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ORIGINAL ARTICLE

Limonin: A Triterpenoid Exerts Protective Effect during Lipopolysaccharide Stimulated Inflammation in BV2 Microglial Cells

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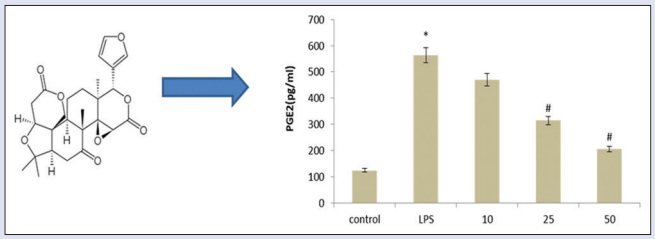
ABSTRACT

Objectives: Neuroinflammation mediated by the reactive oxygen and nitrogen species (ROS and RNS, respectively) is responsible for the production of cytokines and chemokines in the central nervous system, which causes glial activation and associated pathology. Limonin is an oxygenated triterpenoid dilactone obtained from the families *Rutaceae* and *Meliaceae*. It exerts anticancer, antioxidant, antiviral, antinociceptive, and anti-inflammatory effects. In this study, we intended to analyze the anti-inflammatory and neuroprotective activities of limonin against neuroinflammation induced by lipopolysaccharide (LPS) in an *in vitro* model with BV2 immortalized murine microglial cells. **Materials and Methods:** Cytotoxicity of limonin was investigated via 3-(4,5-dimethylthiazolyl-2)-2,5-dipheniltetrazolium bromide tetrazol assay, and anti-inflammatory activity was assessed by analyzing the status of ROS, nitric oxide (NO), prostaglandin E2 (PGE2), interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α using ELISA. The expression of inducible nitric oxide synthase (iNOS), TNF-α, IL-1β, and Cyclooxygenase (COX-2) was studied through immunoblot analysis. **Results:** Our results showed that pretreatment of LPS-induced microglial cells with limonin appreciably prevented the formation of ROS and prevented the accretion of NO, PGE2, IL-1β, IL-6, and TNF-α. Furthermore, limonin pretreatment significantly reduced the expressions of iNOS and COX-2. **Conclusion:** Our results indicate that limonin inhibits inflammatory response through repressing the formation of ROS, accretion and discharge of cytokines, and activation of inflammatory mediators. Limonin can be used as a neuro-protecting agent.

Key words: Astrocytes, BV2 microglia, glial cells, inflammation, LPS, neuroprotection

SUMMARY

- Limonin has gained considerable interest due to its diverse biological functions such as anti-inflammatory, antitumor, and antimicrobial activities.
- Limonin significantly attenuated reactive oxygen species-mediated oxidative stress and microglial activation, thereby showing offers a cytoprotective effect against lipopolysaccharide -induced cytotoxicity.



Abbreviations used: GST: Glutathione S-transferase; COX-2: Cyclooxygenase; PGs: Prostaglandins; NO: Nitric oxide; AD: Alzheimers disease; PD: Parkinson's disease; LPS: Lipopolysaccharide.

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INTRODUCTION

Inflammation of the nervous system (neuroinflammation) ensures homeostasis and it localizes toxic materials, fights pathogens, and prevents tissue injury,[1] thereby benefiting tissue repair. On the contrary, excessive, and uncontrolled inflammation leads to tissue injury which in turn leads to various disorders.[2-4] Excessive production of reactive oxygen species (ROS) in the mitochondria and excessive accumulation of cytokines was previously perceived as responsible for the pathogenesis of brain damage, inflammatory condition, and neurodegenerative ailments.[5-8] According to the World Health Organization (WHO), approximately 70 million people will experience neurodegenerative diseases by the year 2030 and approximately 106 million people by the year 2050,[9] and it might cause major socio-economic burden in the future.

The central nervous system (CNS) comprises the brain, the spinal cord, and the peripheral nervous system, which in turn comprise neurons, endothelial cells, astrocytes, and glial cells. Stimulated glial cells, (called microglia) and stimulated astrocytes (called astroglia) are responsible for the pathogenesis of neurodegeneration through the stimulation of inflammatory pathways. Gliosis is an inflammatory condition exemplified through astrocytic multiplication and hypertrophy of the cell body. Glial cells activated via toxins such as lipopolysaccharide (LPS), interleukin (IL)-1β, and tumor necrosis factor (TNF-α) are responsible for the inflammatory pathogenesis.[10,11] Furthermore, disorders such as Alzheimer disease (AD), Parkinson

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disease (PD), Huntington disease (HD), and trauma activate microglial cells, which produce inflammatory mediators such as cyclooxygenase (COX-2)/prostaglandins (PGs), inducible nitric oxide synthase (iNOS)/nitric oxide (NO), or cytokines over and above neurotoxic substances, which mediates brain tissue injury.[12,13] Furthermore, accumulating evidence also implicates that inflammation contributes to diverse brain pathologies, apparently killing neurons via activation of glia and inflammatory pathways.[14] Hence, research focusing on developing strategies that target the activation of glial cells and associated inflammatory pathways might help in the clinical management of inflammation-associated neurodegenerative pathologies.

Limonin is a limonoid glycoside isolated from leaves, fruits, and seeds of citrus plants and belongs to the class of furanolactones.[15] According to the literature, about 300 limonin analogs have been extracted from various natural resources. Recently, limonin has gained considerable interest due to its diverse biological functions such as anti-inflammatory, antitumor, antimicrobial, and antifeedant activity.[16] Furthermore, research shows that limonin induces and maintains the levels of glutathione S-transferase (GST).[17] In addition, it acts as a hepatoprotective agent against ischemia and prevents reperfusion mediated- and D-galactosamine-mediated hepatic injury.[18] However, to the best of our knowledge, studies analyzing the effect of limonin against CNS pathologies and inflammatory degenerative conditions are scarce. Because inflammation is highly regarded as the crucial mediator of the initiation and succession of neurodegenerative conditions,[19] herein, we aimed to explore the anti-inflammatory effects of limonin against LPS-induced inflammation using BV2 microglia cells. In summary, our results displayed that limonin reveals a defensive outcome via inhibiting the generation of ROS and by regulating the production and release of inflammatory cytokines, which can be attributed to its neuroprotective property.

MATERIALS AND METHODS

Chemicals

LPS and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 and sodium pyruvate penicillin/streptomycin, and trypsin-EDTA were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India) and fetal bovine serums (FBS) was purchased from Gibco (Germany). All other reagents were of diagnostic grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sisco Research Laboratories (SRL Pvt Ltd, India).

BV2 microglial cell culture

BV2 microglial cell line was purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were routinely cultured on Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL of streptomycin. The cells were grown in an incubator under 5% CO_2/95% atmospheric air at 37°C.

Cell viability test

We tested the cell viability via 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide tetrazol (MTT) test. MTT is reduced by the mitochondrial dehydrogenases in living cells to yield purple formazan crystals. Cells were plated in 24-well plates (50,000–75,000 cells/well). After 12 h, the media was discarded and the adherent cells were washed with buffered saline. Then, the cells were incubated in a serum-free medium (SFM) for 12 h. Then, SFM was replaced with medium containing limonin at concentrations in the range of 10–100 mg/mL (limonin was prepared in 0.01% DMSO). Control cells were incubated with 0.01% DMSO, which having SFM. After 24 h, cells were washed with phosphate-buffered saline (PBS) and 5 mg/mL of MTT (prepared in PBS) was added and incubated at 37°C for 2 h. Next, 500 mL of DMSO was added to dissolve the formazan crystals formed. Then, the absorbance of the color formed was read at 570 nm on ELISA reader. Cell viability was calculated as per the given formula:

% cell viability = (absorbance of the treated cells/absorbance of the control cells) × 100%.

Measurement of intracellular reactive oxygen species

Intracellular ROS was measured by utilizing an oxidation-sensitive probe, dichloro-dihydro fluorescein diacetate (DCFH-DA). DCFH-DA was dissolved in SFM in a ratio of 1:1000. To test the antioxidant activity, BV2 microglial cells were pre-exposed to different concentrations of limonin by incubating for up to 12 h. Then, the cells were washed and DCFH-DA (20 μM) was added to each well. The cells were incubated at 37°C for 20 min. DCFH-DA was removed by washing the cells with twofold PBS. Then, the plate was stirred for about 3 to 5 min and then, the cells were washed with serum culture media to discard any unreacted DCFH-DA dye. Tests dissected by stream cytometry (FACS Calibur, Becton Dickinson, 530 nm).

Enzyme-linked immunosorbent assay

In this study, we tested the presence of IL-1β, IL-6, and TNF-α in cell culture supernatants via ELISA packs (R&D Systems, Minneapolis, MN, USA) as per manufacturer's guidelines.

Determination of nitric oxide

NO was determined by utilizing the Griess reagent. BV2 microglial cells were grown on regular DMEM with no phenol red. Briefly, after 16 h of LPS challenge, cells (50 μL) were seeded on to 96-well plates. Then, 50 μL of Griess reagent A was added [1% (w/v) sulfanilamide in 5% phosphoric acid] and incubated at 37°C for 10 min in dark. Next, an equal volume of Griess reagent B (0.1%, w/v, N-(1-napthyl)-ethylenediamine dihydrochloride) was added and incubated at 37°C for 10 min in dark. Next, the absorbance was read at 543 nm with the aid of Synergy-4 plate reader. A standard curve was prepared using sodium nitrite (0–100 μM) in the culture medium.

Immunoblot analysis

To determine the levels of protein expression, the BV2 microglial cells were washed thrice with PBS. Then, the cell suspension was centrifuged at 6000 rpm for 15 min, and the pellet was homogenized with RIPA buffer supplemented with protein lyase inhibitors. Then, the homogenized cell suspension was centrifuged at 13,000 rpm for 20 min. About 40 μg of proteins from each example were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% separating gel). The separated bands were transferred on to the polyvinylidene difluoride (PVDF) layers. PVDF film was blocked with 5% skimmed milk followed by incubating it in respective probes. Antibodies utilized for iNOS and hostile to COX-2 against IκBα, phospho-IKKα/β and phospho-IκBα against α-tubulin (Sigma). The PVDF membrane was washed with Tris-buffered saline-Tween 20 afterward hatched with optional antibodies. Finally, the proteins were visualized by the aid of ChemiScope 3300 Mini (CLINX, Shanghai, China).

Data analysis

For multi-assemble correlation, the information was broken down via a one-way analysis of variance trailed through Student Newman Keul's posthoc technique. $P < 0.05$ was considered as significant.

RESULTS

Effects of limonin on BV2 microglial cell viability

Cytotoxicity of limonin at different concentrations was evaluated via MTT assay [Figure 1]. Limonin at 100 μM concentration showed a 25% reduction in the viable cells after 24 h of incubation. The survival rate of cells did not decline rapidly with an increase in the concentration of limonin (up to 100 μM). These findings show that the IC_{50} values of limonin were greater than 100 μM. Therefore, further analysis was conducted with a concentration ranging from 10 μM to 50 μM limonin.

Effects of limonin on the production of reactive oxygen species status from lipopolysaccharide-induced BV2 microglial cells

Figure 2 presents the effects of limonin on the ROS production in LPS-induced BV2 microglial cells. LPS challenge remarkably increased ($P < 0.05$) the production of ROS in BV2 microglia cells. However, pretreatment of cells with limonin markedly decreased the ROS production in a dose-dependent manner ($P < 0.05$). This shows that limonin exerts beneficial effects by decreasing the production of ROS during the incubation of cells with LPS.

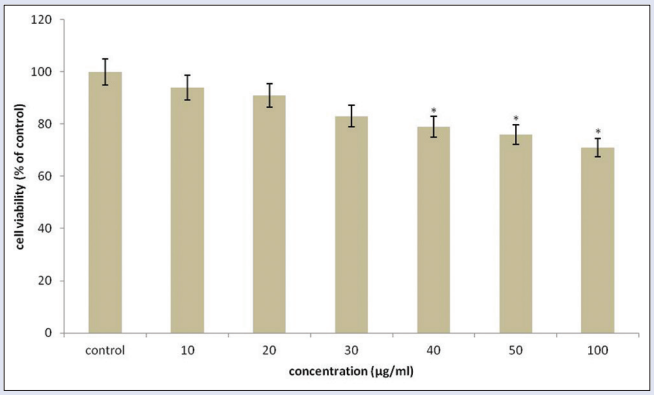

Figure 1: The effect of Limonin on viability of BV-2 microglial cells. *Significantly different ($P < 0.05$) as compared to control group. Data are represented as mean ± standard error of mean of three individual experiments

Effects of limonin on nitric oxide levels during lipopolysaccharide treatment in BV2 microglial cells

In this study, the effect of limonin on the intracellular accumulation of NO was investigated in BV2 microglial cells after induction with LPS [Figure 2]. Pretreatment with LPS significantly increased NO production in BV2 microglia cells. However, limonin (10, 25, 50, and 100 μg/mL), significantly decreased the production of NO after stimulation with LPS in a dose-dependent manner ($P < 0.05$). This shows that limonin effectively suppressed the inflammatory process and also quenched the existing ROS.

Effects of limonin on the levels of prostaglandin E2 levels in lipopolysaccharide-induced BV2 microglial cells

Figure 3 shows the accumulation of prostaglandin E2 (PGE2) after limonin treatment in BV2 microglial cells induced with LPS. Treatment with LPS markedly increased the accumulation of PGE2 than that of control cells. Pretreating the cells with limonin surprisingly attenuated the levels of LPS-induced PGE2 expression in a dose-dependent manner. The highest level of attenuation was recorded for a concentration of 100 μg/mL.

Effects of limonin on the secretion of interleukin-1β, interleukin-6, and tumor necrosis factor-α from lipopolysaccharide-induced BV2 microglial cells

Figure 4 shows the amount of secretion of IL-1β, IL-6, and TNF-α into the medium from LPS-induced BV2 microglia cells. Stimulation of BV2 microglia cells with LPS resulted in increase in the secretion of TNF-α, IL-1β, and IL-6. Pretreatment with limonin resulted in a decrease in the levels of these inflammatory cytokines in a dose-dependent manner ($P < 0.05$). According to our results, limonin potentially attenuated the secretion of pro-inflammatory mediators (TNF-α, IL-1β, and IL-6) which shows that limonin is a good anti-inflammatory agent.

Effect of limonin on the activation of nuclear factor kappa B in lipopolysaccharide-induced BV2 microglial cells

Figure 5 shows the effect of limonin in modifying the nuclear factor kappa B (NFκB) pathway during LPS stimulation in BV2 microglial cells. We analyzed whether limonin pretreatment regulates LPS-stimulated phosphorylation of IKKα and IKKβ and IκB-α levels in BV2 microglia cells. LPS induction significantly increased the expressions levels of pIKKα, pIKKβ, COX-2, and iNOS, whereas the levels of Iκβ-α is

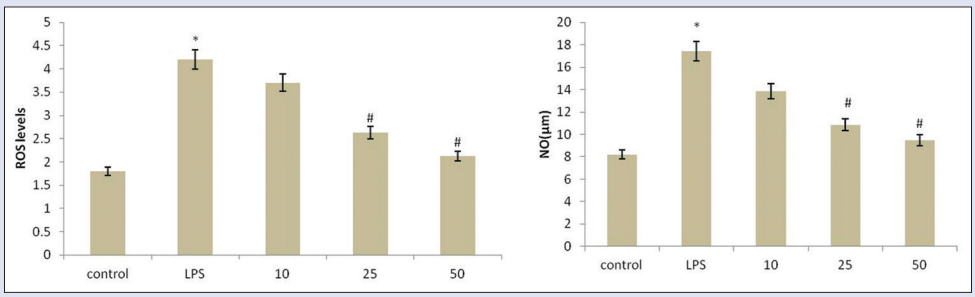

Figure 2: The effect of Limonin on reactive oxygen species and nitric oxide levels in lipopolysaccharide-induced BV-2 cells. *Significantly different ($P < 0.05$) as compared to control group. #Significantly different ($P < 0.05$) as compared to lipopolysaccharide alone treated group. Data are represented as mean ± standard error of mean of three individual experiments

markedly reduced. However, pretreatment with limonin significantly reduced the phosphorylation of IκB-α and increased the expression of Iκβ-α after LPS stimulation. Our results showed that limonin pretreatment significantly decreased the activation of the NFκB pathway during LPS stimulation in BV2 microglial cells.

DISCUSSION

Similar to macrophages of the CNS, the occupant immune cells, microglias, was known to perform a protective role in the CNS. Microglia safeguards the cells against and/or or during toxic insults.[20] In certain conditions, too much stimulation of acute or chronic microglial cells can elicit neuronal injury via secretion cytotoxic mediators (e.g., NO, ROS, TNF-α, and IL-1β).[21] (Surace and Block, 2012) Various stimuli such as heavy metals or aggregated proteins induce the transition of microglia from the resting state to the activated state and are characterized by the accumulation of various cytokines in addition to the induction of inflammatory mediators such as iNOS and COX-2.[22] Research on neurodegeneration, toxicology, and immunity, which investigates the role of microglia, requires a large number of animals. Therefore, research utilizing the microglial cells will effectively reduce the necessity of animal experimentation, provided that the cell line of our interest replicates the *in vivo* niche we are looking for.

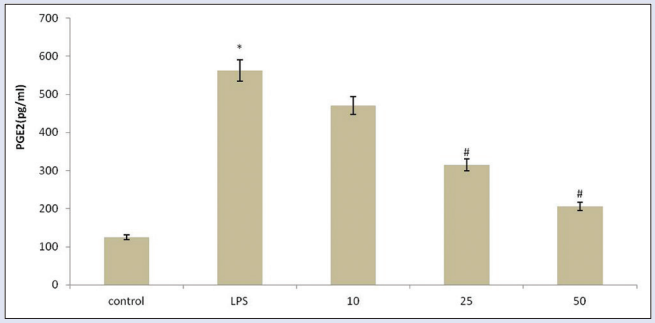

Figure 3: The effect of Limonin on prostaglandin E2 levels in lipopolysaccharide-induced BV-2 cells. *Significantly different ($P < 0.05$) as compared to control group. #Significantly different ($P < 0.05$) as compared to lipopolysaccharide alone treated group. Data are represented as mean ± standard error of mean of three individual experiments

The stimulation of inflammatory cells contributes to the initial inflammatory response in brain injury.[23] This initial microglial activation in the CNS is an effort to safeguard the neurons. On the contrary, too much stimulation of microglia eventually leads to the necrosis of neurons.[24]

Recent scientific research has enlightened the application of medicinal plants due to their significant pharmacological activity, low cytotoxicity, and economic feasibility. In this context, the role of limonin as an anticancer, antiapoptotic, and chemoprotective agent has been highlighted, but the reports are scarce. Among the majority of the citrus limonoids studied, limonin, and nomilin. Limonin is a triterpenoid aglycone derivative containing a furan ring linked to D-ring at C-17 in addition to oxygen-containing functional groups at C-3, C-4, C-7, C-16, and C-17 and an epoxide group at C-14 and C-15. Limonin has been reported as an abortifacient mediator,[25] amoebicidal essence,[26] chemopreventive agent, and prevents oral carcinoma.[27]

In this study, limonin did not exert any cytotoxicity toward BV2 cells at concentrations up to 100 μM. Our results suggest that limonin (20, 40, 60, 80, and 100 μg/mL) slightly decreased cell proliferation after incubation of cells for 24 h; however, the tested concentrations were not significantly cytotoxic. Hence, we tested concentrations ranging from 10 μM to 50 μM in further experiments.

Excessive production of ROS or low level of antioxidant defense system can critically induce the inflammatory system. Inflammatory stimuli can result in the increased accumulation of ROS which can significantly reduce the macromolecular content within the cells. This can lead to genomic instability, genomic repair inhibitions, chromosomal aberrations, and eventually leads to genetic mutations. Excessive accumulation of ROS increases oxidative stress and inflammatory reactions, which eventually promotes pathophysiological conditions.[28]

In neurons, NF-κB has been linked to the various inflammatory conditions during ischemia. After activation, the canonical NF-κB pathway prompts

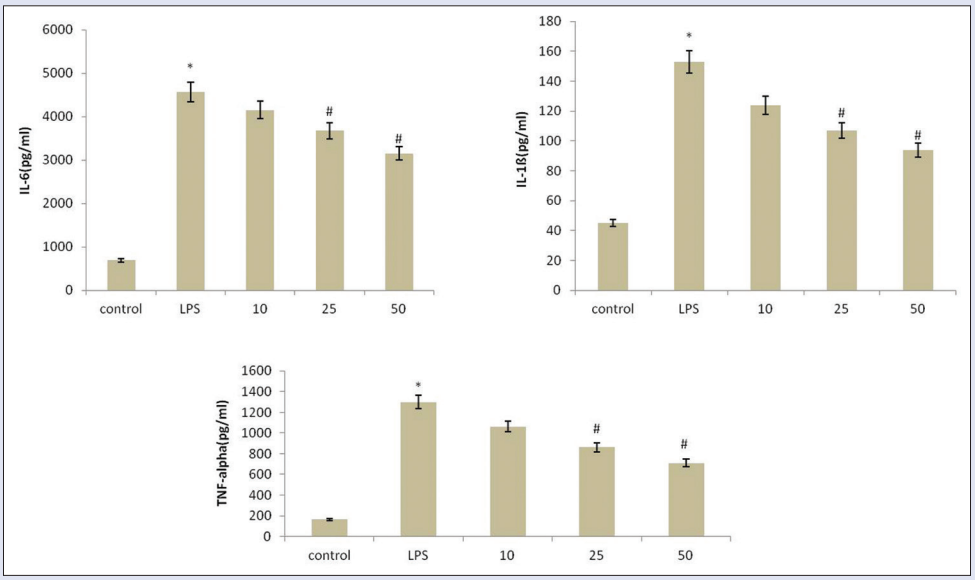

Figure 4: Effects of Limonin on interleukin-1β, interleukin-6 and tumor necrosis factor-α release in lipopolysaccharide stimulated BV2 microglia cells. *Significantly different ($P < 0.05$) as compared to control group. #Significantly different ($P < 0.05$) as compared to lipopolysaccharide alone treated group. Data are represented as mean ± standard error of mean of three individual experiments

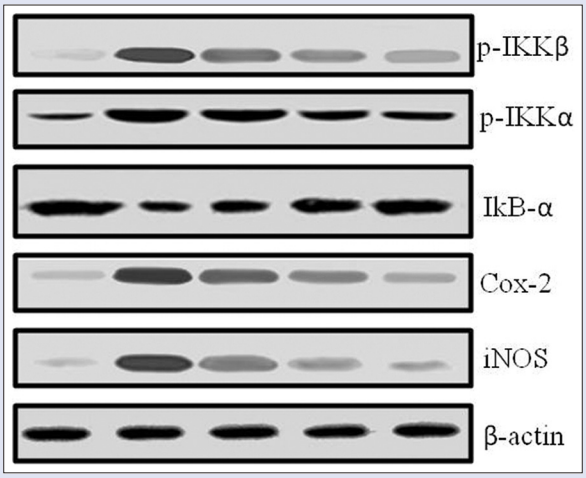

Figure 5: Western blot analysis on the effect of Limonin on the expressions of inflammatory mediator proteins in lipopolysaccharide-induced BV-2 cells. β-actin was used as internal control. Representation of three individual experiments is shown

IκB kinase (IKK) function, which directs to its phosphorylation and subsequent dilapidation of IκB proteins and discharging or translocation of NF-κB p65/p50 heterodimers. Heterodimeric p65/p50 NF-κB upon translocation to the nucleus activates a particular DNA sequence thereby initiating the transcription of genes.[29] In this study, BV2 microglial cells supplemented with limonin regulates IKK, IκB, and pIκB expressions.

The modulators of inflammation (e.g. NO and PGE2) have been known to be significantly increased during inflammation, which is mediated by iNOS and COX-2.[30] In this study, the expression of iNOS and COX-2 was significantly upregulated after the stimulation of BV2 microglial cells with LPS. However, when the cells were preincubated with limonin, the expression of iNOS and COX-2 was found to be significantly reduced when compared with LPS-induced cells. Usually, high quantities of NO are produced by inflammatory cells that revert to secrete iNOS. NO, a gaseous inflammatory molecule, is required in the mediation of delayed expression of *COX-2*, an essential mediator on neuroinflammation.[31] In this study, the possible mechanism for decreased accumulation of NO was found to be due to decreased expression of iNOS protein after pretreatment of cells with limonin. According to the literature, iNOS and COX-2 enzymes can upregulate the stimulatory response.[32] In this study, NF-κB, iNOS, and COX-2 levels were downregulated by limonin, which shows that limonin exerts significant anti-inflammatory activity against LPS-induced neuroinflammation.

In this study, we observed a significant increase in the level of PGE2 after LPS stimulation in comparison to control. Pretreatment of cells with limonin significantly ameliorated the LPS-induced PGE2 expression in a dose-dependent manner (100 μg/mL showed the highest activity). TNF-α, IL-1β, and IL-6 upregulated the secretion of inflammatory mediators produced by macrophages, which are important regulators of the human immune defense system.[33] IL-6 is synthesized mainly by macrophages and is a very powerful regulator of acute-phase inflammatory response.[34] IL-1β is yet another important pivotal pro-inflammatory cytokine of inflammatory response.[35] In this study, LPS stimulation of BV2 microglial cells increased the secretion of TNF-α, IL-1β, and IL-6. Limonin pretreatment markedly reduced the inflammation in a dose-dependent manner. This shows that limonin attenuates the accumulation of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6.[36]

CONCLUSION

Glial cells provide support to brain parenchymal cells, which provide plasticity and safeguard the brain against damages. Overactivation of glial cells promotes inflammation and causes neurodegeneration, which will eventually lead to cell death. Moreover, redox signaling and microglial activation are considered two crucial events in several inflammatory diseases, such as AD and PD. Promising remedial methods aimed at redox-susceptible molecules, microglial activation during brain inflammation, and neurodegenerative disorders can serve as potential avenues in the search for neuroprotective therapies. In this study, treatment with limonin significantly attenuated ROS-mediated oxidative stress and microglial activation, thereby offering cytoprotection against LPS-induced cytotoxicity. Hence, we endorse that limonin can be regarded as a remedial agent in avenue for the medication of neurodegenerative conditions, with further in-depth analysis.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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