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Anti-inflammatory effects of TP1 in LPS-induced Raw264.7 macrophages

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Abstract

Inflammation is an essential defense mechanism in health; however, excessive inflammation contributes to the pathophysiology of several chronic diseases. Although anti-inflammatory drugs are essential for controlling inflammation, they have several side effects. Recent findings suggest that naturally derived compounds possess physiological activities, including anti-inflammatory, antifungal, antiviral, anticancer, and immunomodulatory activities. Therefore, this study aimed to investigate the anti-inflammatory effects and molecular mechanisms of 2,5,6-trimethoxy-p-terphenyl (TP1), extracted from the Antarctic lichen *Stereocaulon alpinum*, using in vitro models. TP1 treatment decreased the production of nitric oxide (NO) and reactive oxygen species (ROS) in LPS-stimulated Raw264.7 macrophages. Additionally, TP1 treatment significantly decreased the mRNA levels of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6) and the mRNA and protein levels of the pro-inflammatory enzymes (inducible nitric oxide synthase and cyclooxygenase-2). Moreover, TP1 suppressed lipopolysaccharide-induced phosphorylation of the NF- κ B and MAPK signaling pathways in Raw264.7 macrophages. Conclusively, these results suggest that TP1 ameliorates inflammation by suppressing the expression of pro-inflammatory cytokines, making it a potential anti-inflammatory drug for the treatment of severe inflammatory diseases.

Keywords TP1, Anti-inflammation, NF- κ B, MAPK

Introduction

Inflammation, the body's "natural defense system" against injury and disease, has five representative signals: fever, redness, dysfunction, swelling, and ache [1]. Although inflammation is essential to maintain health, excessive

inflammation can damage host cells and cause diseases [2]. Pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), specialized motif of bacteria, viruses, and fungi, and trigger inflammatory responses through intracellular signaling [3]. Toll-like receptors (TLRs) are the most well-known members of the PRR family [4]. TLR4 recognizes lipopolysaccharides (LPS) [5]. LPS is a major component of the cell wall of gram-negative bacteria and can trigger the release of numerous inflammatory cytokines, resulting in an acute inflammatory response [6]. The binding of LPS to TLR4 induces the formation of myeloid differentiation primary response protein 88 (MyD88), interleukin-1 receptor-associated kinases (IRAKs), and TNF receptor-associated factor 6 (TRAF6) complex [7]. These processes can trigger intracellular signaling cascades by stimulating the AKT/NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways, including

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ERK1/2, JNKs, and p38 MAPKs [8]. Stimulation of these pathways can induce the expression of pro-inflammatory mediators, such as pro-inflammatory cytokines (interleukin-6 [IL-6], IL-1 β , and tumor necrosis factor- α [TNF- α]) and pro-inflammatory enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [9].

Sepsis, one of the deadliest diseases worldwide, is an acute inflammatory disease caused by an uncontrolled inflammatory response [10]. Over the years, the incidence of sepsis has increased, and LPS is a major cause of sepsis [11, 12]. Although extensive studies have been performed to treat sepsis, no effective treatment has been developed [13]. Currently, sepsis is mainly managed using antibiotics, intravenous fluids, and vasoactive drugs [14]. Although ceftriaxone and vancomycin are commonly used antibiotics for sepsis treatment [15, 16], they have several side effects, including urolithiasis, nephrolithiasis, and maculopapular or erythematous rashes [17, 18]. Therefore, it is crucial to develop novel anti-inflammatory drugs with low toxicity for treating sepsis.

Natural compounds are recognized as sources of therapeutic agents owing to their structural diversity [19]. Moreover, 13 drugs based on natural products were approved for clinical use between 2005 and 2007 [20]. Antarctic plants produce secondary metabolites with various physiological activities as a survival strategy in extreme environments with low temperatures and high ultraviolet rays [21]. For example, lobaric acid, a metabolite of *Streocaulon alpinum*, inhibits protein tyrosine phosphatase 1B (PTP1B) [22]. Libertellenone G and libertellenone H, isolated from the Arctic fungus *Eutypella sp.*, have high cytotoxicity and antibacterial activity [23]. New steroid compounds isolated from the Antarctic October coral *Anthomastus bathyproctus* exhibited anticancer effects on the human cancer cell lines MDA-MB-23, A-549, and HT-29 [24]. However, there is limited understanding of the biological activities of samples from the polar regions [25]. Therefore, natural compounds from polar regions are untapped resources for the development of natural compound-based medicines [26].

2,5,6-trimethoxy-p-terphenyl (TP1) is isolated from the Antarctic lichen *S. alpinum*, with a p-terphenyl backbone. p-Terphenyls are 1,4-diphenyl benzene derivatives with three phenyl rings connected by a single C–C bond [27]. p-Terphenyls possess several biological activities, including neuroprotective, antithrombotic, and anti-coagulant activities [28]. However, studies have not yet investigated the anti-inflammatory effects and molecular mechanisms of several bioactive p-terphenyls. Therefore, this study aimed to investigate the anti-inflammatory effects and molecular mechanisms of TP1 in LPS-stimulated Raw264.7 macrophages.

Materials and methods

Reagents

TP1 was extracted by the Korea Polar Research Institute and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM; high glucose) was obtained from Hyclone Laboratories Inc. (Marlborough, MA, USA), Fetal bovine serum (FBS) was purchased from Corning (Corning, NY, USA). Penicillin–streptomycin–glutamine (PSQ) was procured from Gibco (Waltham, MA, USA). MTT and LPS from *Escherichia coli* O127:B8, N-(1-naphthyl) ethylenediamine dihydrochloride, phosphoric acid, sulfanilic acid, and nitrite ion standard solutions were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (DPBS) and Hank's balanced salt solution (HBSS) were obtained from WELGENE (Gyeongsan, Korea). 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) was procured from Cayman Chemical Company (Ann Arbor, MI, USA). Primers for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were purchased from Macrogen (Seoul, Korea). Primary antibodies against iNOS (#13120), COX-2 (#12282), phospho-p65 (#13346), phospho-JNK (#9255), phospho-ERK (#4370), and β -actin (#8457) were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Goat anti-rabbit IgG (5220-0036) and goat anti-mouse IgG (5220-0341) antibodies were purchased from SeraCare Life Sciences, Inc. (Gaithersburg, MD, USA).

Cell culture

The mouse macrophage cell line RAW264.7 was provided by Dr. Sung Ho Ryu's lab (POSTECH, Korea). The cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ at 37 °C in a 5% CO₂ incubator.

Cell viability

Raw264.7 macrophages were seeded in a 48-well plate (3×10^4 cells/well). After 24 h, cells were pretreated with TP1 for 30 min, treated with 0.1 μ g/mL of LPS, and incubated for 24 h at 37 °C under 5% CO₂. After removing the culture medium, the cells were treated with the MTT solution (1 mg/mL) for 2 h. Thereafter, the MTT solution was removed, followed by the addition of 200 μ L of DMSO to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific Co.).

Measurement of nitric oxide production

Nitric oxide (NO) levels in the cell culture supernatants were measured using the Griess assay. Briefly, Raw264.7 macrophages were seeded in a 24-well plate (6×10^4 cells/well). After 24 h, the cells were pretreated with 25 μ M of

TP1 for 30 min, and then treated with LPS (0.1 µg/mL) and incubation at 37 °C in a 5% CO₂ incubator for 24 h. The supernatants were collected from each well, transferred to a 96-well plate, and Griess reagent was added (reagents A and B at a 1:1 ratio). After 15 min, absorbance was measured at 550 nm using a microplate reader.

Measurement of reactive oxygen species production

The DCF-DA assay was used to measure reactive oxygen species (ROS) production. Briefly, Raw264.7 macrophages were seeded in a 24-well (6 × 10⁴ cells/well) and incubated for 24 h. Thereafter, the cells were pretreated with TP1 (25 µM) for 30 min, and then treated with LPS (0.1 µg/mL) and incubated at 37 °C in a 5% CO₂ incubator for 24 h. Cells were treated with DCF-DA for 30 min at 37 °C, followed by three washes with HBSS. ROS levels were detected by a fluorescence microscope (Zeiss, Jena, Germany) and analyzed using FACS (BD FACSVerse™, BD Biosciences).

qRT-PCR

Raw264.7 macrophages were seeded in a 12-well plate (3 × 10⁵ cells/well). After 24 h, cells were pretreated with TP1 (25 µM) for 30 min, and then treated with 0.1 µg/mL of LPS. After 24 h, the cells were washed thrice with DPBS and harvested. Total RNA was isolated from the cells using TRIzol reagent and reverse-transcribed to generate cDNA using a SimpliAmp Thermal Cycler (Applied Biosystems Co., Waltham, MA, USA). qRT-PCR was performed on a StepOnePlus Real-Time PCR System Cycler (Applied Biosystems Co.) using a Power SYBR Green PCR mix Cycler (Applied Biosystems Co.) and specific primers. The threshold cycle (Ct) values for the genes of interest were standardized with respect to the β-actin reference. The primer sequence for *iNOS* (Forward 5′-TGAAGAAAACCCCTTGTGCT-3′, Reverse 5′-TTCTGTGCTGTCCAGTGAG-3′), *COX-2* (Forward 5′-GAAGATTCCCT CCGGTGTTT-3′, Reverse 5′-CCCTTCTCACTGGCTTATGTAG-3′), *Tnf-α* (Forward 5′-ACGTGGAAGTGGCAGAAGAG-3′, Reverse 5′-GGTCTGGGCCATAGAAGTGA-3′), *Il-1β* (Forward 5′-AGGTCAAAGGTTTGGAAGCA-3′, Reverse 5′-TGAAGCAGCTATGGCA ACTG-3′), *Il-6* (Forward 5′-TCTGAAGGACTCTGGCTTTG-3′, Reverse 5′-GATGGAT GCTACCAAAGTGA-3′), and *β-actin* (Forward 5′-ATGGAGGGGAATACAGCCC-3′, Reverse 5′-TTC TTT GCAGCTCCTTCGTT-3′).

Western blot analysis

Raw264.7 macrophages were seeded in a 60 mm plate (6 × 10⁵ cells/well). Cells were pretreated with TP1 (25 µM) for 30 min, and then treatment with 0.1 µg/mL of LPS. Proteins were isolated from 100 µL of each sample

at 30 min and 24 h intervals using RIPA buffer (10 mM Tris [pH 7.4], 150 mM of NaCl, 1 mM of EDTA [pH 8], 1% Triton X-100, 1% sodium deoxycholate, 30 mM of NaF, 1.5 mM of NaVO₄, 1 mM of PMSF, and 1 mg/mL each of aprotinin, leupeptin, and pepstatin A), followed by sonication for 5 s thrice. The cell lysate was centrifugated at 130,000 rpm for 10 min at 4 °C, and the protein content of the supernatant was collected and quantified using Bradford assay (Abcam, Cambridge, UK). Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose (NC) membranes. Thereafter, the membranes were blocked with skim milk (5%) in 1X TBS-T buffer (Tris-buffered saline with 0.1% Tween-20) containing NaN₃ for 30 min, followed by overnight incubation with primary antibodies at room temperature (RT). After washing with TBS-T for 30 min, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG antibodies for 1 h at RT. Finally, the antibody reactions were detected using an ECL reagent (Cytiva, Malborough, MS, USA), and the bands were visualized using Chemidoc (iBright™ CL1500, Invitrogen). The intensities of the bands were analyzed using ImageJ software, with β-actin as the loading control.

Immunocytochemistry

Raw264.7 macrophages were seeded in a 6-well plate (5 × 10⁵ cells/well). Cells were pretreated with TP1 (25 µM) for 30 min, and then treated with 0.1 µg/mL of LPS. After 30 min, the cells were washed with DPBS, followed by fixation with 4% paraformaldehyde (PFA, Sigma-Aldrich; Merck KGaA) for 20 min. Subsequently, they were incubated with 0.2% Triton X-100 and 0.1% citrate (Sigma-Aldrich, Merck KGaA) in PBS for 5 min. Thereafter, the cells were blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 30 min and probed with rabbit anti-p65 NF-κB antibody (1:500; cat. no. 8242; Cell Signaling Technology) for 2 h. Subsequently, washed the cells with 2% BSA, and then probed with fluorescein goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 555 (4 µg/mL; cat. no. A-21428; Invitrogen Inc., Middlesex, MA, USA) for 1 h. After washing, the nuclei were stained with 1 mg/mL of DAPI solution for 5 min. Finally, fluorescence was visualized by a fluorescence microscope (Zeiss Microscope, Carl Zeiss AC).

Data analysis

The results are presented as mean ± standard deviation (SD). The student's t-test for comparisons between two groups. Statistical significance was defined at p < 0.05 (*).

Results

TP1 inhibits the differentiation of Raw264.7 macrophage without inducing cytotoxicity

p-Terphenyls (Fig. 1A) are cytotoxic owing to their biological activities [29]. Therefore, we investigated the potential cytotoxic effects of TP1 in Raw264.7 macrophages using an MTT assay and found that TP1 treatment (5, 15, and 25 μM) for 24 h was not cytotoxic to Raw264.7 macrophages (Fig. 1B). Macrophage morphology has been linked to inflammation [30]. LPS treatment activates Raw264.7 macrophages, resulting in an increase in cell size and the formation of pseudopodia [31]. Macrophages, characterized by numerous fibrous cytoplasmic projections on their cell surface, are associated with the induction of inflammation [32, 33]. TP1 treatment significantly suppressed the LPS-induced transformation of Raw264.7 macrophages into the inflammatory phenotype compared with that in the LPS-only group (Fig. 1C).

TP1 suppresses LPS-induced NO and ROS production in Raw264.7 macrophages

ROS and NO serve as central mediators of inflammatory response [34]. Excessive ROS production leads to endothelial dysfunction and tissue injury [35]. NO plays multiple roles in inflammatory responses, including the regulation of leukocyte activity and tissue cytotoxicity, which leads to vasodilation and edema formation [36]. To examine the anti-inflammatory effects of TP1, we measured the NO and ROS levels in Raw264.7 macrophages. TP1 treatment caused a significant dose-dependent decrease in LPS-induced NO production in Raw264.7 macrophages compared with that in the LPS-only group (Fig. 2A). Additionally, FACS (Fig. 2B and C) and fluorescence microscopy (Fig. 2D) confirmed that TP1 decreased ROS production in a dose-dependent manner. Collectively, these data demonstrate that TP1 can decrease NO and ROS production in LPS-induced Raw264.7 macrophages.

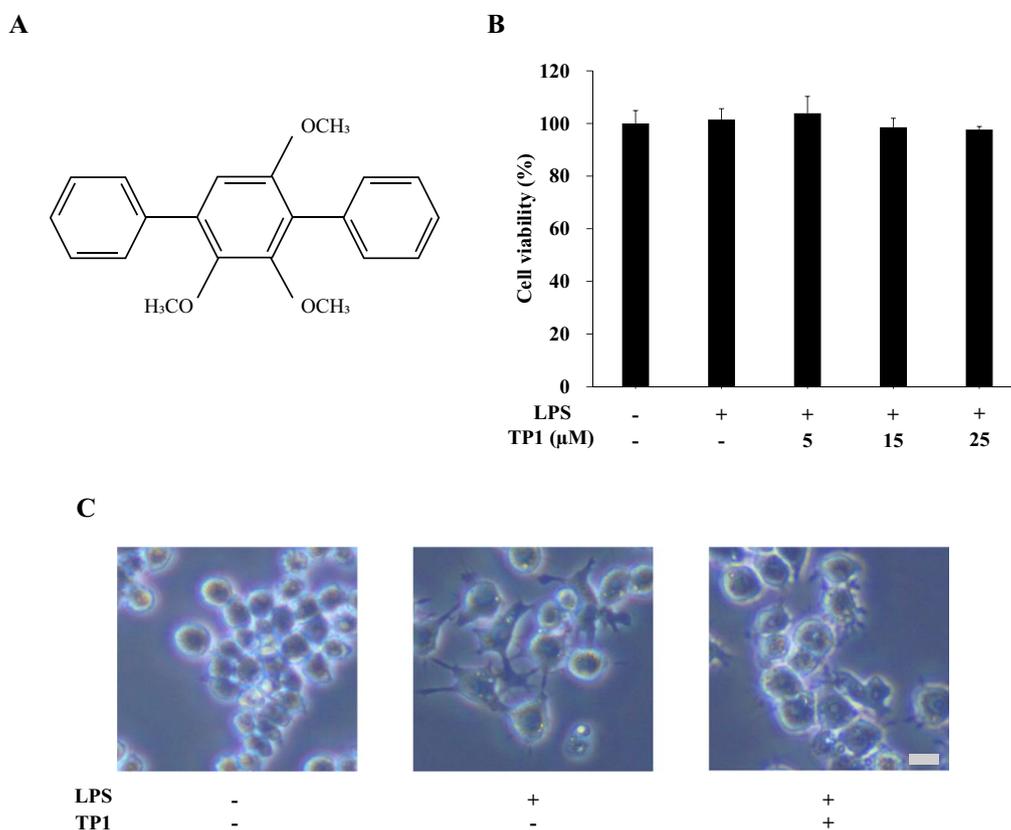


Fig. 1 Effects of TP1 on the viability and morphology of Raw264.7 macrophages. **a** Chemical structure of TP1. **b** The cells were pretreated with TP1 (0, 5, 15, and 25 μM) for 30 min, followed by treatment with LPS (0.1 $\mu\text{g}/\text{mL}$) for 24 h, after which cell viability was measured using MTT assay. The data are presented as mean \pm standard deviation (SD). **c** To assess the anti-inflammatory effects of TP1, the cells were pretreated with TP1 (25 μM) for 30 min. Afterwards, morphological changes in Raw264.7 macrophages were examined after treatment with LPS (0.1 $\mu\text{g}/\text{mL}$) for 24 h. The data are presented as mean \pm SD. Three independent experiments were performed, and representative data are presented

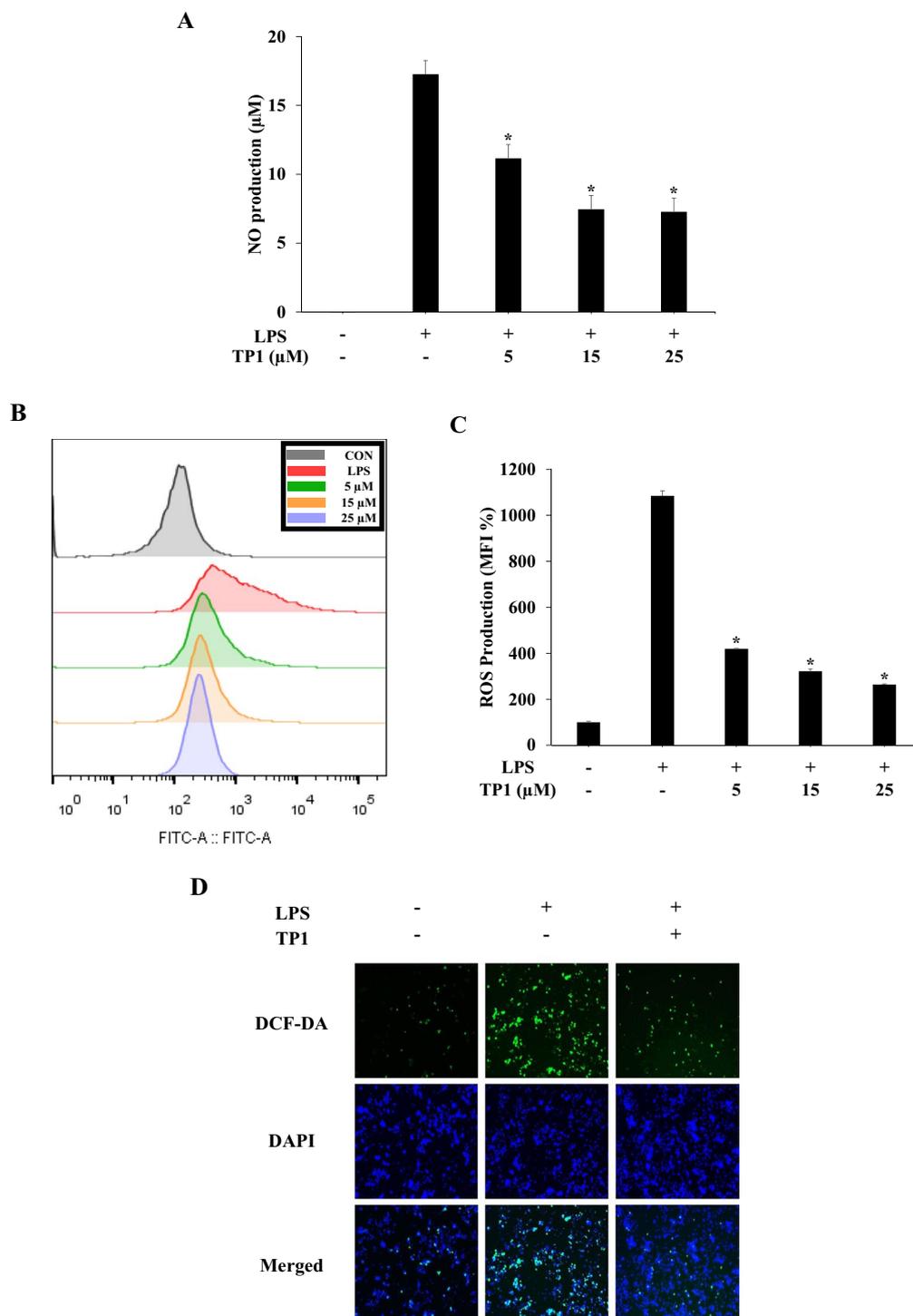


Fig. 2 Effects of TP1 on nitric oxide (NO) and reactive oxygen species (ROS) production in LPS-induced Raw264.7 macrophages. The cells were pretreated with TP1 (0, 5, 15, and 25 µM), followed by treatment with LPS (0.1 µg/mL) for 24 h. **a** The NO level of the cell culture supernatant was measured. **b, d** ROS production was measured by FACS (**b**) and fluorescence microscopy (**d**). **c** Quantitative analysis of panel **b**. * $p < 0.05$ compared with the LPS group. The data are presented as the means \pm standard deviation (SD). Three independent experiments were performed, and representative data are presented

TP1 decreases the mRNA expression of pro-inflammatory enzymes and cytokines in LPS-induced Raw264.7 macrophages

LPS-induced activation of macrophages promotes the production of various inflammatory enzymes, such as iNOS and COX2, and inflammatory cytokines, including IL-1 β , TNF- α , and IL-6 [37]. iNOS is an important marker of inflammation that leads to NO production [38]. COX-2 produces prostaglandin E2 (PGE₂), which mediates the inflammatory cascade [39]. These components play pivotal roles in mediating inflammatory processes [40]. Therefore, we investigated the effects of TP1 on the mRNA expression of pro-inflammatory enzymes (iNOS and COX-2) and cytokines (IL-1 β , TNF- α , and IL-6) using qRT-PCR. TP1 pretreatment significantly inhibited LPS-induced increase in the mRNA expression of pro-inflammatory enzymes (iNOS and COX-2) and cytokines (IL-1 β , TNF- α , and IL-6) 24 h after LPS treatment (Fig. 3A–E). Overall, these data show that TP1 downregulates the gene expression of pro-inflammatory enzymes and cytokines in LPS-induced Raw264.7 macrophages.

TP1 reduces the protein expression of pro-inflammatory enzymes in LPS-induced Raw264.7 macrophages

iNOS and COX-2 are crucial pro-inflammatory enzymes that play central roles in mediating inflammatory processes [41]. The final products of iNOS and COX2 (NO and PGE₂, respectively) can accelerate inflammation and are involved in the pathogenesis of inflammatory diseases [42]. Given that the mRNA levels of iNOS and COX-2 were decreased by TP1, we examined the effect of TP1 on the protein levels of iNOS and COX-2 in LPS-induced Raw264.7 macrophages. Consistent with the mRNA expression levels, TP1 treatment significantly decreased iNOS and COX-2 protein expression levels compared with those in the LPS group (Fig. 4). Collectively, these data show that TP1 downregulates the protein expression of pro-inflammatory enzymes.

TP1 modulates the phosphorylation of AKT/NF- κ B pathway proteins in LPS-induced Raw264.7 macrophages

NF- κ B is a key transcription factor that modulates immune cells, including inflammatory T cells, and triggers the expression of pro-inflammatory enzymes and cytokines after LPS stimulation [43, 44]. AKT activation can trigger the degradation of the inhibitor of nuclear factor B (I κ B) protein and enhance the phosphorylation of the p65 NF- κ B subunit, facilitating its translocation to the nucleus. Moreover, inhibiting AKT downregulates the nuclear translocation of NF- κ B [45]. Therefore, we investigated the effects of TP1 on AKT and p65 phosphorylation in LPS-induced Raw264.7 macrophages.

Additionally, immune-cytochemistry was performed to assess the nuclear translocation of p65. TP1 treatment significantly decreased AKT and p65 phosphorylation and suppressed p65 nuclear translocation (Fig. 5A–E). Overall, these results indicate that TP1 effectively downregulates the AKT and NF- κ B signaling pathways.

TP1 modulates the phosphorylation of MAPK pathway proteins in LPS-induced Raw264.7 macrophages

The MAPK signaling pathway plays an important role in initiating cellular inflammatory responses, including the production of pro-inflammatory cytokines, in response to LPS stimulation [46]. ERK1/2, JNK, and p38 phosphorylation regulates the generation of pro-inflammatory cytokines, such as TNF- α and IL-6 [47, 48]. Therefore, we examined the effects of TP1 on ERK, JNK, and p38 phosphorylation and found that TP1 treatment reduced LPS-induced phosphorylation of ERK in Raw264.7 macrophages (Fig. 6). Overall, these results suggest that TP1 exerts its anti-inflammatory effects by suppressing the ERK signaling pathway.

Discussion

Strategies for ameliorating inflammation may be effective in protecting host tissues from the negative effects of long-term or excessive inflammation [49]. Currently available anti-inflammatory drugs show diverse side effects depending on the individual and treatment duration and concentration [50]. Nonsteroidal anti-inflammatory drugs (NSAIDs) used in the treatment of rheumatoid arthritis exert their therapeutic activity by inhibiting COX-2 [51]. For example, acetaminophen, a member of the NSAIDs family, selectively inhibits COX-1/2. However, it is associated with serious side effects, such as upper gastrointestinal complications [52].

Polar organisms produce various secondary metabolites for survival and adaptation to harsh environments, and these secondary metabolites possess several biological activities, such as antioxidant, anti-inflammatory, and antiparasitic activities [25]. Therefore, polar regions are considered large reservoirs of bioactive compounds [53]. However, studies on the anti-inflammatory effects of compounds derived from these organisms are limited. In the present study, TP1 treatment inhibited the expression of iNOS and COX2, crucial inflammatory enzymes, at both the mRNA and protein levels without causing any cytotoxic effects. The stimulation of PRRs on innate immune cells following pathogen infection or tissue damage triggers the activation of MAPKs, including the p38, ERK, and JNK subfamilies, resulting in the expression of multiple inflammatory genes [54]. To elucidate the potential anti-inflammatory mechanism of TP1, we examined

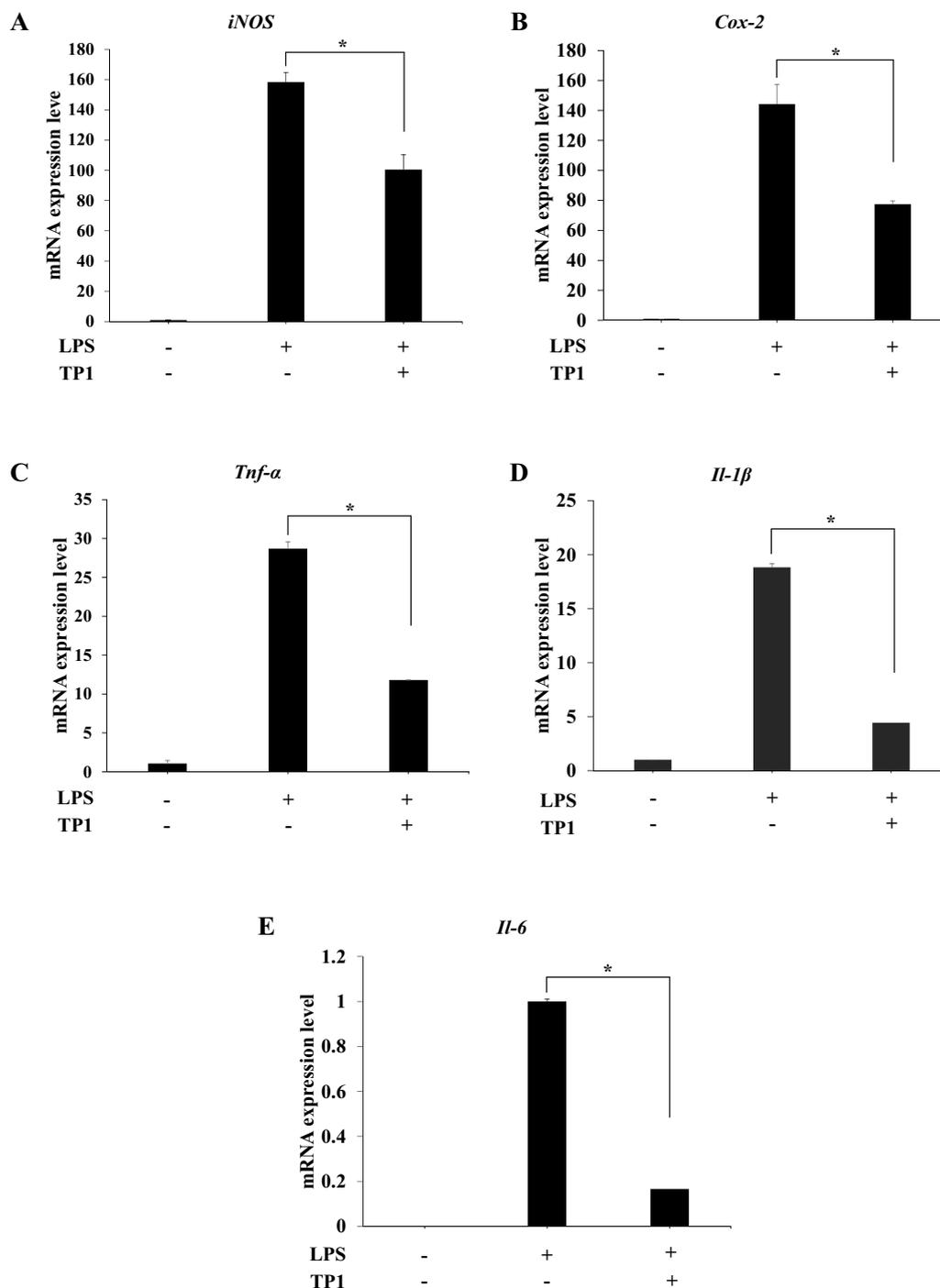


Fig. 3 Effects of TP1 on the mRNA expression of pro-inflammatory mediators. The cells were pretreated with TP1 (25 μM), followed by treatment with LPS (0.1 μg/mL) for 24 h. **a** iNOS, **b** COX-2, **c** TNF-α, **d** IL-1β, and **e** IL-6 mRNA expression levels were measured using qRT-PCR. * $p < 0.05$ compared with the LPS group. The data are presented as mean ± standard deviation (SD). Three independent experiments were performed, and representative data are presented

the phosphorylation of MAPKs (p38, ERK, and JNK). TP1 treatment decreased LPS-induced phosphorylation of ERK but did not affect LPS-induced JNK and

p38 phosphorylation. The MAPK signaling cascade comprises three main kinases: MAPK kinase kinase (MAP3K), which activates MAPK kinase (MAP2K),

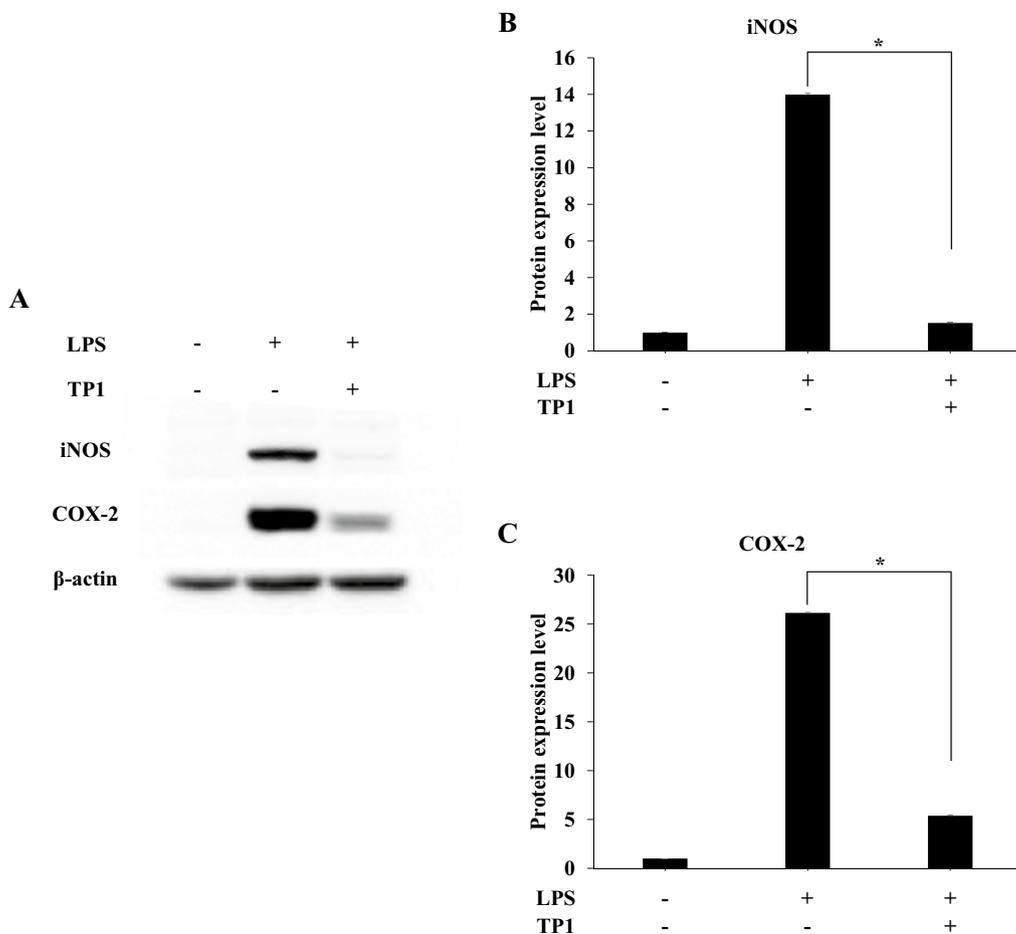


Fig. 4 Effects of TP1 on the protein expression levels of pro-inflammatory enzymes. Raw264.7 macrophages were pretreated with TP1 (25 μM), followed by treatment with LPS (0.1 μg/mL) for 24 h. **a** Protein expression levels were measured using western blot analysis. **b** and **c** iNOS/β-actin and COX-2/β-actin expression levels were quantified by ImageJ software. **p* < 0.05 compared with the LPS group. The data are presented as mean ± standard deviation (SD). Three independent experiments were performed. Among them, representative data are presented

which in turn activates MAPK [55]. Each MAPK (ERK, JNK, and p38) is activated by distinct upstream MAP3K and MAP2K [56]. Therefore, the results of the present study suggest that TP1 can downregulate upstream molecules, including kinases that affect ERK but not p38 or JNK. Similar to TP1, tetrandrine derived from *Stephania Tetrandra* S. Moore inhibits inflammatory responses by modulating the ERK signaling pathway in primary rat mesangial cells [57].

Recently, several natural compounds have been studied for their ability to modulate inflammation and treat sepsis [58]. For example, 7-MCPA extracted from *E. longifolia* protected C57BL/6 mice against LPS-induced

death [59]. Additionally, mangiferin from mangoes and papaya alleviates sepsis by reducing acute lung injury [60]. Moreover, silymarin isolated from milk thistle is effective in protecting mice against LPS-induced sepsis [61]. In line with these previous studies, it is anticipated that TP1 treatment could exert an anti-inflammatory effect in a murine model with LPS-induced sepsis.

Conclusively, the results suggest that TP1 exhibits anti-inflammatory effects in LPS-stimulated macrophages via MAPK pathway-mediated suppression of pro-inflammatory mediators. Overall, TP1 has potential applications in the development of anti-inflammatory drugs for treating inflammatory diseases such as sepsis.

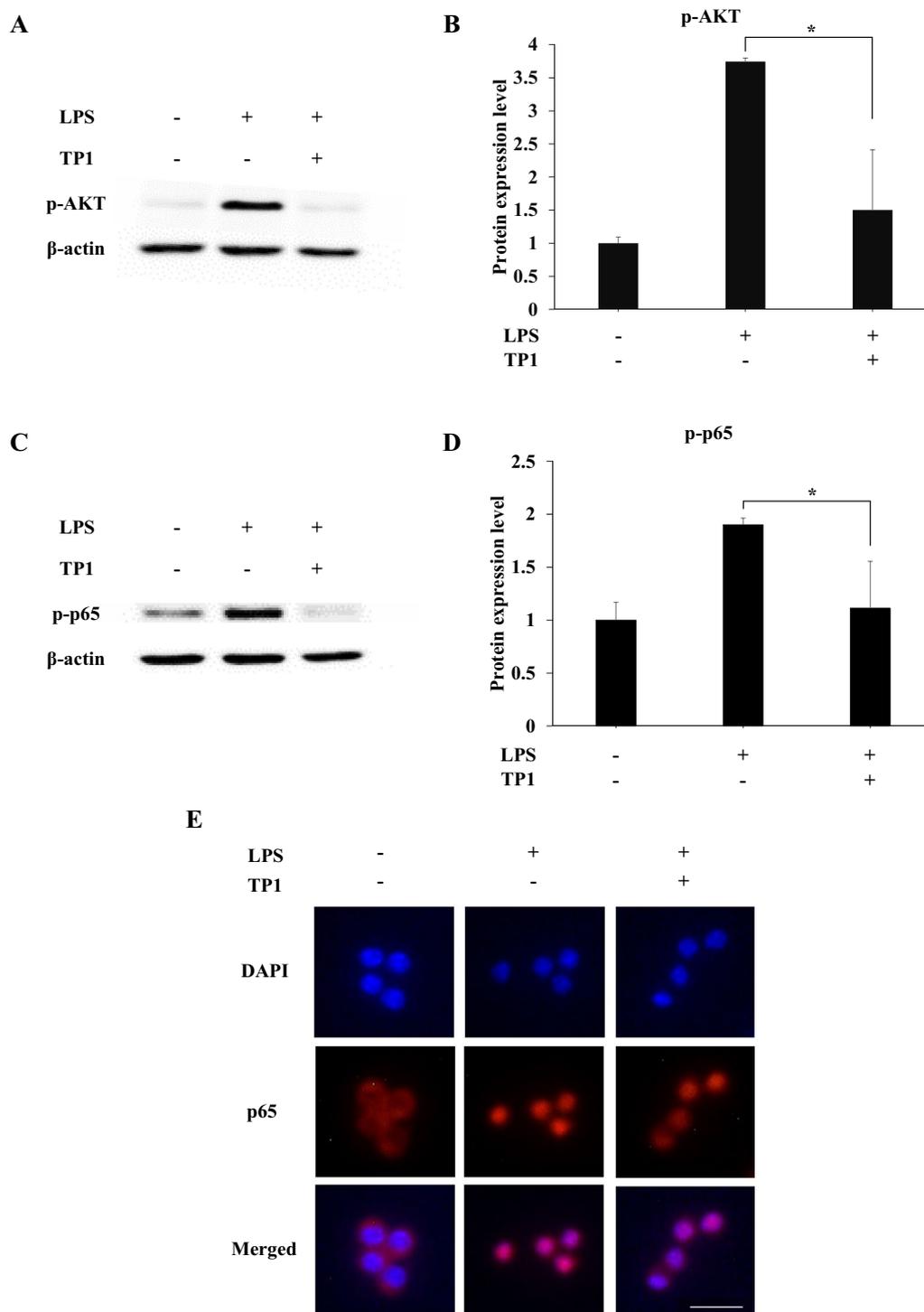


Fig. 5 Effects of TP1 on the phosphorylation of proteins of the AKT/ NF-κB pathway. Raw264.7 macrophages were pretreated with 25 μM of TP1, followed by treatment with LPS (0.1 μg/mL) for 2 h. **a** AKT and **(c)** p65 phosphorylation levels were measured by western blot analysis. **b** p-AKT/β-actin and **d** p-p65/β-actin expression levels were quantified using ImageJ software. **e** p65 translocation was visualized using an immunofluorescence assay. * $p < 0.05$ compared with the LPS group. The data are presented as mean ± standard deviation (SD). Three independent experiments were performed. Among them, representative data are presented

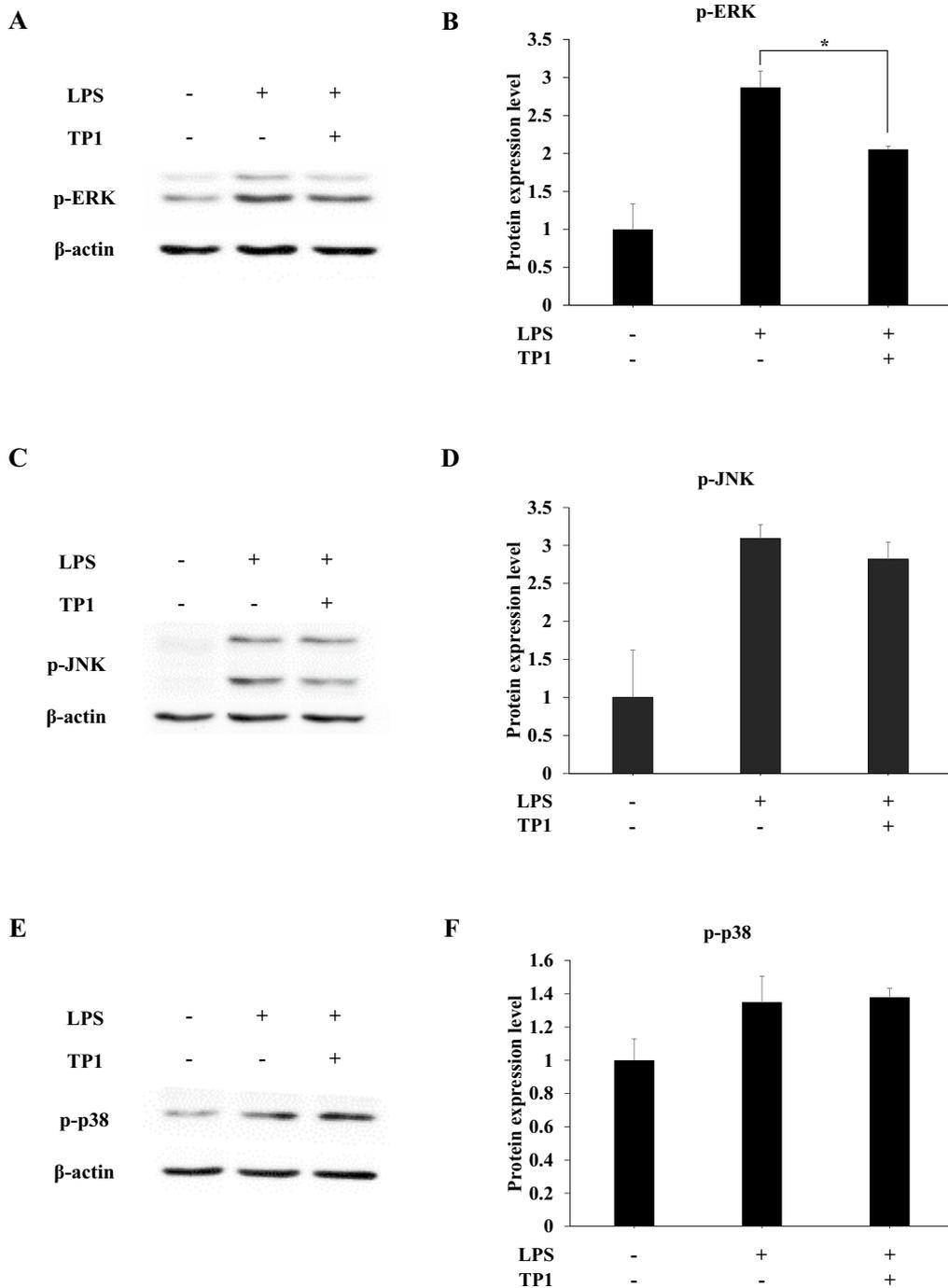


Fig. 6 Effects of TP1 on the phosphorylation levels of MAPK pathway proteins. Raw264.7 macrophages were pretreated with TP1 (25 μM), followed by treatment with LPS (0.1 μg/mL) for 30 min. **a** ERK, **c** JNK, and **e** p38 phosphorylation levels were measured by western blot analysis. **b** p-ERK/β-actin, **d** p-JNK/β-actin, and **f** p-p38/β-actin expression levels were quantified by ImageJ software. **p* < 0.05 compared with the LPS group. The data are presented as mean ± standard deviation (SD). Three independent experiments were performed. Among them, representative data are presented

Acknowledgements

Not applicable.

Author contributions

MK, JA, SAS, MK, SC, HK, and KHP performed the experiments. MK wrote the manuscript under the guidance of CSL. SYM, JHL, UJY, and HHP contributed intellectually to the present study. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets that support the finding of this study are available from the corresponding author on reasonable request.

Declarations**Competing interests**

The authors declare no competing interest.

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