal remedy. It has been extensively utilized across Southeast Asia for many years [10]. It has been reported that *O. Indicum* contains chemical compounds such as baicalein, baicalin, chrysin, oroxylin A, and oroxylin B [11,12]. The plant has received attention due to its numerous health benefits such as anti-inflammatory, neuro-protective, antioxidant-boosting, and blood sugar-lowering properties [13-16]. Animal experiments on *O. Indicum* have demonstrated its positive safety profile, with rats tolerating well oral doses of 250 mg/kg b.wt for 28 days [15]. Previous studies have demonstrated the potential of extracts of the fruit and seed of *O. indicum* to reduce neuroinflammation in BV2 microglial cells [17-19]. However, not much is known about the effect of *O. indicum* leaf extract on microglial activation. The objective of this study was to investigate the antioxidant and anti-inflammatory effects of an ethanol leaf extract of *O. indicum* on LPS-activated BV2 microglial cells.

EXPERIMENTAL

Preparation of *O. indicum* leaf extract

Oroxylum indicum leaf extract was prepared as described previously [18].

Cell cultures and treatments

Murine BV2 microglial cells were kind gifts from Dr. James R. Connor of Department of Neurosurgery, Pennsylvania State University College of Medicine, Hershey, PA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C under humidified atmosphere of 95 % O_2 and 5 % CO_2. The medium was refreshed twice a week. All cell culture reagents were purchased from HyClone, South Logan, UT. To begin the experiments, BV-2 cells were plated at a density 1 x 10^4 cells/well in 96-well plates and grown in 5 % FBS-supplemented DMEM. When cells reached 80 % confluence, the medium in each well was completely removed. In assay of cell viability, the cells in each well were gently washed once with serum-free DMEM before adding freshly prepared serum-free DMEM containing specified concentrations of *O. indicum* leaf extract ranging from 0 to 50 µg/mL in the presence or absence of LPS. Prior to determination of levels of NO, IL-6, and ROS, the growth medium in each well was completely removed and replaced with a medium containing LPS, with or without the specified concentrations of *O. indicum* leaf extract. Cells in serum-free DMEM served as the untreated control.

Cell viability assay

After 24 h of treatment, the medium was removed from each well and replaced with 0.1 mL of MTT reagent (0.4 mg/mL; Sigma Co, St Louis, MO, USA) in serum-free DMEM, followed by incubation at 37 °C for 2 h. After the incubation period, the MTT solution was removed from each well, followed by the addition of dimethyl sulfoxide to solubilize the resultant precipitate of formazan crystals. After each well was placed on a microplate mixer for 10 min, the absorbance (A) of the formazan solution was read at 540 nm using a plate reader (Spectramax 340 PC).

Nitric oxide (NO) assay

The levels of NO were determined by measuring nitrite accumulation in the cell culture supernatants using Griess reagent (1 % sulfanilamide/0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5 % phosphoric acid) (Invitrogen, Carlsbad, CA). In this assay, the nitrite-containing sample (150 µL/well) was placed in a 96-well plate, mixed with 20 µL of Griess reagent and 130 µL of deionized

water, and incubated for 30 min at room temperature. Absorbance values were read at 540 nm using a microplate reader (BiO-Tek Instruments Inc., Winooski, VT).

Measurement of ROS generation

2',7'-Dichlorofluorescein diacetate (DCFH-DA) is a sensitive and widely used compound for the determination of intracellular oxidant production. The cells were incubated with 10 µM DCFH-DA for 20 min at 37 ^{o}C in a CO_2 incubator. Then, the cells were treated with LPS in the presence or absence of *O. indicum* leaf extract in a serum-free medium for 24 h. Fluorescence intensity was measured with a fluorescence microplate reader at an excitation wavelength of 495 nm and a 525 nm emission filter.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6 in culture supernatants were measured using ELISA kits (R & D Systems, Minneapolis, MN) in line with the kit manufacturer's instructions. In this method, 50 µL of assay diluent was added to each well, followed by addition of 50 µL of each sample or standard sample to the antibody-coated plates. The plates were covered, incubated at room temperature for 2 h, and washed four times with rinsing buffer before adding biotinylated antibody. Thereafter, the plates were incubated at room temperature for an additional 2 h. Then, substrate solution was added to each well and incubated at room temperature for 30 min, after which the enzyme reaction was stopped by addition of a stop solution to each well. The absorbance of each well was read at 450 nm in a microplate reader (BiO-Tek Instruments Inc., Winooski, VT). The concentration of IL-6 in each sample was calculated with reference to a standard curve.

Assays of FRAP, DPPH and ABTS radical scavenging activity

The total free radical scavenging capacity of *O. indicum* leaf extract was determined in terms of FRAP, DPPH and ABTS assays according to the method of Yang *et al* [20].

Determination of total flavonoid and total phenolic contents

The levels of total flavonoids and total phenolics in *O. indicum* leaf were determined as described in a previous study [20].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of three independent experiments. Multi-group comparisons were done using one-way ANOVA, followed by Bonferroni post-hoc test. Differences among the means were considered statistically significant at $p < 0.05$.

RESULTS

Effect of *O. indicum* leaf extract on cell viability

The MTT technique was used to determine whether or not the leaf extract of *O. indicum* was cytotoxic to BV2 cells. The results of the study demonstrated that exposure to *O. indicum* leaf extract at doses as high as 50 µg/mL did not result in any toxicity in BV2 cells, as depicted in Figure 1. Consequently, the *O. indicum* leaf extract was used at concentrations of 12.5, 25, and 50 µg/mL in the follow-up studies.

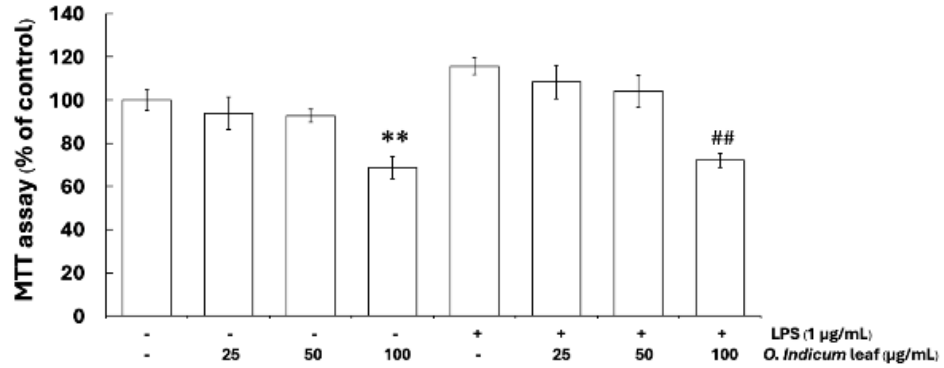

Figure 1: Effect of *O. indicum* leaf extract on the viability of BV2 microglial cells. BV2 cells were treated with *O. indicum* leaf extract alone or with LPS (1 µg/mL) in serum-free media for 24 h. Cell viability was determined using the MTT assay. $^{**}P < 0.01$ vs control group; $^{\#\#}p < 0.01$ vs LPS-treated group

Effect of *O. indicum* leaf extract on LPS-induced NO production

To evaluate the potential of *O. indicum* leaf extract as an anti-inflammatory agent, its effect on LPS-induced NO production was investigated, and the results obtained are presented in Figure 2. When BV2 microglia were exposed to LPS, NO levels in the culture media were significantly higher than in the untreated control group ($p < 0.01$). This showed that the microglia were activated. However, concurrent administration of *O. indicum* leaf extract during LPS-induced microglial activation resulted in a concentration-dependent reduction in NO levels in the culture medium, with significant decrease observed at a concentration as low as 12.5 µg/mL ($p < 0.01$). These data indicate that *O. indicum* leaf extract reduced inflammation in activated microglia by lowering NO generation.

Effect of *O. indicum* leaf extract on ROS production

The intracellular levels of ROS during microglial activation were used as a measure of the antioxidant properties of *O. indicum* leaf extract. As shown in Figure 3, exposure of BV2 microglial cells to LPS significantly increased ROS accumulation, when compared to untreated control cells ($p < 0.01$), indicating oxidative stress. However, co-treatment with LPS and *O. indicum* leaf extract led to concentration-dependent decreases in ROS levels within BV2 cells. Notably, the reduction in ROS levels was significant even at extract concentration of 25 µg/mL, an indication of the potent antioxidant effect of the extract. These data suggest that *O. indicum* leaf extract effectively mitigated oxidative stress in activated microglia.

Effect of *O. indicum* leaf extract on LPS-induced IL-6 production

The anti-inflammatory potential of *O. indicum* leaf extract in LPS-activated BV2 microglial cells was determined by measuring IL-6 levels in the cell culture medium using ELISA assay. As seen in Figure 4, the treatment of BV2 cells with LPS markedly increased IL-6 level in the culture medium, when compared to the control group ($p < 0.01$).

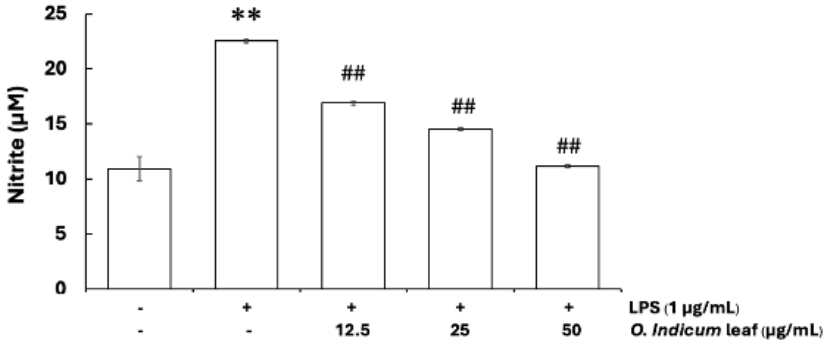

Figure 2: Effect of *O. indicum* leaf extract on LPS-induced NO production in BV2 microglial cells. The cells were treated with LPS (1 µg/mL) in the presence or absence of *O. indicum* leaf *extract* in serum-free medium for 24 h. ***P* < 0.01 vs control group; $^{\#\#}p < 0.01$ vs LPS-treated group

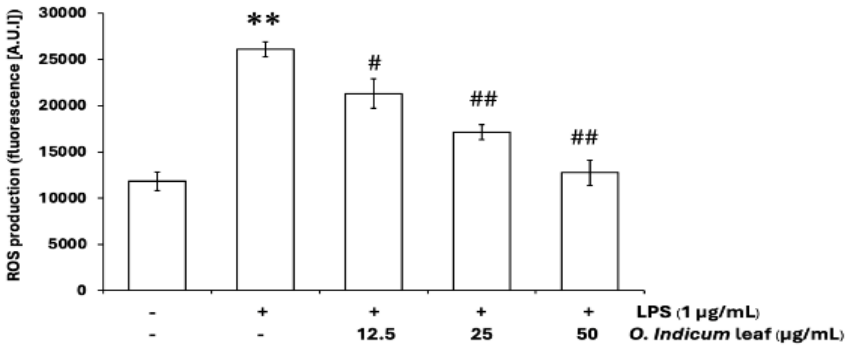

Figure 3: Effect of *O. indicum* leaf extract on ROS production in LPS-activated BV2 microglial cells. The cells were treated with LPS (1 µg/mL) in the presence or absence of *O. indicum* leaf extract in serum-free medium for 24 h. ***P* < 0.01 vs control group; $^{\#}p < 0.05$ vs LPS-treated group; $^{\#\#}p < 0.01$ vs LPS-treated group

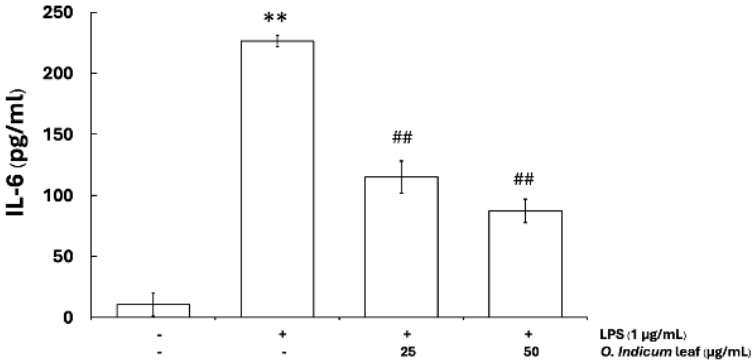

Figure 4: Effect of *O. indicum* leaf extract on LPS-induced IL-6 production. Cells were treated with LPS (1 µg/mL) in the presence or absence of *O. indicum* leaf in a serum-free medium for 24 h. ***P* < 0.01 vs control group; $^{\#\#}p$ < 0.01 vs LPS-treated group

Table 1: *In vitro* antioxidant effect of leaf extract of *O. indicum*

Sample	FRAP *(mg TE/g extract)*	DPPH IC_{50} *(mg/mL)*	ABTS IC_{50} *(mg/mL)*
O. indicum leaf extract	0.44±0.011	0.1973±0.00895[b]	0.0043±0.00075
Ascorbic acid		0.0020±0.00004	0.0011±0.00001
Trolox		0.0026±0.00008[a]	0.0011±0.00002

Note: [a]*P* > 0.05; [b]*p* < 0.05 vs ascorbic acid. Values are presented as mean ± SEM of triplicate determinations

This indicated a strong inflammatory response. However, when the cells were co-treated with LPS and *O. indicum* leaf extract, there were concentration-dependent decreases in IL-6 levels. This reduction was significant even at the lowest extract concentration, thereby demonstrating its effective anti-inflammatory properties.

In vitro antioxidant potential of *O. indicum* leaf extract

The *in vitro* antioxidative potential of *O. indicum* leaf was assessed using FRAP, DPPH radical scavenging and ABTS assays. The results of these assays are presented in Table 1.

Total phenolic and flavonoid contents of *O. indicum* leaf extract

The total phenolic and flavonoid contents of *O. indicum* leaf extract were determined using colorimetric methods. Gallic acid was used as the standard phenolic compound, while rutin was used as the standard flavonoid compound. The analyses revealed that the total phenolic content of the extract was 82.58 ± 1.77 mg GAE/g of dried extract (mg gallic acid equivalent/g dry weight), while the total flavonoid content was 13.25 ± 0.16 mg RE/g of dried extract (mg rutin equivalent/g dry weight). These values indicate high concentrations of bioactive compounds in the leaf extract, which may contribute to its therapeutic properties.

DISCUSSION

Microglia, the primary immune cells in the CNS, are crucial for maintenance of neural homeostasis and defense against pathogens [5]. However, prolonged activation of microglia results in persistent neuroinflammation which exacerbates neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease [6,7]. Oxidative stress is characterized by an imbalance between ROS production and antioxidant defense, and it is a significant factor in neurodegenerative diseases. Excessive ROS production damages cellular components, leading to neuronal dysfunction and cell death [8,9]. In the context of neuroinflammation, activated microglia are a major source of ROS which further exacerbate oxidative stress and neuronal injury [6,7].

The goal of this study was to find out if the leaf extract of *O. indicum* (L.) Kurz reduced inflammation and free radicals in BV2 microglial cells activated with LPS. The results obtained indicate that the leaf extract of *O. indicum* effectively and concentration-dependently decreased the LPS-induced production levels of ROS, NO, and IL-6. These data suggest that the extract has the potential to effectively reduce microglial activation and the resultant neuroinflammatory response.

The administration of *O. indicum* leaf extract resulted in decreased ROS levels, indicating

strong antioxidant activity. This effect is essential for reducing oxidative stress in the CNS. The antioxidant property of the extract is most likely due to the significant presence of phenolic and flavonoid compounds (82.58 ± 1.772 mg GAE/g and 13.25 ± 0.163 mg RE/g of dried extract, respectively). Phenolic compounds and flavonoids are known for their effectiveness in scavenging free radicals and inhibiting oxidative stress, thereby protecting neural cells from oxidative damage.

The anti-inflammatory properties of *O. indicum* leaf extract may be mediated through the modulation of key signaling pathways involved in microglial activation. Flavonoids such as baicalein, baicalin and oroxylin A which are present in *O. indicum*, have been shown to inhibit the nuclear factor-kappa B (NF-κB) pathway, a critical regulator of inflammation [21-23]. By inhibiting NF-κB activation, these compounds decrease the expressions of pro-inflammatory cytokines and enzymes, thereby attenuating inflammatory response [21-23]. Furthermore, the decrease in NO production caused by *O. indicum* leaf extract treatment indicates inhibition of inducible nitric oxide synthase activity which is often upregulated in activated microglia and contributes to neuroinflammation and oxidative stress.

The dual antioxidant and anti-inflammatory effects of *O. indicum* leaf extract makes it a promising candidate for the development of neuroprotective therapies. By mitigating both oxidative stress and neuroinflammation, the extract has the potential to slow the progression of neurodegenerative diseases and improve neuronal survival. Future studies should focus on elucidating the precise molecular mechanisms underlying these effects, as well as evaluating the effectiveness of *O. indicum* leaf extract in *in vivo* models of neurodegeneration.

CONCLUSION

The results obtained in this study strongly indicate the therapeutic potential of *O. indicum* leaf extract, and that it may be beneficial for targeting the fundamental pathogenic mechanisms associated with neurodegenerative disorders. The marked decreases in LPS-mediated production of ROS, NO, and IL-6 in BV2 microglial cells highlight its potential as a promising new agent for combating neuroinflammation and oxidative stress. This study has established a basis for further investigation into the utilization of *O. indicum* leaf extract as a natural treatment alternative for neurodegenerative disorders.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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