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Original Research Article

Antioxidant and anti-inflammatory activities of *Oroxylum indicum* Kurz (L.) fruit extract in lipopolysaccharide-stimulated BV2 microglial cells

Nootchanat Mairuae[1]*, Poonlarp Cheepsunthorn[2], Benjaporn Buranrat[1]
[1]Faculty of Medicine, Mahasarakham University, Maha Sarakham 44000, [2]Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

For correspondence: **Email: mairuae.n@gmail.com, nootchanat.m@msu.ac.th; **Tel:** 6643-021021-992; **Fax:** 6643-021021*

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Abstract

Purpose: *To investigate the anti-inflammatory effects and antioxidant activity of Oroxylum indicum (L.) Kurz fruit extract in lipopolysaccharide (LPS)-stimulated BV2 microglia.*
Methods: *BV2 cells were treated with LPS for 24 h in the presence or absence of O. indicum fruit extract. Then, nitric oxide (NO), reactive oxygen species (ROS) and interleukin 6 (IL-16) levels were measured using Griess reagent assay, CM-H2DCFDA and enzyme-linked immunosorbent (ELISA) assays, respectively. The in vitro antioxidant property of the extract was also investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays.*
Results: *Levels of IL-6, NO, and ROS in LPS-treated BV2 cells were significantly higher than those in control ($p < 0.01$). However, exposure of LPS-treated BV2 cells to O. indicum extract led to a marked decrease in the levels of these parameters, when compared to the untreated cells ($p < 0.01$). Results from DPPH and ABTS assays showed that the O. indicum extract exhibited good antioxidant properties, with total flavonoid and total phenolic contents of 115.58 ± 1.09 and 131.04 ± 2.37 mg/g of dried extract, respectively.*
Conclusion: *The results demonstrate that O. indicum fruit exerts anti-oxidant and anti-inflammatory effects in LPS-stimulated BV2 cells. Thus O. indicum fruit might be beneficial in the development of novel anti-oxidative and anti-neuroinflammatory herbal medicines. However, the mechanisms by which O. indium fruits reduces NO and IL-6 needs to be further investigated.*

Keywords: *BV2 microglia, IL-6, Nitric oxide, Oroxylum indicum, Reactive oxygen species, Anti-oxidative, Anti-neuroinflammatory*

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INTRODUCTION

Neuroinflammation and oxidative stress mediated by activated microglia have been implicated in the onset and the progression of many neurodegenerative diseases [1]. Chronic microglial activation participates in inflammatory response by producing ROS and releasing NO and various pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 1

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beta (IL-1β), and IL-6 [2,3]. The overproduction of these agents reduces neuronal population. Therefore, the control of microglial activation is a beneficial approach in the treatment and prevention of neuroinflammation and neurodegenerative disorders.

Oroxylum indicum (L) Kurz, a plant in the Bignoniaceae family is distributed throughout India and South East Asia. This plant has been applied as a traditional herbal medicine for treatment illnesses in China and Japan [4]. The fruits of *O. indicum* are rich in nutrients [4] and they have been used for thousands of years as part of plant-based diet and herbal medicines in several countries, without any known adverse effects [4-6]. Various parts of the plant are sources of several medicinally important compounds. The fruit of *O. indicum* contain flavonoids such as oroxylin A, baicalein, and chrysin, all of which have been reported to have multiple biological effects.

The pharmacological effects of fruit extract of *O. indicum* and compounds isolated from the fruit exhibit hepatoprotective, antidiabetic, anti-adipogenesis antioxidant and anti-inflammatory properties [5,7-9]. A previous study demonstrated that *O. indicum* fruit extract protected SH-SY5Y cells against Aβ25-35-induced cell injury [10]. Although many medicinal properties of *O. indicum* fruit have been reported, the scientific data on the effect of *O. indicum* fruits extract on microglial activation has not yet been reported.

Therefore, the present study was carried out to determine the anti-inflammatory and antioxidant effects of *O. indicum* fruits extract on BV2 microglial cells stimulated with LPS.

EXPERIMENTAL

Preparation of *O. indicum* fruit extract

The fruit extract of *O. indicum* was prepared as described previously [10,11]. The fruits were dried, weighed, chopped, and macerated in 95 % (v/v) ethanol, followed by filtration. The ethanol solvent was evaporated from the crude extract obtained, and the residue was lyophilized.

Cell culturing and treatments of murine BV2 cell

The BV2 cells were obtained from the Department of Neurosurgery, Hershey Medicine Center (Professor James R Connor's laboratory). All reagents for the cell culture were purchased from HyClone (South Logan, UT). The cells were incubated in Dulbecco's adjusted Eagle's medium (DAEM) containing 5 % fetal bovine serum (FBS), at 37 °C in a 5 % CO_2 incubator. To determine the viability of cells, BV-2 cells were seeded in 96-well plates. When cells reached 80 % confluence, the medium in each well was removed, and replaced with new serum-free DAEM containing various concentrations of *O. indicum* fruit extract (0 - 50 µg/mL) in the presence or absence of LPS.

In order to quantify the levels of NO, IL-6 and ROS, the growth medium was completely removed and replaced with new medium containing LPS, with or without various concentrations of *O. indicum* fruit extract. Cells in serum-free DAEM served as untreated control.

Evaluation of cell viability

After 24 h of treatment with *O. indicum* fruit extract at concentrations of 0 - 50 µg/mL, with or without LPS, the incubation medium was discarded, and 0.4 mg/mL MTT reagent (Sigma Co St. Louis, MO) in serum-free DAEM was added to each well. After incubation for 2 h, the MTT medium was removed, and the purple formazan crystals formed were solubilized in dimethyl sulfoxide (DMSO). The optical density of each well was read at 570 nm in a plate reader (Spectramax 340 PC).

Measurement of nitric oxide (NO) assay

To quantify the nitrite accumulation, NO levels in the cell culture supernatants were estimated using Griess reagent (Invitrogen, Carlsbad, CA) as described previously [11]. In this assay, 150 µL of sample was seeded per well in a 96-well plate. Then, 20 µL of Griess reagent was added and mixed with 130 µL deionized water. Incubation was carried out for 30 min at room temperature, after which absorbance of each well was read at 540 nm using a microplate reader (Bi0-Tek Instruments Inc., Winooski, VT).

Determination of ROS generation

The oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to determine intracellular levels of ROS. The cells were cultured with 10 µM DCFH-DA for 20 min at 37 ^{o}C in a 5 % CO_2 incubator. Then, the cells were treated with a medium containing LPS, in the presence or absence of various concentrations of *O. indicum* fruit extract for 24 h. Subsequently, fluorescence intensity was measured at excitation and emission wavelengths of 495 nm and 525 nm, respectively.

Assay of IL-6

The IL-6 levels in the culture supernatant were determined using enzyme-linked immunosorbent assay (ELISA) kits (Minneapolis, MN). The IL-6 levels in the supernatant were quantified by measuring absorbance at 450 nm using Microplate reader (Bi0-Tek Instruments Inc., Winooski, VT). The concentration of IL-6 in each sample was calculated with reference to IL-6 standard curves.

Determination of DPPH and ABTS radical scavenging activity assay

Total free radical scavenging capacity of *O. indicum* fruit extract was determined with DPPH and ABTS assay according to the method of Yang *et al* [12,13].

Determination of total flavonoid and total phenol contents

Total flavonoid and total phenol contents of *O. indicum* fruit extracts were determined as described in a previous study [10].

Statistical analysis

Data are expressed as mean ± S.E.M. One-way ANOVA and Bonferroni post-tests were used for analysis of statistical significance of differences amongst multiple groups. Values of $p < 0.05$ were taken as indicative of statistical significance of differences.

RESULTS

Effect of *O. indicum* fruit extract on the viability of BV2 cells

In this study, MTT assay was applied to determine the effect of *O. indicum* fruit extract on the viability of BV2 cells. The results demonstrated that *O. indicum* fruit extract at concentrations up to 50 µg/mL exhibited no toxicity against BV2 cells (Figure 1). Thus, *O. indicum* fruit extract was used at concentrations of 12.5, 25, and 50 µg/mL in the subsequent studies.

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

To investigate the anti-inflammatory effect of *O. indicum* fruit extract, its effect on LPS-induced NO production was examined. The result demonstrates that LPS treatment caused significant increase in NO level in culture media of BV2 microglia, when compared to untreated control. However, treatment with *O. indicum* led to significant and concentration-dependent decrease in NO levels (Figure 2).

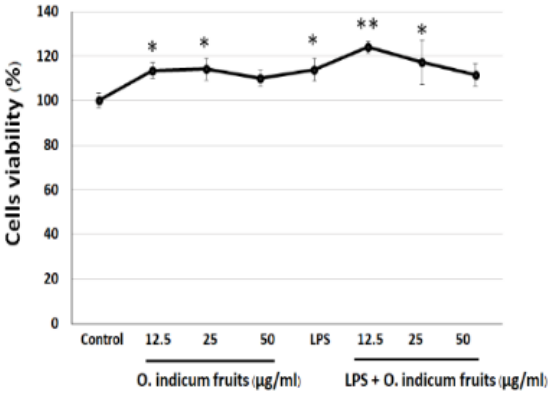

Figure 1: Effect of *O. indicum* fruit extract on BV2 cells viability using MTT assay. BV2 cells were incubated with *O. indicum* fruits extract, alone or with 1 µg/mL of LPS. Cell viability was determined after 24 h of treatment. Data from three independent experiments are expressed as percentage of control, and presented as mean ± SEM; *$p < 0.05$, vs. control group; **$p < 0.01$, vs. control group

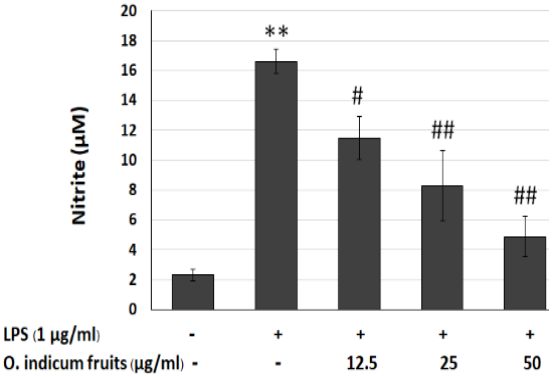

Figure 2: Effect of *O. indicum* fruits on NO levels. The BV2 cells were treated as described earlier-on. After 24 h of treatment, NO level was determined in the culture medium using the Griess assay. Data are presented as mean ± SEM. Values are mean of three independent experiments. **$P < 0.01$, vs. control group; #$p < 0.05$, ##$p < 0.01$ vs. LPS-treated group

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

To determine the antioxidant capacity of *O. indicum* fruit extract in activated BV2 cells, the intracellular level of ROS was determined. There was significant increase in level of ROS in LPS-treated cells, when compared to control. However, ROS level was significantly decreased in *O. indicum* extract-treated BV-2 cells (Figure 3).

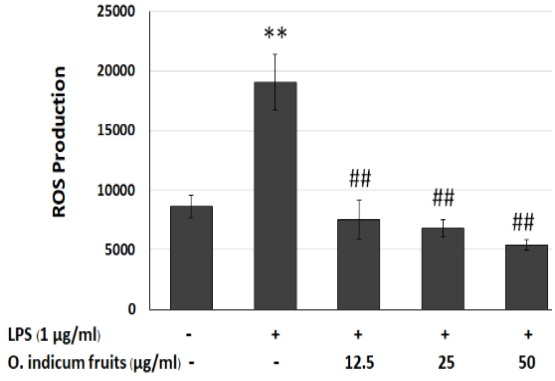

Figure 3: Effect of *O. indicum* fruit extract on ROS production. Cells were treated with LPS (1 µg/mL) in the presence or absence of *O. indicum* fruit extract. Data are presented as mean ± SEM. Values are mean of three independent experiments. ***P* < 0.01, vs. control group; ##*p* < 0.01 vs. LPS-treated group

Effect of *O. indicum* fruit extract on IL-6 levels

To determine the anti-inflammatory properties of *O. indicum* fruit extract in LPS-activated BV2 microglial cells, IL-6 level was determined using ELISA. As shown in Figure 4, IL-6 level in culture media of BV-2 cells was significantly increased after treatment with LPS, relative to control (untreated cells). However, when the cells were co-treated with LPS and *O. indicum* extract, the level of IL-6 was significantly decreased in a concentration-dependent manner (Figure 4).

In vitro antioxidant properties of *O. indicum* fruit extract

The antioxidative potential of *O. indicum* fruit is shown in Table 1.

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

The phenolic content of the extract was 131.04 ± 2.37, while the flavonoid content was 115.58 ± 1.09 mg/g of dried extract. These results are presented in Table 1.

DISCUSSION

It has been reported that microglia-mediated neuroinflammation and oxidative stress are linked to neurodegenerative diseases [14]. Therefore, inhibition of microglia activation may have significant impact on the treatment of neurodegenerative diseases.

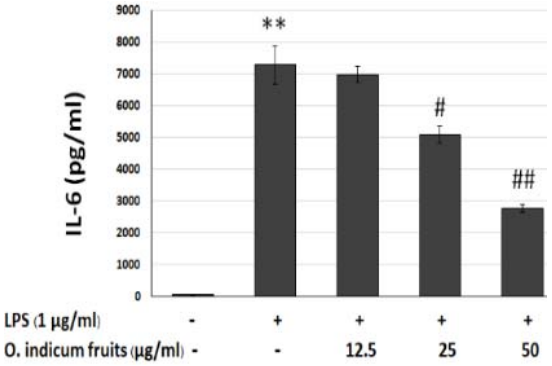

Figure 4: Effect of *O. indicum* fruits on IL-6 levels. Cells were treated with LPS (1 µg/L) in the presence or absence of *O. indicum* fruit extract. The expression of IL-6 was determined after 24 h of treatment using ELISA kits. Data represent mean ± SEM values from three independent experiments. ***P* < 0.01 vs control group; #*p* < 0.05 vs. LPS-treated group, ##*p* < 0.01 vs. LPS-treated group

Using DPPH and ABPS assays, this study showed that *O. indicum* fruit extract has *in vitro* antioxidant effects. The extract inhibited ROS production in LPS-activated BV2 microglia. Furthermore, it suppressed inflammation by decreasing the levels of NO and IL-6. Reactive oxygen species (ROS) trigger neuroinflammation by regulating NF-κB transcriptional factors, leading to enhanced expressions of pro-inflammatory genes. This study has demonstrated that *O. indicum* fruit extract exerted *in vitro* antioxidant effects by decreasing LPS-induced ROS levels in activated BV-2 microglia. This result is consistent with a previous report which showed that *O. indicum* suppressed LPS-activated ROS in RAW264.7 macrophages, and *in vitro* antioxidant assay [15]. Therefore, it is possible that *O. indicum* reduced ROS levels due to suppression of oxidation.

In the central nervous system, NO is the inflammatory mediator derived from activated microglia.

Table 1: *In vitro* antioxidant property of *O. indicum* fruit extract, and total phenolic and flavonoid contents

DPPH assay (mg Trolox equivalent/g extract)	43.79±0.35
ABTS assay (mg Trolox equivalent/g extract)	105.64±0.368
Total phenolic content[a]	131.04 ±2.37
Total flavonoid content[b]	115.58 ±1.09

[a]mg gallic acid equivalent/g dry weight; [b]mg rutin equivalent/g dry wt. Values are presented as the mean of three biological replicates

High concentrations of NO are involved in the onset of multiple sclerosis, AD and PD and other neurodegenerative diseases. It has been established that NO rapidly reacts with *superoxide* anion to *form* the highly toxic *peroxynitrite* anion [16]. Peroxynitrite reacts directly with various *biological molecules* including *lipids*, nucleic *acids*, and proteins [16]. In the present study, Griess reagent was used to assay the secretion of NO by BV-2 cells. The results demonstrated that LPS significantly increased NO production, but this was reversed by *O. indicum* fruit extract, indicating that the extract can mitigate inflammation caused by excessive NO. This result is consistent with a report showing that *O. indicum* fruit suppressed the production of NO in LPS-activated RAW264.7 macrophages [15].

Studies have shown that high IL-6 level is a hallmark of neuroinflammatory diseases [17]. Therefore, the effect of *O. indicum* fruit extract on IL-6 production in activated BV-2 cells was investigated. Treatment with *O. indicum* extract significantly decreased IL-6 levels in LPS-activated BV-2 cells. This result is in line with a previous report showing that *O. indicum* treatment decreased IL-6 production in LPS-activated RAW264.7 macrophages [15]. It has been reported that in *O. indicum*, flavonoid compounds such as apigenin, baicalein, chrysin, luteolin and oroxylin exerted anti-inflammatory effects by modulation of ROS generation and suppression of the proinflammatory cytokines IL-6, TNF-α and IL-1β [18]. In this study, it was demonstrated that total flavonoid and total phenolic contents of *O. indicum* fruit extract were 115.58 ± 1.09 and 131.04 ± 2.37 mg/g of dried extract, respectively. Thus, a flavonoid-rich extract from *O. indicum* could potentially act as a source of anti-inflammatory compounds.

CONCLUSION

The results obtained in the present study indicate that *O. indicum* fruit extract mitigates oxidative stress and inflammation by reduction of LPS-induced increases in levels of IL-6, NO, and ROS in BV2 microglial cells. Therefore, the extract has potential for use in development of herbal medicine or food supplement for combating oxidative stress and neuroinflammation.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We 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 the authors.

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