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ORIGINAL ARTICLE

Oroxylum indicum (L.) Kurz Seed Extract Prevents LPS-Mediated BV2 Microglial Activation through NF-κB Nuclear Translocation and Activation of the Akt/ERK1/2 Signaling Pathways

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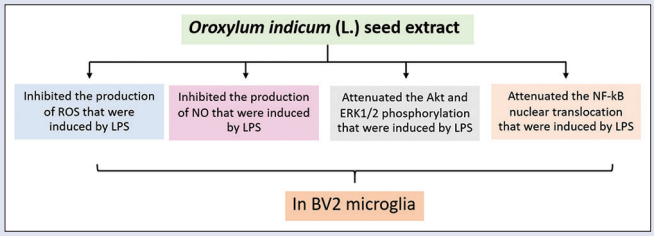
ABSTRACT

Background: *Oroxylum indicum* (L.) Kurz is an edible plant that is widely consumed in several Asian countries. In our previous study, it was demonstrated that *Oroxylum indicum* (L.) Kurz seed (OIS) extract attenuated oxidative stress and proinflammatory cytokines in activated microglia. However, the molecular mechanism remains unknown. **Objectives:** To confirm the antiinflammatory and antioxidative effects of OIS extract on and molecular mechanisms of OIS in lipopolysaccharide (LPS)-induced microglial activation. **Materials and Methods:** Nitrite and Chloro methyl derivative of 2′, 7′-dichlorodihydrofluoresceindiacetate (CM-H_2DCFDA) assays were performed to examine the effect of OIS extract on LPS-induced nitric oxide (NO) and reactive oxygen species (ROS) production, respectively. Immunofluorescence and ELISA were performed to detect the levels of nuclear factor-κB (NF-κB) in the nucleus. Western blot analysis was performed to detect the phosphorylated (p-) form of Akt and extracellular-signal-regulated kinase 1/2 (ERK1/2). **Results:** OIS extract exhibited a significant and dose-dependent suppressive effect on LPS-induced NO and ROS production. Mechanistically, OIS extract attenuated the p-Akt and p-ERK1/2 signaling pathways, thereby inhibiting the LPS-induced NF-κB nuclear translocation. **Conclusion:** OIS extract exerts anti-oxidative effects by suppressing ROS production, and anti-inflammatory effects by inhibiting NO in activated BV2 microglial cells. Mechanistically, OIS extract could inhibit the Akt/ERK1/2-mediated NF-κB pathway. OIS extract may act as a promising functional food for the alleviation of neuroinflammation in neurodegenerative diseases.

Key words: Akt, BV2 cells, ERK1/2, NF-κB, neuroinflammation, *Oroxylum indicum* (L.) Kurz seed

SUMMARY

- This study aimed to explore the antioxidant and antiinflammatory activities of OIS extracts in LPS-stimulated BV2 microglia.
- OIS extract inhibited the LPS-induced ROS production in a dose-dependent manner.
- OIS extract inhibited NO production in LPS-stimulated BV2 microglia in a dose-dependent manner.
- OIS extract inhibited the phosphorylation levels of Akt and ERK1/2 in LPS-treated BV2 microglia.
- OIS extract inhibited the nuclear translocation of NF-κB in LPS-stimulated BV2 microglia.



Abbreviations used: DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; LPS: Lipopolysaccharide; MSU: Mahasarakham University; NF-kB: Nuclear factor-kappa B; NO: Nitric oxide; OIS: *Oroxylum indicum* (L.) Seed; ROS: Reactive oxygen species; SD: Standard deviation.

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INTRODUCTION

Neuroinflammation is a process that can contribute to the onset and progression of several neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease.[1-4] Microglial cells, the resident immune cells of the brain, have been reported to play a central role in this process.[1,3-6] Microglia can be either neuroprotective or neurotoxic. In their resting state, microglia maintain the brain's normal physiological conditions.[7,8] In the case of brain injury, infection or brain diseases, microglia are activated and release various proinflammatory mediators and cytotoxic factors, including tumor necrosis factorα, interleukin1β, nitric oxide (NO), and reactive oxygen species (ROS).[9-11] These factors are responsible for neuronal injury in several neurodegenerative diseases. Currently, searching for natural anti-oxidant compounds with a strong antiinflammatory activity is an attractive therapeutic strategy for the treatment of neurodegenerative diseases mediated by activated microglia.

Oroxylum indicum (L.) Kurz, a plant from the Bignoniaceae family, is widely used throughout India and Southeastern Asia. *Oroxylum*

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indicum (L.) Kurz has antioxidant, antiarthritic, anticancer, antiulcer, antiproliferative, antimutagenic, antimicrobial, hepatoprotective, photocytotoxic and immunostimulant properties.[12] In Ayurveda and folk medicine, several parts of *Oroxylum indicum*, including the seeds, stem, fruits, leaves, roots and bark have been used to treat diarrhea, fever, ulcer, jaundice and cancer.[12] A previous study reported that *Oroxylum indicum* extract can decrease the death of neuronal SH-SY5Y cells following treatment with Aβ25-35.[13] In our recent study, *Oroxylum indicum* (L.) seed (OIS) extract exerted anti-inflammatory and anti-oxidant effects in activated BV2 cells.[14] However, its molecular mechanisms on microglial activation have not yet been investigated. The aim of the present study was to investigate the effects of an OIS extract on the production of NO and ROS, as well as the transcription factor that regulates pro-inflammatory gene expression, with a focus on the underlying molecular mechanisms in LPS-treated BV2 microglial cells. A high-performance liquid chromatographic (HPLC) method was also used for quantitative analysis of the contents of the flavones in the extracts.

EXPERIMENTAL

Preparation of OIS extract

The OIS extract was prepared as previously described.[14]

Cell culture

A murine BV2 microglial cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin in 5% fetal bovine serum, as previously described.[14] All cell culture reagents were obtained from HyClone (Cytiva, Logan, UT, USA).

Evaluation of cell viability

A day after treatment with different concentrations of OIS extract (0–50 μg/mL) with or without LPS the incubation medium was discarded. MTT reagent (0.4 mg/mL; MilliporeSigma, Burlington, MA, USA) in serum-free DMEM was added to each well. The MTT medium was then discarded following incubation for 2 hr. The purple formazan crystals that formed were solubilized in dimethyl sulfoxide and the optical density of each well was then measured at 570 nm using a microplate reader.

Nitric oxide assay

NO levels in BV2 cells were assessed using a Griess reagent, as previously described.[14]

Intracellular reactive oxygen species assay

After a day of treatment, intracellular ROS was detected using Chloro methyl derivative of 2', 7'-dichlorodihydrofluoresceindiacetate (CM-H2DCFDA), as previously described.[14]

Western blot analysis

Treated cells were lysed with RIPA-containing protease and phosphatase inhibitors and then centrifuged at 12,000 ×*g* for 5 min at 4°C to collect the supernatant. Western blot analysis was performed as previously described.[15] All antibodies were obtained from Santa Cruz Biotechnology, Inc. Data are presented as fold changes compared with the controls.

Immunofluorescence

BV2 cells were seeded on an adherent 24-well plate in a growth medium. After a day of seeding, cells were treated for 4 hr, and nuclear factor-κB (NF-κB) nuclear translocation was evaluated using immunofluorescent staining.[16]

ELISA

ELISA was performed as previously described.[16]

High-performance liquid chromatographic (HPLC)

The contents of flavonoid contents in the OIS were quantitatively analyzed using the validated HPLC method modified from the previous report.[17] An HPLC analysis was performed using a Shimadzu LC-20AC pump, SPD-M20A system (Shimadzu, Kyoto, Japan) equipped with a diode array detector and a column heater (Shimadzu, Kyoto, Japan). An InertSustain® C18 column (250 mm × 4.6 mm i.d., 5 μm, GL Sciences Inc., Tokyo, Japan) is protected with a guard column. Gradient elution was performed with a water – 0.01% phosphoric acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.8 mL/min. The column temperature was 40°C with an injection volume of 20 μL; detection was performed at 285 nm. Spectra were recorded from 200 to 600 nm. Flavonoid contents in the samples were identified by comparing their relative retention times and UV spectra with those of authentic compounds and were detected using an external standard method.

Statistical analysis

Data are shown as the mean ± SEM from at least three independent experiments performed in triplicate. Multiple comparisons of data were performed using one-way ANOVA and Bonferroni post-hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

BV-2 cell viability following treatment with LPS and OIS extract

BV-2 cells were treated with varying concentrations of LPS and OIS extract for 24 hr [Figure 1]. Following treatment, an MTT assay was performed. The results showed that BV2 cells exhibited no toxicity after 24 hr up to 50 μg/mL [Figure 1]. Therefore, 25 and 50 μg/mL were selected as the concentrations of OIS extract for the following experiments.

Attenuation of NO production in LPS-induced microglial activation following OIS treatment

To examine the effects of OIS extract on NO level in LPS-treated BV2 microglia, a Griess reagent assay was performed. BV-2 microglia were treated with LPS and OIS extract for 24 hr. Following treatment, NO production was measured. The results demonstrated that the NO levels

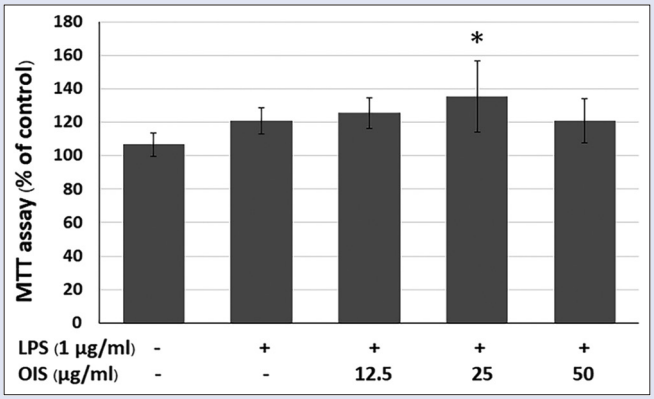

Figure 1: Effects of OIS extract on BV2 cell viability using an MTT assay. BV2 cells were incubated with OIS extract alone or with 1 μg/ml LPS. Cell viability was determined 24 hr after treatment. Data are presented as the mean ± SEM; *$P < 0.05$, compared with the control group

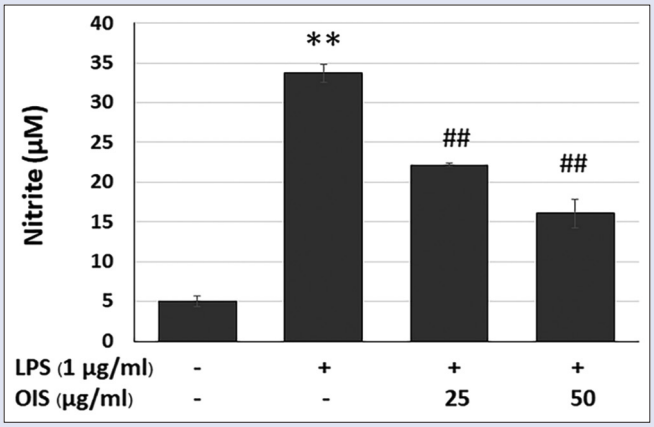

Figure 2: Effect of OIS extract on LPS-induced NO production. After 24 hr of treatment, the culture media was harvested to examine NO production. Data are presented as the mean ± SEM ($n = 3$). $^{**}P < 0.01$, compared with the control group; $^{\#\#}P < 0.01$ compared with the LPS-treated group

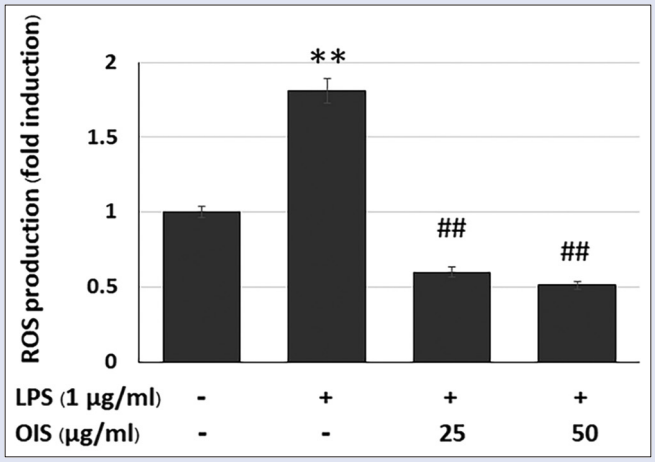

Figure 3: Effect of OIS extract on LPS-induced intracellular ROS levels. BV2 microglia were treated as indicated for 24 hr, and ROS accumulation was measured. Data are presented as the mean ± SEM ($n = 3$). $^{**}P < 0.01$, compared with the control group; $^{\#\#}P < 0.01$, compared with the LPS-treated group

of LPS-treated BV2 microglia were significantly increased from basal levels ($P < 0.01$). Co-OIS treatment extract inhibited the NO levels effectively and in a concentration-dependent manner [$P < 0.01$; Figure 2].

Attenuation of ROS production in LPS-induced microglial activation following OIS treatment

The levels of ROS were measured following the administration of OIS and LPS. The results demonstrated that the levels of ROS in the BV-2 cells following LPS treatment were significantly increased (1.8-fold) compared with the untreated group [Figure 3]. However, OIS treatment significantly reduced ROS levels in LPS-treated BV2 cells in a concentration-dependent manner. These results highlighted the ability of OIS to reduce LPS-induced intracellular ROS production.

Attenuation of NF-κB nuclear translocation in microglia activated by LPS following OIS treatment

NF-κB is a key transcription factor that controls the expression of several inflammation-related genes. Therefore, in this experiment, the nuclear translocation of NF-κB was assessed using immunofluorescent staining and ELISA. Immunofluorescence showed that the NF-κB p65 subunit was mainly located in the cytoplasm in the untreated group under normal physiological conditions, and is translocated into the nucleus following LPS treatment. When studying the suppression of NF-κB activation following OIS treatment in LPS-treated BV2 microglia, it was found that OIS extract could reduce the high nuclear NF-κB p65 translocation in these cells [Figure 4]. ELISA demonstrated that the NF-κB p65 level in the nuclear extract significant increased following LPS treatment. However, NF-κB p65 in the nuclear fraction decreased following OIS treatment of microglia activated by LPS [Figure 4b].

Attenuation of p-Akt and p-extracellularsignal-regulated kinase 1/2 (ERK1/2) in LPS-induced microglial activation following OIS treatment

The expression of p-Akt and p-ERK1/2 was determined by western blotting 2 h after treatment. As shown in Figure 5a, following LPS stimulation, Akt phosphorylation was markedly increased. However, following OIS treatment, a significant decrease in LPS-induced Akt phosphorylation was observed. Following the stimulation of BV-2 cells with LPS, p-ERK1/2 levels were also significantly increased. OIS treatment markedly suppressed p-ERK1/2 [Figure 5b] phosphorylation.

Analysis of flavonoid contents in OIS extract by HPLC

As shown in Figure 6, HPLC analysis of OIS extract showed specific chromatographic fingerprints with the presence of the peaks that corresponded to baicalein and chrysin at the retention times of 8.27 and 14.43 min, respectively, according to the standard mixture HPLC chromatogram in Figure 7. From the optimized and validated HPLC method, the baicalein and chrysin contents in OIS extract were 263.06 and 12.80 μg/mg extract, respectively.

DISCUSSION

OIS has been widely used in folk medicine and health-promoting food in several Asian countries.[12] In our previous study, it was demonstrated that LPS treatment increased NO and ROS production in microglia can be attenuated by OIS extract; however, its mechanisms of action were unclear.[14] Therefore, the aim of the present study was to elucidate the mechanisms underlying the antiinflammatory effects of the OIS ethanol extract. Based on previous studies, the antiinflammatory effect of OIS extract on LPS-activated microglia was first confirmed. The results showed that the proinflammatory mediator's NO and ROS were markedly increased following exposure to LPS alone. By contrast, OIS extract treatment significantly attenuated the production of NO and ROS in a concentration-dependent manner. This result was consistent with that of a previous study.[14] Mechanistically, OIS extract was found to attenuate NF-κB activation through the regulation of the Akt/ERK signaling pathway in LPS-induced BV2 microglial activation.

Activated microglia are considered to be an important hallmark of brain inflammation, and play a key role in regulating neuroinflammatory reactions.[18] Chronic microglial activation is known to release a variety of neurotoxic pro-inflammatory mediator effects in neuronal cell death.[19] NO is a neurotoxic mediator that is widely accepted to result in the formation of reactive nitrogen species and induce neuronal cell death

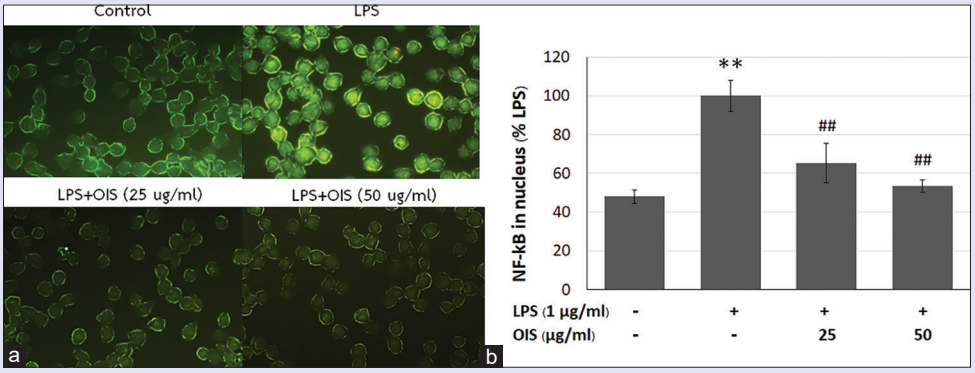

Figure 4: Effect of OIS extract on NF-κB nuclear translocation in LPS-treated BV2 microglia. (a) Immunofluorescence. (b) ELISA. BV2 cells were stimulated with LPS in the presence or absence of OIS, as indicated. Following 4 h of treatment, cells were examined using immunofluorescence. All data are presented as the mean ± SEM ($n = 3$). $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the LPS-treated group

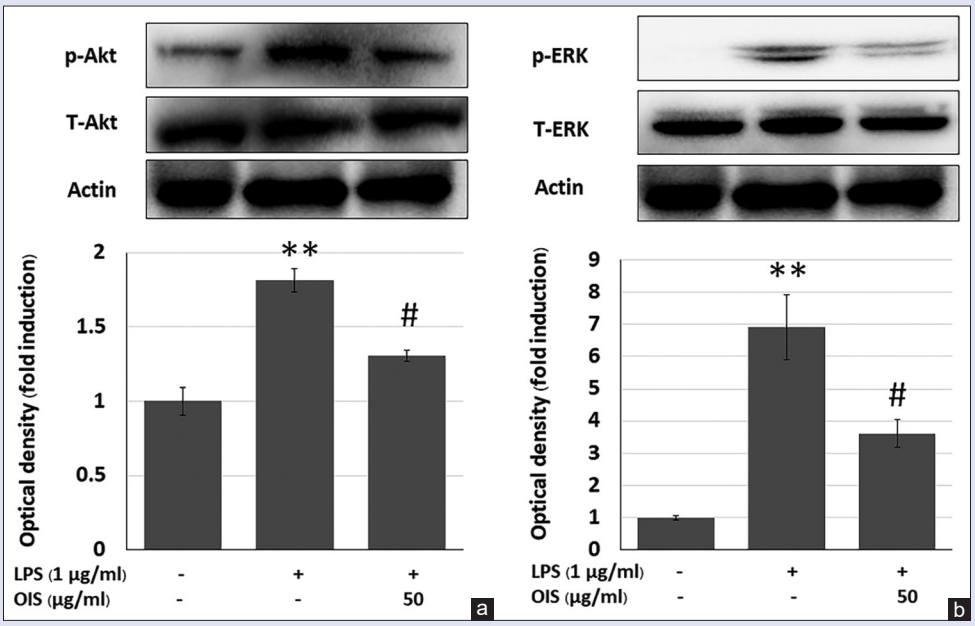

Figure 5: Effect of OIS extract on p-Akt (a) and p-ERK1/2 (b) in LPS-treated BV2 microglia. After 2 hr of treatment, total cell lysate was harvested and the anti-Akt, p-Akt, ERK1/2, and p-ERK1/2 were determined using western blot analysis. Histograms are represented as fold changes of p-Akt (a) and p-ERK1/2 (b) compared with the untreated control. All data are presented as the means ± SE ($n = 3$). $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$ compared with the LPS-treated group

via activation of microglia during neuroinflammation.[20] ROS have also been implicated in neuronal cell death.[21] Therefore, OIS extract suppresses NO and ROS production, suggesting that the inhibition of microglial activation by OIS may prevent inflammation-related neuronal death.

NF-κB activation has been reported to control the expression of pro-inflammatory cytokines and ROS during neuroinflammation to trigger a self-perpetuating process that results in progressive neuronal cell death.[22] Under physiological conditions, NF-κB is bound to the inhibitory IκB protein in the cytoplasm. However, IκB is phosphorylated and subsequently degraded in response to stimuli, and NF-κB is released and translocates to the nucleus, where it promotes the expression of pro-inflammatory cytokines and enzymes.[23] Therefore, to further investigate the mechanisms underlying the inhibitory effects of OIS on proinflammatory cytokines in BV-2 cells, the effect of OIS on NF-κB activation was examined using immunofluorescence and ELISA. The results revealed that OIS attenuated the nuclear translocation of NF-κB in LPS-treated microglia, as shown by immunofluorescence and ELISA. *Oroxylum indicum* (L.) Kurz has been found to be a potent NF-κB inhibitor,[24] It has been also reported to contain baicalein and chrysin which are known to suppress the activation of NF-κB.[25,26] Since OIS extract has been reported to contain the baicalein and chrysin content, the reduction of NF-κB nuclear translocation in LPS-activated microglia following OIS extract treatment in the present study may have been due to the baicalein and chrysin in OIS. The activation of NF-κB has been found to be controlled by the PI3K/Akt and ERK1/2 pathways.[27] The inhibitory effect of OIS extract on the levels of the Akt and ERK phosphorylation was further confirmed herein. The phosphorylation levels of Akt and ERK1/2 pathways were markedly suppressed following treatment with OIS extract in LPS-activated BV2 cells. Because baicalein and chrysin have also been reported to inhibit PI3K/Akt signaling pathway,[28-30] therefore, the reduction of Akt phosphorylation in LPS-activated microglia following OIS extract treatment might be due to the baicalein and chrysin in OIS.

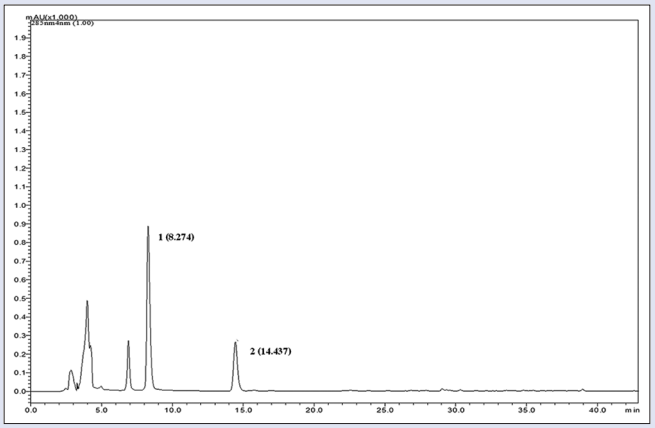

Figure 6: HPLC chromatograms of OIS extract; peak 1 = baicalein, peak 2 = chrysin

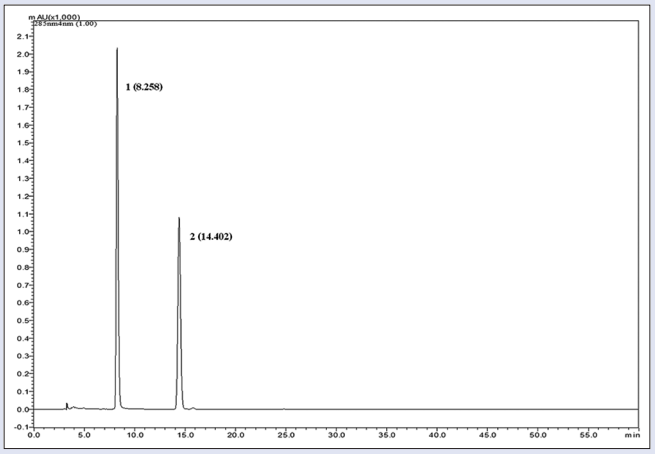

Figure 7: HPLC chromatograms of standard compounds; 1 = baicalein, 2 = chrysin

HPLC was performed to investigate the flavonoid contents in OIS extract. From our study, it was clearly shown that the major flavonoid in the OIS was baicalein, and chrysin according to the standard mixture HPLC chromatogram. However, in OIS sample, there was a peak that appeared at the retention time before the peak of baicalein which maybe other major compounds in OIS such as baicalin and oroxin as a previous study.[31]

CONCLUSION

In conclusion, the present study provided evidence that OIS extract attenuated NO and ROS production in LPS-treated microglia. Mechanistically, the inhibition of NF-κB translocation from the cytoplasm to the nucleus was due to OIS extract treatment, which is associated with the inactivation of the Akt and ERK1/2 signalling pathways. These results suggested that OIS extract might have the potential to prevent and treat inflammatory diseases and several neurodegenerative diseases that involve microglial activation.

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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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Conflicts of interest

There are no conflicts of interest.

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