Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.104221

Zingiber officinale attenuates neuroinflammation in LPS-stimulated mouse microglia by AKT/STAT3, MAPK, and NF-κB signaling

Suping HAO[1,2], Byoung Ok CHO[3,4], Feng WANG[1], Jae Young SHIN[4], Da Jeong SHIN[4], Seon Il JANG[1,4]*

Abstract

To explore the protective activity of ginger (*Zingiber officinale*) root ethanol extract (GRE) on the neuroinflammation induced by lipopolysaccharide in microglial cells. Ginger has been investigated as a neuroprotective and anti-aging agent. Nevertheless, ginger extract attenuates neuroinflammation in microglia have not been discovered in depth. The results showed that GRE had high total phenolic and (55.63 ± 0.16 mg GAE/g) and total flavonoid content (4.33 ± 0.17 mg QUE/g), and antioxidant activity. GRE inhibited the release of cytokines and inflammatory mediators including COX-2, PGE_2, Nitric oxide, interleukin-6, TNF-α, and iNOS. GRE ameliorated microglia-mediated neuronal insults via upregulating the expression of Bax and reducing the expression of Bcl-2. GRE suppressed NF-κB and AKT/STAT3, and the MAPK pathway in the neuroinflammatory response. In conclusions, GRE positively affected anti-neuroinflammatory and neuroprotective activity without serious side effects, which might be used as a functional food additive and/or therapeutic material for the management and prevention of neurodegenerative diseases.

Keywords: *Zingiber officinale*; neuroinflammation; microglia; NF-κB; MAPK.

Practical Application: Ginger can not only be applied to cooking spices and medicine but also processed into functional food.

1 Introduction

Ginger *(Zingiber officinale*), is the family Zingiberaceae, has been employed as a food, medicine and spice for more than 2000 years (Mao et al., 2019; Shahrajabian et al., 2019). Ginger is cultivated around the world including in India, China, Korea, and Australia (Beristain-Bauza et al., 2019). Ginger has been considered safe for use in food processing and medicine (Beristain-Bauza et al., 2019). Ginger rhizome is applied for the prevention and treatment of numerous common diseases, such as nausea, emesis, dysmenorrhea, carsickness, headaches and colds (Beristain-Bauza et al., 2019). Recently, ginger has been proven to possess multiple biological activities, including anti-inflammatory (Lantz et al., 2007), antioxidant (Si et al., 2018; Stoilova et al., 2007), anticancer (Habib et al., 2008), antimicrobial (Sebiomo et al., 2011), neuroprotective (Hussein et al., 2017; Sahardi & Makpol, 2019), cardiovascular protective (Attyah & Ismail, 2012), and anti-obesity activities (Kim et al., 2018). Ginger's therapeutic effect mainly stems from its active constituents, which include paradols, shogaols, and gingerols (Mao et al., 2019). Ginger has been investigated as a neuroprotective and anti-aging agent that protects against inflammation and oxidative stress in neurodegenerative and aging diseases (Sahardi & Makpol, 2019). 6-Gingerol (6-G), a ginger compound, has been used as an in *vitro* and in *vivo* neuroprotective agent in the lipopolysaccharide (LPS)-induced microglia neuroinflammation model (Zhang et al., 2018). Therefore, ginger might be a good natural food material to relieve neuroinflammation of LPS-induced neurodegeneration.

Currently, the aging of the population is intensifying worldwide due to reduced fertility and increased life expectancy (Lunenfeld & Stratton, 2013). Increases in neurodegenerative diseases accompany aging. For example, Parkinson's disease (PD) together with Alzheimer's disease (AD) are becoming major health issues (Pogačnik et al., 2020). These diseases have the main biological mechanisms, including oxidative stress, neuroinflammation, and protein misfolding, as well as mitochondrial dysfunction. Previous studies have confirmed that anti-inflammatory drugs and antioxidants could help treat these diseases (Pogačnik et al., 2020). Microglia play pivotal roles in the central nervous system (CNS). The activation of microglia results in the accumulation of pro-inflammatory factors and cell death in neurodegenerative diseases (Oh et al., 2021). LPS is an endotoxin inducing inflammation and is used to create neurodegenerative *in vitro* models (Oh et al., 2021). LPS induces microglial cells activate inflammation through AKT/STAT, NF-κB, and MAPKs signaling pathways, these cells generate too many inflammatory mediators for instance NO and PGE_2, at the same time pro-inflammatory cytokines TNF-α, IL-6, and IL-1 (Wang et al., 2021a; Wang et al., 2021b; Jung et al., 2009; Ryu et al., 2019; Zhao et al., 2019). Thus, it is significant to regulate MAPK, NF-κB, and the AKT/STAT pathways for treating neuroinflammatory diseases and controlling microglial

Received 01 Oct., 2021
Accepted 05 Nov., 2021
[1] *Department of Health Management, Jeonju University, Jeonju-si, Jeollabuk-do, Korea*
[2] *Department of Biological Science and Engineering, Xingtai University, Xingtai, Hebei, China*
[3] *Institute of Health & Science, Jeonju University, Jeonju, Korea*
[4] *Research Institute, Ato Q&A Co., LTD, Jeonju-si, Jeollabuk-do, Korea*
*Corresponding author: sonjjang@jj.ac.kr

activation can be used to treat apoptosis and inflammation in neurodegenerative diseases.

The purpose of this work was to explore the inhibition of neurodegeneration and inflammation by GRE and its mechanisms in LPS-stimulated microglial cells.

2 Materials and methods

2.1 Reagents

6-gingerol (6-G), Folin-Ciocalteu's phenol reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Griess reagent and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Ascorbic acid (VC), butylated hydroxytoluene (BHT), LPS, and antibodies for β-actin, phospho-AKT, phospho-IκBα, phospho-p38, phospho-JNK, phospho-NF-κB, phospho-ERK1/2, AKT, NF-κB, IκBα, JNK, p38, Bax, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). STAT3, p-STAT3, iNOS, ERK1/2, horseradish peroxidase (HRP) labeled-IgG secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Bradford's reagent was obtained from Bio-Rad (Hercules, CA, USA). RIPA buffer and chemiluminescent Western blotting detection reagents were purchased from Thermo Scientific™ (Rockford, IL, USA). The Quanti-Max™ WST-8 cell viability assay kit and stripping buffer were purchased from Biomax, Co. Ltd. (Seoul, Korea). IL-6, TNF-α, and PGE_2 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), and penicillin-streptomycin (P/S) were obtained from GIBCO BRL (Grand Island, NY, USA).

2.2 Plant material and extract preparation

Ginger came from Bongdong traditional market (Wanju-gun, Jeonbuk, Korea). The ginger rhizome were washed, the peel has been scraped with a knife, and then cut into pieces of uniform size, about 1 cm thick, dried at 40 °C for 48 h in a dry oven (LabTech, Korea). The rhizome of ginger extracted (20 g) in 50% (V/V) ethanol for 2 days at 40 °C. The extraction was filtered and evaporated and freeze-dried into powder. An exact concentration of 200 mg/mL was made with DMSO and distilled water (1:1).

2.3 Total Polyphenol (TP) content in ginger extract

The TP *content* in GRE was measured by the method of Folin-Ciocalteau (Cho et al., 2017). The GRE was diluted into distilled water (1 mg/mL), standard (gallic acid) was made with distilled water prepared various concentrations (1000 - 0 μg/mL). The Folin-Ciocalteu's phenol reagent (0.1 mL) was mixed with standard and GRE (100 μL). After five minutes, 4% Na_2CO_3 (1 mL) was added and mixtured. Samples were cultivated for half an hour. At 750 nm, the absorbance was detected. The gallic acid was utilized as a standard curve (r^2 =0.9991). The TP contents was described as the gallic acid equivalents GAE/g of ginger extract**.**

2.4 Total Flavonoid (TF) content in ginger extract

The content of TF in GRE was measured with the aluminum chloride colorimetric approach (Abessolo et al., 2021). Briefly, the GRE was diluted into 1 mg/mL with distilled water, standard Quercetin was made with ethanol prepared various concentrations (1000 - 0 μg/mL). A 0.5 mL of GRE and standard was mixed with 10% $AlCl_3$ for 5 min. At 405 nm, the absorbance was determined. The calculation of the TF content was conducted from a standard curve (r^2 =0.9991) utilizing Quercetin Equivalent (QUE).

2.5 DPPH radical scavenging activity

DPPH was applied to detect the free radical scavenging ability of GRE (Cho et al., 2017). Briefly, 0.3 mM DPPH (0.1 mL) was mixed with GRE (0.1 mL) having various concentrations, cultured in darkness for half an hour with 540 nm absorbance. The lower the absorbance at 540 nm, the higher the antioxidant and free radical scavenging capacity of extract. Using BHT as standard. The calculation of activity was carried out as below: DPPH radical scavenging activity (%) = [1-(sample absorbance/blank absorbance)] ×100.

2.6 ABTS radical scavenging activity

The ABTS assay was conducted to detect free radical scavenging (Cho et al., 2017). The ABTS stock solution made by the mixture of potassium persulfate (2.6 mM) and ABTS (7.4 mM) (1:1), stocked in darkness under a temperature of 4 °C for 12-24 h. Working solution dilute the stock solution of ABTS through utilizing distilled water before the absorbance is between 0.7 ± 0.02 and 0.75 ± 0.02 at 750 nm. The GRE and standard VC were made with distilled water prepared various concentrations (1000 - 0 μg/mL), respectively. 50 μL of different concentrations of GRE and VC and ABTS working solution 950 μL mixed, in dark for half an hour. At 750 nm, the absorbance was determined. All of the experiments were implemented in quadruplicate. The calculation of activity was conducted as below: ABTS radical scavenging activity (%) = [1-(sample absorbance/blank absorbance)] ×100

2.7 Cell culture

SIM-A9 microglial cells (CRL-3265) were provided by (ATCC, Manassas, VA, USA). The cells were the cell lines of mouse microglia, which were grown in the DMEM adding with heat-inactivated HS (5%), heat-inactivated FBS (10%), and 1% P/S, in an incubator under a temperature of 37 °C, with 5 percent CO_2. They were cultivated in the cell culture dishes (SPL Life Sciences, gveonggi-do, Korea). The microglial cells of SIM-A9 (2×10^5 cells per mL) were cultivated for 24 h and pretreated with GRE (100, 200 μg/mL) and 6-G (100 μM) for 1 h and then stimulated through using LPS (2 μg/mL). After LPS stimulation, the cells were incubated for either 24 h or 30 min depending on the experiment to be carried out.

2.8 Cell viability

The microglia (1×10^5 cells per mL) were cultivated in the cell culture plate (96-well) for one day. The cells were pretreated

using or not using GRE (0–1000 μg/mL) for 1 h and subsequently stimulated using or not using 2 μg/mL of LPS for 24 hours. The cells after cultivating were added with WST-8 (10 μL) into each well and next cultured for four hours. At 450 nm, the absorbance was determined. The absorbance correlated with the number of live microglia.

2.9 Nitric oxide measurement

The Griess reaction was employed for the measurement of NO (Min et al., 2010). 100 μL culture media was mixed with the reagent of Griess (100 μL) for ten minutes. At 540 nm, the absorbance could be tested. Using nitrite ion standard solution ($NaNO_2$) as standard curve.

2.10 PGE_2, TNF-a, and IL-6 ELISA assays

The levels of IL-6, TNF-a, and PGE_2 in the cell culture supernatants were examined through applying the TNF-α, IL-6 and PGE_2 ELISA Kits in accordance with the protocol of manufacturer.

2.11 Whole-cell protein extraction and Western blots

Whole-cell protein extraction was done using RIPA buffer according to the manufacturer's instruction. Bradford's reagent was applied to determine the concentration of proteins. Each sample 20 micrograms protein and 5× SDS-PAGE loading buffer (Biosesang, Korea) was mixed and heated to 95 °C, 5min. Running on a SDS-PAGE gel (15% or 10%) and transferred onto the membranes of PVDF, then incubated in 10 mL blocking buffer for 5 min blocking to decreases non-specific antibody binding to reduce background. The membranes were incubated with a variety of primary antibodies overnight at 4 °C with shaking. After washing, they were cultivated via the secondary antibodies combined with corresponding HRP. After washing, the membranes were then exposed to the WestGlow™ FEMTO Chemiluminescent substrate reagent (Biomax, Korea) and visualized on an ultraviolet detection imaging system. Then, the membranes were stripped using stripping buffer and reprobed with other primary antibodies applying protocol mentioned previously. The band density were using ImageJ analysis software program.

2.12 Statistical analysis

Student's t-test was exploited to analyze the data. p-value of < 0.001, 0.01, and < 0.05 was set to confirm the statistically significant outcomes.

3 Results and discussion

3.1 The total phenolic and flavonoid content and and antioxidant activities of GRE

Free radical scavenging activity is an indicator of antioxidant activity. The polyphenols and flavonoids in plants are highly reactive to free radicals, so determining the content of these compounds in selected plant extracts is also indicative of antioxidant activity (Aryal et al., 2019; Chavez-Santiago et al., 2021; Zapata et al., 2021). The TP and TF content of GRE is reflected in Table 1. The TP content of GRE was 55.63 ± 0.16 mg GAE/g and the TF content was 4.33 ± 0.17 mg QUE/g.

In vitro antioxidant activity is commonly measured by the DPPH and ABTS methods (Olszowy & Dawidowicz, 2018) To further investigate the more potent antioxidant activity of GRE, the DPPH and ABTS methods were used. BHT and VC were used as the reference standards, respectively. DPPH and ABTS free radical scavenging activity are usually described as IC50 (sample concentration producing a 50% decrease in free radicals). Lower IC50 values indicate higher antioxidant activity. The antioxidant activity of GRE is shown in Table 1. The GRE IC50 was 552.52 ± 3.71 μg/mL compared to the IC50 of BHT of 652.72 ± 10.20 μg/mL, indicating that GRE was more potent DPPH than BHT. The ABTS free radical scavenging IC50 of GRE was 1665.98 ± 16.20 μg/mL, whereas that of the VC reference was 79.13 ± 0.18 μg/mL, indicating that VC had a significantly greater free radical scavenging capacity than GPE. The results showed the GRE had a high antioxidant capacity.

3.2 Effects of GRE on cell viability and apoptosis

Before investigating the latent anti-inflammatory effects of the ginger extract, we first determined the effect of GRE on microglia activity. According to the Figure 1A, the various concentrations of GRE (including 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL) or 6-G (100 μM) were employed, treatment with GRE alone for 24 h, the results neither caused no cytotoxicity nor reduced neuron viability, and at concentrations of ≥ 400 μg/mL and 6-G group has slight proliferation ($p < 0.01$). No more than 200 μg/mL observed no significant changes in cell viability.

Then the GRE effects on inflammation and cell viability stimulated by LPS were investigated. The cell viability did not affect in protreatment with 100 or 200 μg/mL of GRE and stimulated by LPS (2 μg/mL) compared to untreated control group, and proliferation was found with the concentrations of ≥ 100 μg/mL and 6-G group compared to LPS group ($p < 0.01$)

Table 1. Antioxidant activity and total flavonoid and phenolic content of GRE.

Sample	Radical scavenging activity (IC50)		Total phenolic content	Total flavonoid content
	DPPH	ABTS		
GRE	552.52 ± 3.71 μg/mL	1665.98 ± 16.20 μg/mL	55.63 ± 0.16 mgGAE/g	4.33 ± 0.17 mgQUE/g
BHT	652.72 ± 10.20 μg/mL			
VC		79.13 ± 0.18 μg/mL		

Results are expressed as the mean ± standard deviation (n = 4). GRE: ginger root 50% ethanol extract; BHT: butylated hydroxytoluene;VC: L-ascorbic acid; GAE: gallic acid; QUE: quercetin.

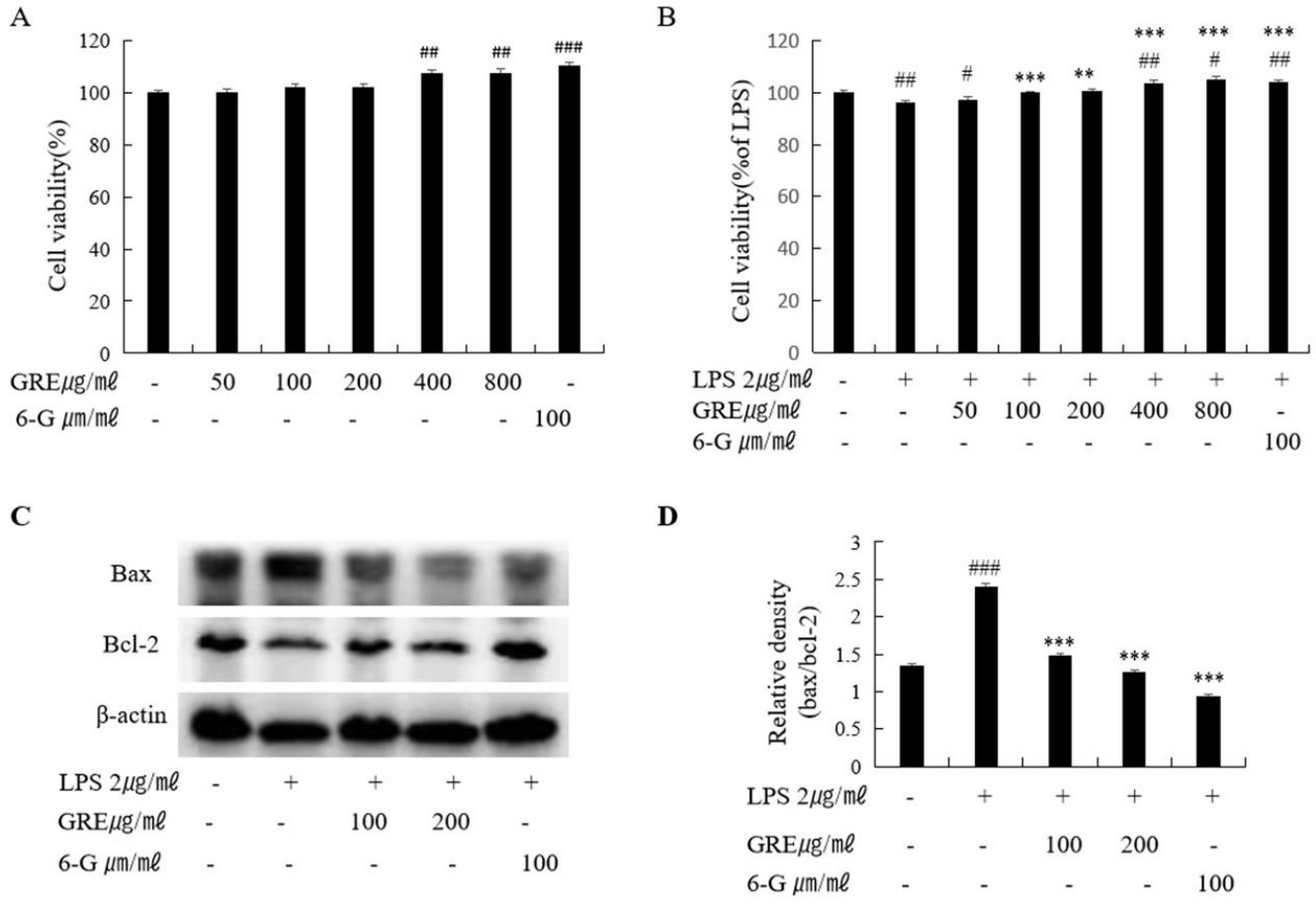

Figure 1. The effects of GRE on cell viability and Bax/Bcl-2 ratio in microglial cells treated with or without LPS. (A) Percentage of viable cells at diverse GRE concentrations without LPS, assessed using WST assays; (B) Percentage of viable cells with LPS stimulated. (C) The assay of western blot was implemented utilizing the specific antibodies on Bax and Bcl-2, show the results of WB on Bcl-2 and Bax. (D) The graphs show the quantitative analysis on the Bax/Bcl-2 ratio. The results are described with the mean ± SD (n = 3). # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ vs. control group. ** $p < 0.10$, *** $p < 0.001$ vs. only LPS group.

(Figure 2B). From the above results, the concentration 100, 200 μg/mL was selected to further investigate.

Excessive inflammatory response and aerobic metabolism may inflict cellular damage and lead to cell apoptosis (Pawlowski & Kraft, 2000). Bax and Bcl-2 are regulators of mitochondrial-mediated anti-apoptotic pathways. In brief, upregulation of the Bax to Bcl-2 ratio leads to cell apoptosis (Guadagno et al., 2013). Neuroinflammation leads to cell death, which is mediated by upregulated Bax levels and reduced Bcl-2 levels (Pawlowski & Kraft, 2000; Subedi et al., 2017). The Bax/Bcl-2 ratio was quantified through Western blotting to evaluate LPS-induced apoptosis. The LPS-induced group showed the upregulated expression of Bax and reduced Bcl-2, indicating increased Bax/Bcl-2 ratios compared to non-stimulated cells ($p < 0.001$). The results suggested that LPS led to microglial cell apoptosis by regulating Bcl-2 and Bax expression levels. However, pretreatment with GRE or 6-G effectively downregulated the Bax/Bcl-2 ratio induced by LPS ($p < 0.001$) (Figure 1C, D). GRE or 6-G lead to alterations in the Bax/Bcl-2 ratio, inhibiting apoptosis. Also, the Bax/Bcl-2 value in the 6-G group was lower than that in the GRE group. The present results indicated that GRE inhibited LPS-induced apoptosis without accompanying cytotoxicity in microglial cells. These results were consistent with the viability assay results that showed cell proliferation.

3.3 Effect of GRE on LPS-induced production of NO and PGE_2 and protein expression of iNOS and COX-2 in microglial cells

NO, PGE_2, iNOS, and COX-2 have been related to pro-inflammatory mediators induced by LPS and exert an essential effect in neuroinflammatory diseases. The COX-2 and iNOS enzymes mediate PGE_2 and NO generation, respectively (Giovannini et al., 2003).

In accordance with the NO detection assay results, NO was strongly increased in microglial cells by LPS stimulation ($p < 0.001$). Pretreatment with GRE strongly inhibited NO production induced by LPS ($p < 0.001$), and GRE at 200 μg/mL produced no significant changes compared to the 6-G group

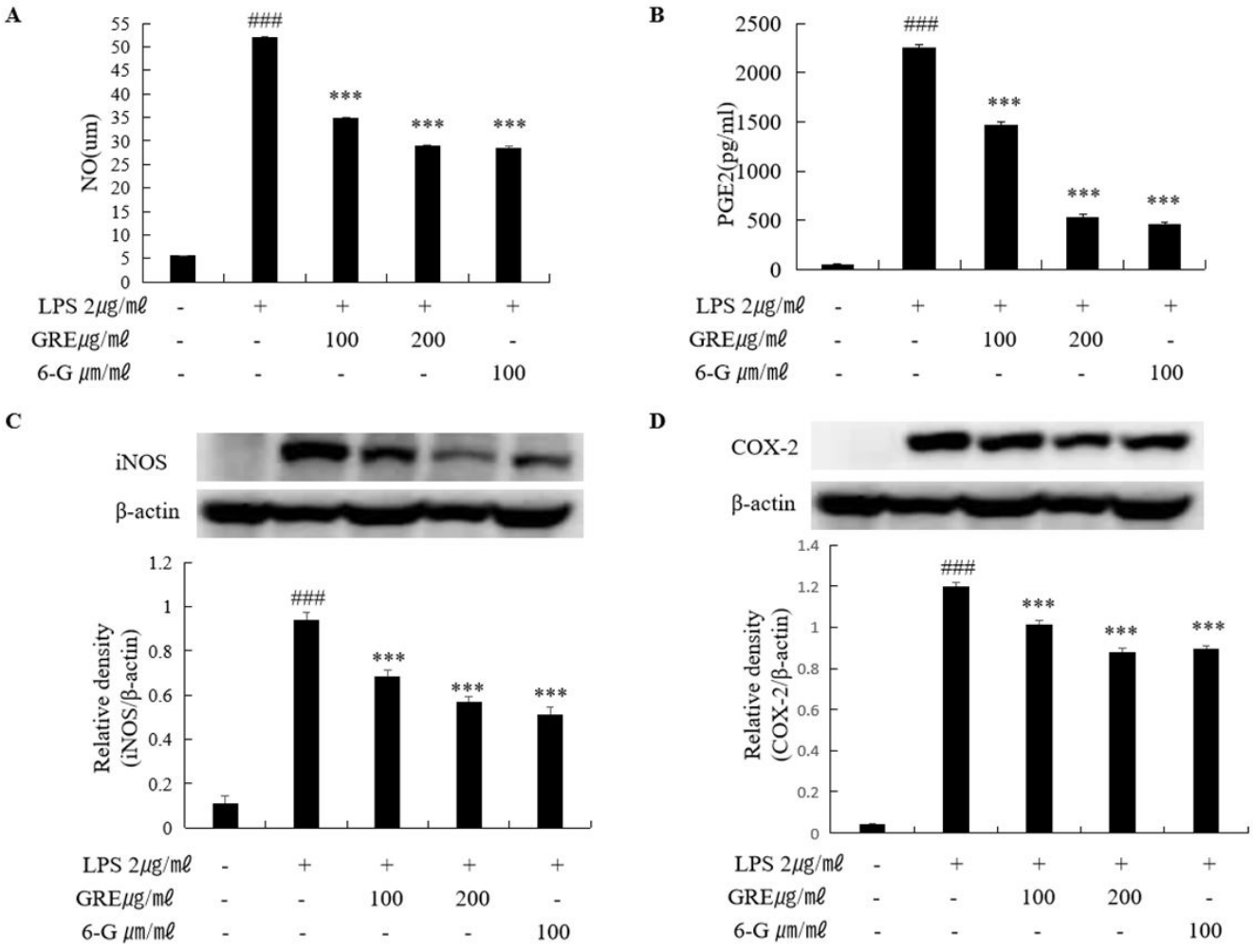

Figure 2. The GRE effects on production induced by LPS of PGE_2 and NO and COX-2 and iNOS protein expression in the microglial cells. Microglial cells were pretreated via using GRE (100 and 200 μg/mL) and 6-G (100 μM) for 60 minutes, and then conducted the incubation for one day using 2 μg/mL LPS. The contents of NO and PGE_2 generation in the cell culture supernatants was detected with Griess assay kit and ELISA Kit. (A) NO production; (B) PGE_2 production; (C) A same amounts of the cell lysates were subjected for electrophoresis. iNOS protein level and expression, utilizing the β-actin as the internal control. (D) COX-2 protein expression and level. The outcomes represent three separated researches. The error bar expresses the mean ± SDs. $^{###}$ $p < 0.001$ vs. control group. *** $p < 0.001$ vs. only LPS group.

(Figure 2A). The PGE_2 results under the same conditions were the same. PGE_2 production was significantly increased by LPS stimulation ($p < 0.001$), and pretreatment with GRE significantly inhibited PGE_2 production in a dose-dependent manner ($p < 0.001$). GRE at 200 μg/mL and the 6-G group showed no significant changes (Figure 2B).

The microglial cells stimulated by LPS the iNOS or COX-2 protein expression highly significantly elevated compared with non-stimulated group ($p < 0.001$), the pretreatment with GRE or 6-G effectively down-regulated protein expression of COX-2 and iNOS ($p < 0.001$). GRE pretreatment attenuated of LPS-stimulated expression of COX-2 and iNOS with a dose-dependent mode. Also findings showed that GRE highly significantly inhibited expression of COX-2 and iNOS and subsequent NO and PGE_2 release (Figure 2C, 2D). So, the GRE could protective effects against inflammation in microglial cells. In summary, GRE could exert protective neuroinflammatory effects by inhibiting the expression of COX-2 and iNOS and the subsequent PGE_2 and NO release.

3.4 Effect of GRE on LPS-stimulated production of IL-6 and TNF-α and protein expression of AKT and STST3

Neurodegenerative diseases are associated with neuroinflammation due to microglial activation in the CNS (An et al., 2020). Therefore, the suppression of neuroinflammation due to microglia over-activation is considered a potential treatment or prevention of inflammation-related brain diseases. Pro-inflammatory cytokines initiate the inflammatory response

and lead to the development of neuroinflammatory diseases (Smith et al., 2012).

The TNF-α and IL-6 levels remarkably up-regulated in the microgl0ia stimulated by LPS ($p < 0.001$). Pretreatment with GRE at 100 or 200 μg/mL markedly reduced IL-6 and TNF-α secretion ($p < 0.001$) (Figure 3A, 3B). So, GRE inhibited inflammatory cytokine secretion in activated microglia. We next examined the possible mechanism of GRE reducing the expression of inflammatory cytokines.

AKT (protein kinase B) and its downstream signaling exert a significant effect in neurodegenerative diseases, influencing the development of the brain and neuronal growth (Manning & Toker, 2017). PI3K/Akt is an important regulator of inflammation and regulates LPS-induced pro-inflammatory cytokine production by microglia (Dong et al., 2014). The recent research confirmed that suppressing microglia-mediated neuroinflammation and pro-inflammatory cytokine and production of NO is regulated by Akt-mTOR-STAT3 pathway. PI3K/Akt signaling pathway

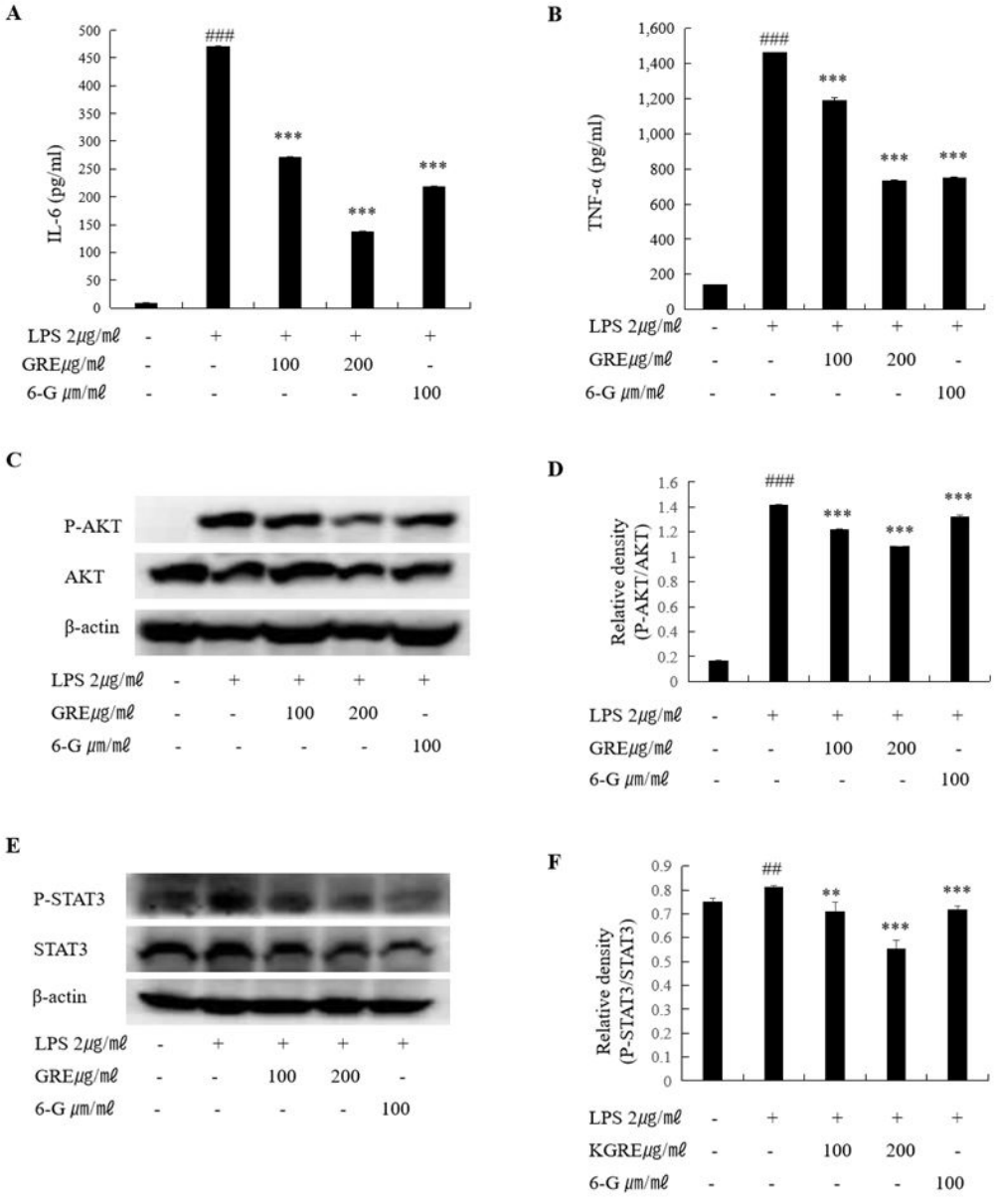

Figure 3. The GRE effects on the production stimulated by LPS of TNF-α and IL-6 and activation of AKT and STAT3 in microglial cells. Microglial cells were subsequently pretreated through GRE for 60 minutes, next 30 min or 24 h of incubation with LPS (2 μg/mL). The supernatants of culture media were harvested and determined TNF-αand IL-6 with ELISA Kit. (A) IL-6, (B) TNF-α. The expression levels of STAT3 and AKT were measured with the analysis of western blot of whole-cell protein extract, where utilizing β-actin as the internal control. (C) The protein expression of AKT and P-AKT; (D) AKT/AKT expression levels. (E) The protein expression of STAT3 and P-STAT3; (F) STAT3/STAT3 expression levels. Error bars represent the means ± SDs. $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ vs. control group. ** $p < 0.10$, *** $p < 0.001$ vs. only LPS group.

triggered inflammatory response by regulating transcription factor such as NF-κB. LR4/PI3K/AKT and STAT3 signaling alters IL-6 (Liu et al., 2020). The GRE may effect anti-neuroinflammation by the AKT-STAT3 pathway. LPS-stimulated microglia increased the phosphorylation of the expression of STAT3 and Akt while GRE or 6-G pretreatment attenuated the phosphorylation of the expression level of STAT3 and Akt with the dose-dependent mode (Figure 3C, 3D, 3E, 3F). Therefore, GRE inhibits the production of TNF-α and IL-6 induced by LPS via Akt-STAT3 pathway. GRE could inhibit neuroinflammation and nerve protection by Akt-STAT3 pathway.

3.5 Effects of GRE on MAPK pathway and NF-κB activation in LPS-stimulated microglial cells

We next examined the latent GRE mechanism in reducing inflammatory cytokine expression. MAPK signaling pathways are typical inflammatory signaling pathways and play important roles in the LPS-induced neuroinflammatory response in microglia (An et al., 2020). To confirm the anti-apoptosis performances and anti-inflammatory of the GRE, we examined the phosphorylation of three MAPK molecules. LPS significantly enhanced the phosphorylation of JNK, ERK, and p38 ($p < 0.01$),

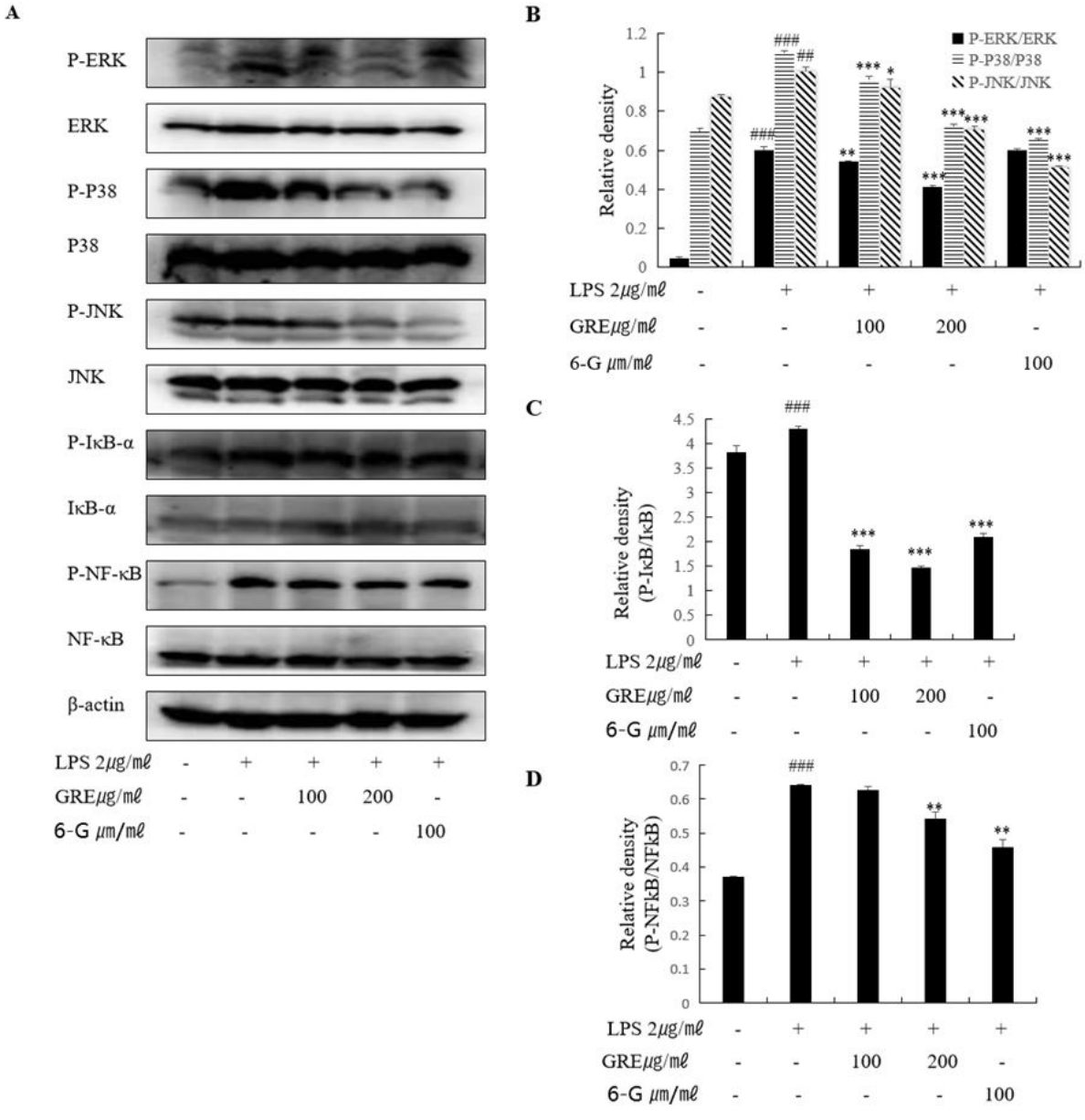

Figure 4. GRE reduced MAPK/NF-κB signaling pathway activation induced by LPS in microglial cells. Microglial cells (2×10^5 cells per mL) were cultured and pre-treated with GRE (100 and 200 μg/mL) and 100 μM 6-G for one hour and subsequently stimulated with LPS for half an hour. (A) The protein expression levels of the phosphorylated or total forms of ERK1/2, p38, JNK, and NF-κB, and IκBα cell signaling kinase were investigated by Western blot assays. (B-D) The band densities were analyzed compared to β-actin using ImageJ software. Error bars represent the means ± SDs. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.10$, and $^{***}p < 0.001$ vs. LPS-only group.

pretreatment with GRE evidently inhibited the phosphorylation up-regulation induced by LPS of JNK, ERK, and p38 with the dose-dependent mode (Figure 4A, 4B).

NF-κB is also well known as a typical inflammatory signaling pathway and a major drug target for inflammatory diseases (Zheng & Wang, 2020). The GRE inhibits inflammatory in microglia activated by LPS with the phosphorylation of IκB and NF-κB. LPS highly evidently up-regulated the phosphorylation of the protein expression of IκB and NF-κB ($p < 0.001$), and GRE or 6-G inhibits the degradation induced by LPS of IκB-α NF-κB and with the dose-dependent mode (Figure 4A, 4C, 4D). As a result, the inhibitory effect of the signaling pathway of NF-κB in microglia via GRE may cause the decreasing pro-inflammatory mediators, thus leading to an anti-inflammatory activity.

4 Conclusions

In conclusion, GRE had a high antioxidant capacity. GRE or 6-gingrol have exert protective neuroinflammatory effects by inhibiting the expression of COX-2 and iNOS and the subsequent PGE_2 and NO release. GRE also inhibited the production of TNF-α and IL-6 induced by LPS. GRE or 6-gingrol ameliorated microglia-mediated neuronal insults by increasing Bax and decreasing Bcl-2. We clearly demonstrated that GRE attenuates neuroinflammation in LPS-stimulated mouse microglia by AKT/STAT3, MAPK, and NF-κB signaling. In summary, GRE positively exerted anti-neuroinflammatory and neuroprotective activity without serious side effects, which might be used to contribute to the management and prevention of neurodegenerative diseases. Also, it can be used as a functional food additive.

References

Abessolo, D. M., Mebale, A. A., Mve, J. A., & Ondo, J. (2021). Phytochemical characterization and of total polyphenols and flavonoids content of the aqueous extract of the seeds of Cucumeropsis edulis (Cucurbitaceae) from Gabon. *Journal of Pharmacognosy and Phytochemistry*, 10(2), 138-141.

An, J., Chen, B., Kang, X., Zhang, R., Guo, Y., Zhao, J., & Yang, H. (2020). Neuroprotective effects of natural compounds on LPS-induced inflammatory responses in microglia. *American Journal of Translational Research*, 12(6), 2353-2378. PMid:32655777.

Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants*, 8(4), 96. http://dx.doi.org/10.3390/plants8040096. PMid:30978964.

Attyah, A. M., & Ismail, S. H. (2012). Protective effect of ginger extract against cisplatin-induced hepatotoxicity and cardiotoxicity in rats. *Iraqi Journal of Pharmaceutical Sciences*, 21(1), 27-33.

Beristain-Bauza, S. D. C., Hernández-Carranza, P., Cid-Pérez, T. S., Ávila-Sosa, R., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2019). Antimicrobial activity of ginger (*Zingiber officinale*) and its application in food products. *Food Reviews International*, 35(5), 407-426. http://dx.doi.org/10.1080/87559129.2019.1573829.

Chavez-Santiago, J. O., Rodríguez-Castillejos, G. C., Montenegro, G., Bridi, R., Valdés-Gómez, H., Alvarado-Reyna, S., Castillo-Ruiz, O., & Santiago-Adame, R. (2021). Phenolic content, antioxidant and antifungal activity of jackfruit extracts (*Artocarpus heterophyllus* Lam.). *Food Science and Technology*. Ahead of print. http://dx.doi.org/10.1590/fst.02221.

Cho, B.-O., Che, D. N., Yin, H.-H., & Jang, S.-I. (2017). Gamma irradiation enhances biological activities of mulberry leaf extract. *Radiation Physics and Chemistry*, 133, 21-27. http://dx.doi.org/10.1016/j.radphyschem.2016.11.013.

Dong, H., Zhang, X., Dai, X., Lu, S., Gui, B., Jin, W., Zhang, S., Zhang, S., & Qian, Y. (2014). Lithium ameliorates lipopolysaccharide-induced microglial activation via inhibition of toll-like receptor 4 expression by activating the PI3K/Akt/FoxO1 pathway. *Journal of Neuroinflammation*, 11(1), 140. http://dx.doi.org/10.1186/s12974-014-0140-4. PMid:25115727.

Giovannini, M. G., Scali, C., Prosperi, C., Bellucci, A., Pepeu, G., & Casamenti, F. (2003). Experimental brain inflammation and neurodegeneration as model of Alzheimer's disease: protective effects of selective COX-2 inhibitors. *International Journal of Immunopathology and Pharmacology*, 16(Suppl. 2), 31-40. PMid:14552702.

Guadagno, J., Xu, X., Karajgikar, M., Brown, A., & Cregan, S. (2013). Microglia-derived TNF α induces apoptosis in neural precursor cells via transcriptional activation of the Bcl-2 family member Puma. *Cell Death & Disease*, 4(3), e538. http://dx.doi.org/10.1038/cddis.2013.59. PMid:23492769.

Habib, S. H. M., Makpol, S., Hamid, N. A. A., Das, S., Ngah, W. Z. W., & Yusof, Y. A. M. (2008). Ginger extract (*Zingiber officinale*) has anti-cancer and anti-inflammatory effects on ethionine-induced hepatoma rats. *Clinics*, 63(6), 807-813. http://dx.doi.org/10.1590/S1807-59322008000600017. PMid:19061005.

Hussein, U. K., Hassan, N. E. Y., Elhalwagy, M. E. A., Zaki, A. R., Abubakr, H. O., Venkata, K. C. N., Jang, K. Y., & Bishayee, A. (2017). Ginger and Propolis Exert Neuroprotective Effects against Monosodium Glutamate-Induced Neurotoxicity in Rats. *Molecules*, 22(11), 1928. http://dx.doi.org/10.3390/molecules22111928. PMid:29117134.

Jung, H. W., Yoon, C. H., Park, K. M., Han, H. S., & Park, Y. K. (2009). Hexane fraction of Zingiberis Rhizoma Crudus extract inhibits the production of nitric oxide and proinflammatory cytokines in LPS-stimulated BV2 microglial cells via the NF-kappaB pathway. *Food and Chemical Toxicology*, 47(6), 1190-1197. http://dx.doi.org/10.1016/j.fct.2009.02.012. PMid:19233241.

Kim, S., Lee, M. S., Jung, S., Son, H. Y., Park, S., Kang, B., Kim, S. Y., Kim, I. H., Kim, C. T., & Kim, Y. (2018). Ginger extract ameliorates obesity and inflammation via regulating MicroRNA-21/132 expression and AMPK activation in white adipose tissue. *Nutrients*, 10(11), 1567. http://dx.doi.org/10.3390/nu10111567. PMid:30360535.

Lantz, R. C., Chen, G. J., Sarihan, M., Solyom, A. M., Jolad, S. D., & Timmermann, B. N. (2007). The effect of extracts from ginger rhizome on inflammatory mediator production. *Phytomedicine*, 14(2-3), 123-128. http://dx.doi.org/10.1016/j.phymed.2006.03.003. PMid:16709450.

Liu, Y., Deng, S., Zhang, Z., Gu, Y., Xia, S., Bao, X., Cao, X., & Xu, Y. (2020). 6-Gingerol attenuates microglia-mediated neuroinflammation and ischemic brain injuries through Akt-mTOR-STAT3 signaling pathway. *European Journal of Pharmacology*, 883, 173294. http://dx.doi.org/10.1016/j.ejphar.2020.173294. PMid:32681941.

Lunenfeld, B., & Stratton, P. (2013). The clinical consequences of an ageing world and preventive strategies. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 27(5), 643-659. http://dx.doi.org/10.1016/j.bpobgyn.2013.02.005. PMid:23541823.

Manning, B. D., & Toker, A. (2017). AKT/PKB signaling: navigating the network. *Cell*, 169(3), 381-405. http://dx.doi.org/10.1016/j.cell.2017.04.001. PMid:28431241.

Mao, Q. Q., Xu, X. Y., Cao, S. Y., Gan, R. Y., Corke, H., Beta, T., & Li, H. B. (2019). Bioactive compounds and bioactivities of ginger (*Zingiber*

officinale Roscoe). *Foods*, 8(6), 185. http://dx.doi.org/10.3390/foods8060185. PMid:31151279.

Min, S.-W., Ryu, S.-N., & Kim, D.-H. (2010). Anti-inflammatory effects of black rice, cyanidin-3-O-β-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. *International Immunopharmacology*, 10(8), 959-966. http://dx.doi.org/10.1016/j.intimp.2010.05.009. PMid:20669401.

Oh, Y. C., Li, W., & Choi, J. G. (2021). Saussureae radix attenuates neuroinflammation in LPS-stimulated mouse BV2 microglia via HO-1/Nrf-2 induction and inflammatory pathway inhibition. *Mediators of Inflammation*, 2021, 6687089. http://dx.doi.org/10.1155/2021/6687089. PMid:33790692.

Olszowy, M., & Dawidowicz, A. L. (2018). Is it possible to use the DPPH and ABTS methods for reliable estimation of antioxidant power of colored compounds? *Chemical Papers*, 72(2), 393-400. http://dx.doi.org/10.1007/s11696-017-0288-3.

Pawlowski, J., & Kraft, A. S. (2000). Bax-induced apoptotic cell death. *Proceedings of the National Academy of Sciences of the United States of America*, 97(2), 529-531. http://dx.doi.org/10.1073/pnas.97.2.529. PMid:10639111.

Pogačnik, L., Ota, A., & Ulrih, N. P. (2020). An overview of crucial dietary substances and their modes of action for prevention of neurodegenerative diseases. *Cells*, 9(3), 576. http://dx.doi.org/10.3390/cells9030576. PMid:32121302.

Ryu, K. Y., Lee, H. J., Woo, H., Kang, R. J., Han, K. M., Park, H., Lee, S. M., Lee, J. Y., Jeong, Y. J., Nam, H. W., Nam, Y., & Hoe, H. S. (2019). Dasatinib regulates LPS-induced microglial and astrocytic neuroinflammatory responses by inhibiting AKT/STAT3 signaling. *Journal of Neuroinflammation*, 16(1), 190. http://dx.doi.org/10.1186/s12974-019-1561-x. PMid:31655606.

Sahardi, N. F. N. M., & Makpol, S. (2019). Ginger (*Zingiber officinale* Roscoe) in the prevention of ageing and degenerative diseases: review of current evidence. *Evidence-Based Complementary and Alternative Medicine*, 2019, 5054395. http://dx.doi.org/10.1155/2019/5054395. PMid:31531114.

Sebiomo, A., Awofodu, A., Awosanya, A., Awotona, F., & Ajayi, A. (2011). Comparative studies of antibacterial effect of some antibiotics and ginger (Zingiber officinale) on two pathogenic bacteria. *Journal of Microbiology and Antimicrobials*, 3(1), 18-22.

Shahrajabian, M. H., Sun, W., & Cheng, Q. (2019). Clinical aspects and health benefits of ginger (*Zingiber officinale*) in both traditional Chinese medicine and modern industry. *Acta Agriculturæ Scandinavica. Section B, Soil and Plant Science*, 69(6), 546-556. http://dx.doi.org/10.1080/09064710.2019.1606930.

Si, W., Chen, Y. P., Zhang, J., Chen, Z. Y., & Chung, H. Y. (2018). Antioxidant activities of ginger extract and its constituents toward lipids. *Food Chemistry*, 239, 1117-1125. http://dx.doi.org/10.1016/j.foodchem.2017.07.055. PMid:28873530.

Smith, J. A., Das, A., Ray, S. K., & Banik, N. L. (2012). Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin*, 87(1), 10-20. http://dx.doi.org/10.1016/j.brainresbull.2011.10.004. PMid:22024597.

Stoilova, I., Krastanov, A., Stoyanova, A., Denev, P., & Gargova, S. (2007). Antioxidant activity of a ginger extract (*Zingiber officinale*). *Food Chemistry*, 102(3), 764-770. http://dx.doi.org/10.1016/j.foodchem.2006.06.023.

Subedi, L., Venkatesan, R., & Kim, S. Y. (2017). Neuroprotective and anti-inflammatory activities of Allyl Isothiocyanate through attenuation of JNK/NF-kappaB/TNF-alpha signaling. *International Journal of Molecular Sciences*, 18(7), 1423. http://dx.doi.org/10.3390/ijms18071423. PMid:28671636.

Wang, F., Cho, B. O., Shin, J. Y., Hao, S., & Jang, S. I. (2021a). Anti-neuroinflammatory activity of Humulus japonicus extract and its active compound luteolin on lipopolysaccharide-induced inflammatory response in SIM-A9 microglia via NF-κB and MAPK signaling pathways. *Food Science and Technology*. Ahead of Print. http://dx.doi.org/10.1590/fst.78621.

Wang, L., Liu, G., Shao, Z., Zhang, Q., Yin, L., Xu, E., Li, B., Cui, X., & Teng, H. (2021b). MicroR-146 protects against rat ischemia-reperfusion injury by targeting NF-κB-mediated PI3K/AKT/mTOR signaling pathway. *Food Science and Technology*. Ahead of Print. http://dx.doi.org/10.1590/fst.36820.

Zapata, J. E., Sepúlveda, C. T., & Álvarez, A. C. (2021). Kinetics of the thermal degradation of phenolic compounds from achiote leaves (*Bixa orellana* L.) and its effect on the antioxidant activity. *Food Science and Technology*. Ahead of Print. http://dx.doi.org/10.1590/fst.30920.

Zhang, F., Zhang, J.-G., Yang, W., Xu, P., Xiao, Y.-L., & Zhang, H.-T. (2018). 6-Gingerol attenuates LPS-induced neuroinflammation and cognitive impairment partially via suppressing astrocyte overactivation. *Biomedicine and Pharmacotherapy*, 107, 1523-1529. http://dx.doi.org/10.1016/j.biopha.2018.08.136. PMid:30257370.

Zhao, D., Gu, M. Y., Xu, J. L., Zhang, L. J., Ryu, S. Y., & Yang, H. O. (2019). Anti-neuroinflammatory effects of 12-Dehydrogingerdione in LPS-activated microglia through inhibiting Akt/IKK/NF-kappaB pathway and activating Nrf-2/HO-1 pathway. *Biomolecules & Therapeutics*, 27(1), 92-100. http://dx.doi.org/10.4062/biomolther.2018.104. PMid:30404129.

Zheng, F., & Wang, Z. (2020). miRNA-1180 suppresses HCC cell activities via TRAF1/NF-κB signaling pathway. *Food Science and Technology*, 40(Suppl. 2), 626-633. http://dx.doi.org/10.1590/fst.26219.

Conflicts of Interest:

The authors declare no conflict of interests.

Funding

This study was financially supported by the Ministry of Small and Medium Enterprise and Startups (MSS), Korea, under the Social Economy Innovation Growth Project (R&D) (Project number P0013037)" supervised by the Korea Institute for Advancement of Technology.