

Enhanced Anti-Neuroinflammatory Effects of KIOM-Patented *Polygonum multiflorum* Root Tuber in LPS-Stimulated BV2 Microglia

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Received: 09 October 2024; Revised: 18 December 2024; Accepted: 22 December 2024

ABSTRACT

Neuroinflammation constitutes the primary pathological mechanism underlying neurodegeneration, with chronic neuroinflammatory responses driving detrimental secondary damage to neural tissues. *Polygonum multiflorum* is a traditional Eastern medicinal herb historically utilized as a hematopoietic agent and applied in the management of diverse pathological conditions. The objective of this study was to evaluate and compare the anti-inflammatory efficacy of standard, commercially available *P. multiflorum* (C1) against a KIOM-patented, *in vitro*-propagated variant of *P. multiflorum* (K1). The K1 variant possesses elevated biomass and a higher concentration of active constituents, and both variants were evaluated under culture and cultivation conditions of lipopolysaccharide (LPS)-induced neuroinflammation. Following LPS stimulation and subsequent treatment with either C1 or K1 in murine microglial BV-2 cells, we evaluated nitric oxide (NO) generation, the secretion of pro-inflammatory cytokines, inducible NO synthase (iNOS) expression, mitogen-activated protein kinase (MAPK) phosphorylation, and transcription factor activity. Additionally, antioxidative capacity was measured via DPPH radical scavenging assays alongside NO production metrics. Both C1 and K1 successfully suppressed the expression of iNOS and cyclooxygenase-2 (COX-2), while also reducing the secretion of pro-inflammatory cytokines. To elucidate the underlying regulatory pathways, Western blotting was employed to quantify inflammatory mediators, including interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , MAPKs, and I κ B α . Furthermore, C1 and K1 attenuated the activation of both p38 and nuclear factor-kappa B (NF- κ B) within the LPS-stimulated BV-2 microglia. Across all experimental parameters, K1 demonstrated superior anti-neuroinflammatory efficacy at a lower dosage compared to C1, a property attributed to its enhanced biomass content. Consequently, K1 displays greater therapeutic potential than C1 and holds promise for broader application in the development of preventative and therapeutic interventions for various inflammation-driven neurodegenerative disorders.

Keywords: KIOM-patented *Polygonum multiflorum*, Lipopolysaccharide, Microglia, Neuroinflammation, Neuroprotection

How to Cite This Article: Zhang W, Hui C, Tan M. Enhanced Anti-Neuroinflammatory Effects of KIOM-Patented *Polygonum multiflorum* Root Tuber in LPS-Stimulated BV2 Microglia. Spec J Pharmacogn Phytochem Biotechnol. 2024;4(2):172-84. <https://doi.org/10.51847/7JehVzWPnW>

Introduction

Concomitant with the global rise in life expectancy, the socioeconomic and healthcare burdens imposed by chronic neurodegenerative disorders—including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis—have escalated substantially [1]. These debilitating conditions involve a progressive loss of function and eventual cellular death within specialized regions of the central nervous system (CNS), driven by an intricate interplay of hereditary liabilities, environmental exposures, and age-dependent endogenous stressors [2]. A pivotal element in the pathogenesis of neurodegeneration is neuroinflammation. When this immune response transforms into a chronic state, the persistent activation of microglia triggers secondary cascades that prove highly

destructive to surrounding neural tissues. Dispersed throughout the brain parenchyma, microglia serve as the primary resident macrophages of the CNS. Upon stimulation by pathogenic triggers or molecular stressors such as lipopolysaccharide (LPS), β -amyloid, thrombin, or interferon-gamma (IFN- γ), these cells initiate localized inflammatory pathways [3, 4]. This process prompts the transcriptional upregulation and subsequent release of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). If this cycle remains unchecked, the resulting hyperactivation of microglia yields an overproduction of immune mediators that disrupt neural homeostasis, thereby accelerating the onset and advancement of multiple sclerosis, AD, PD, and various autoimmune neurodegenerative states [5]. Consequently, modulating the inflammatory agents discharged by aberrantly activated microglia and rectifying the innate immune dysregulation caused by microglial overactivation represent viable therapeutic strategies for slowing down chronic disease progression and mitigating tissue injury.

The root tuber of *Polygonum multiflorum* Thunb., a member of the Polygonaceae family, represents a botanical drug traditionally prescribed in Traditional Korean Medicine (TKM) to enrich and tonify the blood [6]. According to the classical TKM compendium *Dong-Eui-Bo-gam*, this herb serves not only as a hematopoietic agent but also as an intervention against aging, a cognitive enhancer, and a treatment to fortify skeletal integrity. Furthermore, empirical literature has documented its strong neuroprotective properties within hippocampal formations [7]. In therapeutic applications, processed variations of *P. multiflorum* are generally preferred over raw preparations due to their superior clinical efficacy.

In earlier work, an optimized *in vitro* propagation methodology was established to generate premium-quality seedlings and rootstocks of *P. multiflorum* [8]. That project aimed to minimize inherent plant toxicity while elevating therapeutic potency through processing methodologies, which is a core tenet of TKM theory. To standardize the application of *P. multiflorum* within TKM frameworks, the Korea Institute of Oriental Medicine (KIOM) secured a patent (no: 10-2019-0120751, September 30, 2019) for a reliable protocol designed for the rapid *in vitro* cultivation of seedlings and expanded roots. Subsequent assessments demonstrated the regulatory capacity of this KIOM-produced *P. multiflorum* on skeletal metabolism, noting enhanced bone development in osteoblast-like Saos-2 cells and modulated bone resorption in bone marrow-derived macrophages [9]. Additionally, this specific processing system proved highly reproducible, allowing researchers to verify variations in active phytochemical yields across different processing conditions and adjunct materials [10, 11].

The current investigation features a direct comparative evaluation between standard commercial *P. multiflorum* and the KIOM-patented, *in vitro*-propagated counterpart, aiming to contrast their anti-inflammatory potencies in neural models and determine the underlying molecular pathways.

Materials and Methods

Dried roots of commercially sourced *P. multiflorum* (2 kg) were obtained from Jirisan Hasuo Farming Co. (Sancheong, Republic of Korea) and verified by the Herbarium of KIOM as commercial raw *P. multiflorum* (C1). Dried root tubers (2 kg) cultivated via the patented KIOM protocol (patent no. 10-1777833) were supplied by the institute and designated as K1. The extraction and preparation processes strictly mirrored those detailed in previous publications [9, 11].

Dulbecco's modified Eagle's medium (DMEM) was procured from Welgene Inc. (Gyeongsan, Korea). Fetal bovine serum (FBS) along with penicillin/streptomycin solutions were sourced from Hyclone (Logan, UT, USA). All experimental culture plates and dishes were manufactured by SPL Life Sciences (Pocheon, Republic of Korea). LPS, glutamate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were acquired from R&D Systems (Minneapolis, Minnesota, 88 USA). Diverse primary antibodies and horseradish peroxidase (HRP)-linked secondary antibodies for immunoblotting applications were purchased from Cell Signaling Technology, Inc. (Boston, 89 MA, USA).

Sample preparation

The wild-type counterpart of the propagated *P. multiflorum* seed served as the negative control group (C1) and was supplied by Chamdeulae Bio (Sancheong-gun, Gyeongsangnam-do, Republic of Korea). The K1 variant was developed via *in vitro* propagation techniques within ethnomedicine to maximize the agricultural accumulation

of root tuber biomass for practical application. Dried *P. multiflorum* tissue (10 g) was suspended in 100 mL of distilled water for a 2-h period at 20 °C, followed by extraction via centrifugation at 200 rpm.

The resulting fluid from each group was passed through filtration paper (ADVANTEC, 110 mm, Toyo Roshi Kaisha, Japan) and concentrated using a rotary evaporator (EYELA, Tokyo Rikakikai, Japan) prior to reconstitution in 70% ethanol. Centrifugal separation was applied to isolate the supernatant, yielding final analytical stock concentrations calibrated to 10 and 20 mg/mL. All solvents used during extraction and reconstitution procedures were of HPLC grade (J.T. Baker Inc., Phillipsburg, NJ, USA), and purification was completed with a 0.2-µm membrane filter (PALL Corporation, Ann Arbor, MI, USA).

DPPH radical scavenging activity

A 0.20 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was prepared in methanol. Test samples (C1 and K1) were diluted across a range of concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/mL). Next, 150 µL of the DPPH mixture was combined with 50 µL of each sample dilution. Following a 15-minute incubation period in a dark environment, the reduction in optical absorbance was documented at 517 nm. The radical scavenging performance against DPPH was calculated as a percentage of inhibition (%) using the following equation:

where A represents the absorbance value of the experimental test sample and A control represents the absorbance value of the control.

Cell culture and maintenance

The murine-derived microglial cell line BV2 was kindly provided by Professor Kyoungso Suk of Kyungpook National University (Daegu, Republic of Korea) and maintained in DMEM supplemented with 10% FBS and 1% antibiotic solution. These cultures were grown inside a humidified environment regulated at 37 °C with an atmospheric composition of 95% air and 5% CO₂.

The growth media was replaced every 2 days, and experimental treatments were initiated once cellular density reached approximately 80–85% confluence. Cells were stimulated using 100 ng/mL of LPS during exposure to either C1 or K1. For all in vitro applications, stock solutions of LPS and glutamate were prepared by dissolving the compounds in sterile distilled water.

Cytotoxicity investigation using MTT assay

To rule out potential cytotoxic reactions from C1 and K1 and establish appropriate dosing parameters, BV2 microglia were seeded into 24-well culture plates for 24 h to reach a density of 2×10^5 . The cells were pre-treated with various concentrations of C1 and K1 for 1 h, followed by the introduction of 0.1 µg/mL of LPS for a 24-h incubation period.

At the end of this phase, the incubation medium was discarded and replaced with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich, St. Louis, MO, USA) for a 2-h metabolic reduction phase. Following this reaction, the resulting formazan crystals were solubilized using a dimethyl sulfoxide solution. Absorbance values were collected at 540 nm via a microplate reader (Molecular Devices, Sunnyvale, CA, USA) and compared against baseline conditions without LPS or drug exposures. Survival rates (%) for each treated group were calculated relative to the untreated control cultures.

Quantification of Nitric Oxide (NO)

Nitric oxide (NO) levels generated by the LPS-stimulated BV2 cells were quantified utilizing the Griess reagent, which detects NO₂⁻ within the culture medium. Briefly, BV2 cells (2×10^5 cells/well) were seeded into a 24-well plate and incubated for 24 h. The cells were subsequently exposed to varying concentrations of either C1 or K1 for 1 h prior to the introduction of LPS (0.1 µg/mL). Following a 24 h incubation period, the culture medium was harvested and combined with an equivalent volume of Griess reagent at ambient temperature. Finally, the optical density of the resulting mixture was determined at 540 nm utilizing a microplate reader.

Assessment of prostaglandin E2 levels

To determine the production of prostaglandin E2 (PGE2) by BV2 cells following LPS stimulation, culture media obtained under identical experimental conditions were harvested and analyzed utilizing a PGE2 enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The final PGE2 levels (pg/mL) within the cultures were calculated and adjusted according to a standard curve.

Extraction of total RNA, cDNA synthesis, and RT-qPCR

BV2 cells were seeded into a 6-well plate at a density of 1×10^6 cells/well. These cells were pre-incubated with either C1 or K1 at concentrations of 100 and 200 $\mu\text{g/mL}$ for 1 h, subsequently stimulated with LPS (0.1 $\mu\text{g/mL}$), and maintained for an additional 6 h. Following media aspiration, total RNA was isolated utilizing the easy-BLUE™ RNA extraction kit (iNtRON Biotech) in accordance with the provided guidelines. The purified RNA served as a template for cDNA synthesis and reverse transcription via the AccuPower® CycleScript RT PreMix (Bioneer). Across all experiments, relative alterations in gene expression were normalized to RNA concentrations using the $2^{-\Delta\Delta\text{CT}}$ approach. The specific nucleotide sequences for all primers are detailed in **Table 1**.

Table 1. Dedicated primer sequences utilized for PCR amplification

Target		Primer sequences	Accession numbers
TNF- α	Forward	5'-ATGAGCACAGAAAGCATGATC-3'	D84199.2
	Reverse	5'-TACAGGCTTGTCCTCGAATT-3'	
IL-6	Forward	5'-AGTTGCCTTCTTGGGACTGA-3'	NM_031168.2
	Reverse	5'-CAGAATTGCCATTGCACAAC-3'	
IL-1 β	Forward	5'-GGGCCTCAAAGGAAAGAATC-3'	NM_008361.4
	Reverse	5'-TACCAGTTGGGGAAGTCTGC-3'	
GAPDH	Forward	5'-CAGCCTCGTCCCGTAGAC A-3'	NM_008084
	Reverse	5'-CGCTCCTGGAAGATGGTGAT-3'	

Cytokine quantification via ELISA

To evaluate the suppressive impact of C1 and K1 on the secretion of pro-inflammatory cytokines, the levels of TNF- α , IL-1 β , and IL-6 released into the BV2 cell culture medium were quantified employing commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the supplier's instructions. Specifically, 50 μL of a $1 \times$ assay diluent was dispensed, followed by the introduction of 50 μL of either the sample medium or respective standard proteins. This preparation was incubated at ambient temperature for 2 h. Following three buffer washes, the corresponding detection antibody was introduced, and the plate was incubated for another 2 h at room temperature. After an additional three washes, 100 μL of a chromogenic substrate was applied. The plate was then kept in the dark at ambient temperature for 30 min, after which 100 μL of a stop solution was incorporated. The resulting absorbance was recorded utilizing a microplate reader to evaluate the reaction intensity. The final concentrations of the cytokines released by the BV2 cells were derived from standard curves generated using the reference proteins.

Western blot evaluation

To assess the influence of C1 and K1 on the expression levels of iNOS, COX-2, mitogen-activated protein kinases (MAPK), I κ B, and NF- κ B, BV2 cells were plated in 6-cm dishes at a density of 5×10^6 cells/dish. Following exposure to C1 or K1, the cells were rinsed with chilled PBS and subsequently washed with a lysis buffer comprising 1-M Tris-HCl and 5-M NaCl. Cellular lysis was achieved utilizing a Triton X-100 buffer supplemented with a protease inhibitor cocktail. The homogenates were centrifuged at 14,000 rpm for 15 min, and the resulting supernatants were collected. Total protein concentrations were determined utilizing a BSA standard curve, and 30 μg of the extracted protein was resolved via 9–12% polyacrylamide gel electrophoresis. The separated proteins were then electrotransferred onto a nitrocellulose membrane, which was subsequently blocked with 5% BSA for 1 h at ambient temperature. Primary antibodies specific to the target proteins were applied for an overnight incubation at 4 °C. After three washes with a PBS-T buffer, an HRP-conjugated anti-IgG secondary antibody was introduced for a 1 h incubation at room temperature. Following three additional PBS-T washes, the protein bands were visualized using enhanced chemiluminescence Western blotting substrates (SuperSignal, Thermo Scientific, Rockford, IL, USA). Final expression levels were captured and analyzed via the ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA).

Immunofluorescence staining

BV2 cells were plated onto 4-well chambered coverslips (Ibidi, Gräfelfing, Germany) at a density of 3×10^3 cells/well and maintained within a CO2 incubator. These cells were pre-exposed to 250 $\mu\text{g/mL}$ of either C1 or K1 for 1 h prior to a 20 min stimulation with LPS (0.1 $\mu\text{g/mL}$). Following this, the cells underwent fixation using 4%

formaldehyde alongside chilled 100% methanol. They were then incubated in a blocking solution comprising 5% FBS and permeabilized using 0.3% Triton X-100 for subsequent p65 detection. To visualize the targets, samples were incubated with an Alexa Fluor® 488-conjugated goat anti-rabbit IgG (H&L) secondary antibody. To differentiate p65 localization between the cytoplasm (red) and the nucleus (blue), the coverslips were mounted using VECTASHIELD Antifade Mounting Medium supplemented with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). The nuclear translocation of p65 was evaluated employing a fluorescence microscope, with photographic data captured via the LAS X software suite (Leica Microsystems, Wetzlar, Hessen, Germany).

Quantitative analysis by HPLC

Quantitative profiling was performed using a high-performance liquid chromatography (HPLC) system (HITACHI L-2000 series, Hitachi, Japan). Chromatographic separation was achieved using an Agilent Zorbax Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) maintained at an operational and sample temperature of 25 °C. The mobile phase consisted of 0.1% acetic acid (solvent A) and 100% acetonitrile (solvent B), delivered at a constant flow rate of 1.0 mL/min with an injection volume of 10 µL. The overall run duration was set to 80 min. The gradient profile optimized for solvent B was structured as follows: 0–5 min at 10%, 5–45 min at 40%, 45–51 min at 50%, 51–58 min at 80%, 58–67 min at 98%, and 67–80 min at 98%. Ultraviolet spectra were recorded at a wavelength of 288 nm following an initial scan across the 190–400 nm range. Standard calibration curves were generated utilizing reference standards of THSG, emodin, and physcion (Sigma Aldrich Co., Inc. FLUKA, TraceSELECT, USA) prepared at concentrations of 7.8125, 15.625, 31.25, 62.5, 125, and 250 µg/mL. All quantitative HPLC determinations were conducted in triplicate, and evaluations were based on the mean concentration values.

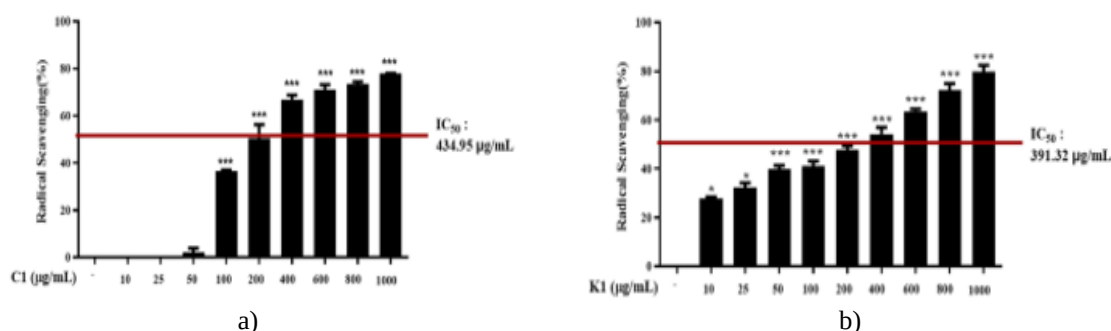
Statistical analysis

Experimental data are expressed as the mean ± SD (or SEM). Statistical evaluations among multiple groups were executed via one-way analysis of variance (ANOVA) utilizing GraphPad Prism version 5.03 for Windows (GraphPad Software Inc., San Diego, CA, USA). Student's t-tests were employed to determine the statistical significance of variations between individual groups.

Results and Discussion

Effects of C1 and K1 on DPPH radical scavenging activity

To evaluate and contrast the antioxidant capacities of C1 and K1, a radical scavenging assay was carried out using DPPH. Both C1 and K1, tested across a concentration spectrum of 10–1000 µg/mL, demonstrated robust, dose-dependent DPPH free radical scavenging efficiency (**Figure 1**). The half-maximal inhibitory concentrations required for 50% clearance of DPPH radicals were determined to be 434.95 µg/mL for C1 and 391.32 µg/mL for K1 ($p < 0.001$).



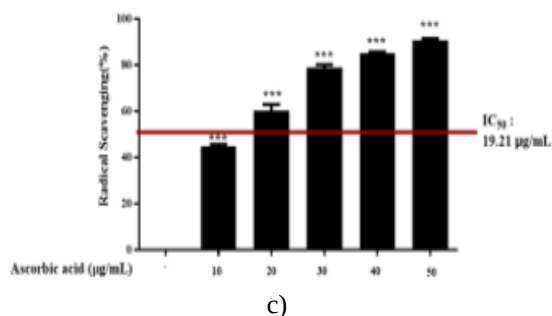


Figure 1. DPPH radical scavenging profiles of C1 (a), K1 (b), and ascorbic acid (c). Data points represent the mean $\pm$ SD obtained from three independent experimental replicates. Statistical significance is defined as $p < 0.05$ and $p < 0.001$ relative to the control (CTL) group.

Effect of C1 and K1 on microglial cell viability

The BV2 cell line retains key functional features of microglia and serves as a well-established *in vitro* surrogate for primary microglial cells in neuroinflammation research. To assess the potential cytotoxicity of C1 and K1 and define an appropriate dosage threshold for this experimental framework, BV2 cells were exposed to a gradient of C1 and K1 concentrations, followed by LPS stimulation in culture for 24 h. Cellular viability was subsequently evaluated using the MTT assay. The results demonstrated an absence of cytotoxic effects up to a concentration of 1.0 mg/mL for both C1 and K1 (**Figures 2a and 2b**). Consequently, subsequent investigations were carried out using C1 or K1 concentrations of ≤ 0.25 mg/mL, while omitting the 0.5 mg/mL dosage.

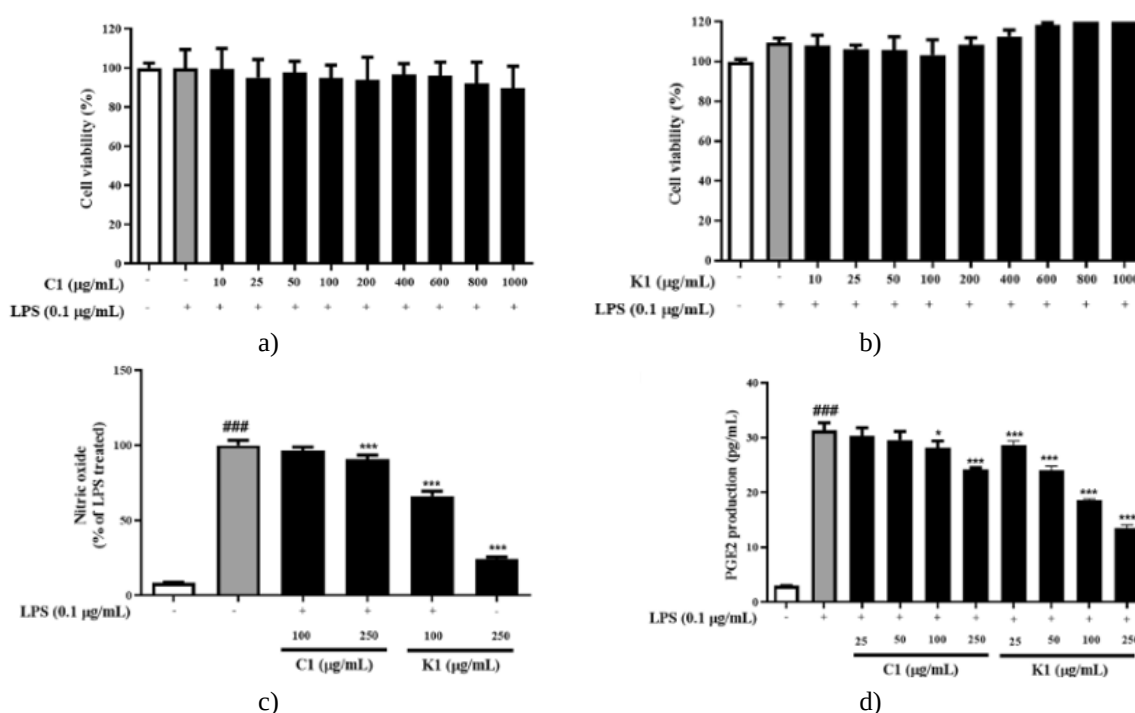


Figure 2. Assessment of cellular viability via MTT assay (a, b), alongside NO (c) and PGE2 (d) synthesis in BV2 cells. Cells were initially seeded into 96-well plates at a density of 1×10^5 cells/well. Following an overnight incubation for cell attachment, the cells were co-incubated with C1, K1, and LPS (0.1 μ g/mL) for an additional 24 h before conducting the MTT assay. Data are expressed as the mean $\pm$ SD of three distinct experimental runs. Statistical variance is indicated as ### $p < 0.001$ versus the untreated control group, and $p < 0.05$, $*p < 0.001$ relative to the LPS-stimulated group.

Effect of inhibitory on NO and PGE2 production by C1 and K1 in LPS-activated BV2 microglial cells

To evaluate the anti-inflammatory capacities of C1 and K1, BV2 microglial cells were challenged with 0.1 μ g/mL of LPS to stimulate the release of the inflammatory mediator nitric oxide (NO). The levels of NO released into the extracellular environment were determined using the Griess assay method. Furthermore, the efficacy of C1

and K1 in dampening the secretion of prostaglandin E2 (PGE2), another pivotal inflammatory mediator produced by BV2 cells, was quantified via ELISA. As illustrated in **Figure 2c**, baseline NO production was minimal in unstimulated cells but surged markedly upon LPS exposure, validating the pro-inflammatory activation of the microglial cells. Pretreatment with C1 at 250 $\mu\text{g/mL}$, as well as K1 at 100 and 250 $\mu\text{g/mL}$, successfully mitigated this LPS-driven NO elevation in a dose-dependent manner. Similarly, extracellular PGE2 concentrations were nominal under control conditions but rose sharply following LPS introduction (**Figure 2d**). Administration of either C1 or K1 at dosages of 25, 50, 100, and 250 $\mu\text{g/mL}$ substantially repressed PGE2 secretion in a concentration-dependent fashion, with K1 displaying higher potency relative to C1.

Inhibitory effects of C1 and K1 on inflammatory cytokine expression in LPS-activated BV2 microglial cells

Prompted by the robust down-regulation of LPS-induced NO and PGE2 synthesis by C1 and K1, their regulatory impact on pro-inflammatory cytokine expression was scrutinized. Specifically, alterations in the transcription and translation of TNF- α , IL-1 β , and IL-6 were characterized via RT-PCR and ELISA, respectively. As shown in **Figures 3a and 3c**, stimulation with LPS (0.1 $\mu\text{g/mL}$) led to a marked up-regulation of the transcripts for all targeted cytokines. Following treatment with C1 and K1, the transcriptional levels of IL-1 β and IL-6 were significantly repressed in a dose-dependent manner. Conversely, TNF- α mRNA levels remained largely unaltered by C1 intervention, whereas high-dose K1 administration (250 $\mu\text{g/mL}$) achieved significant transcriptional inhibition. ELISA data (**Figures 3d and 3f**) mirrored these trends: baseline secretion of TNF- α , IL-1 β , and IL-6 was negligible in resting cells but rose dramatically to 4.3 pg/mL, 7.1 pg/mL, and 288.7 pg/mL, respectively, upon isolated LPS challenge. Consistent with the genetic expression dynamics, the secretion of TNF- α , IL-1 β , and IL-6 was progressively diminished by K1 treatment in a concentration-dependent fashion, whereas C1 failed to induce a notable suppressive response.

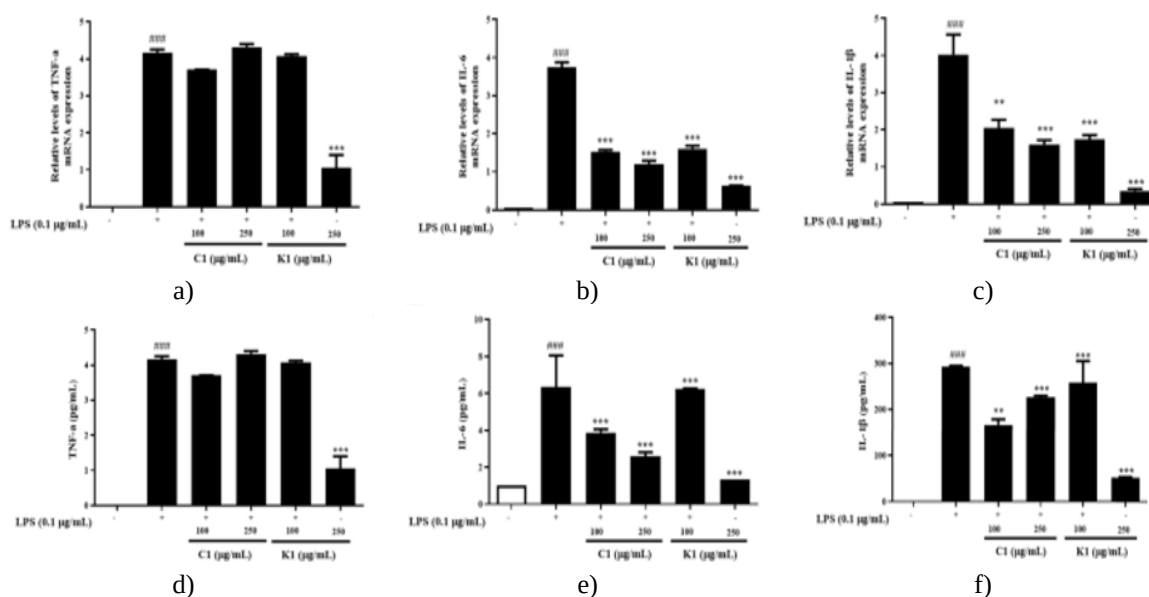


Figure 3. Transcriptional levels of TNF- α (a), IL-6 (b), and IL-1 β (c) obtained via real-time PCR, along with the extracellular production of TNF- α (d), IL-6 (e), and IL-1 β (f) in BV2 cells. Cells were cultured in 6-well plates at an initial density of 3×10^6 cells/well. Following an overnight incubation, the cells were incubated with C1, K1, and LPS (0.1 $\mu\text{g/mL}$) for a 24 h period. Messenger RNA levels for TNF- α , IL-6, and IL-1 β were subsequently quantified via real-time qPCR (a–c), and pro-inflammatory cytokine secretion was evaluated using ELISA (d–f). Data are presented as the mean $\pm$ SD of three distinct experimental replicates. Statistical thresholds are indicated as ###p < 0.001* relative to the control group, and *p < 0.001 relative to the LPS-treated cohort.

Impact of C1 and K1 on iNOS, COX-2, and MAPK activities in LPS-stimulated BV2 microglial cells

Building upon the previously noted substantial suppression of LPS-stimulated NO and PGE2 generation, Western blot analysis was employed to assess the impact of C1 and K1 administration on the expression of iNOS (an NO-synthesizing enzyme) and COX-2 (the primary enzyme responsible for PGE2 synthesis). In untreated cultured

BV2 cells, baseline iNOS and COX-2 levels were minimal; however, these levels rose dramatically following exposure to LPS (0.1 $\mu\text{g/mL}$) (**Figure 4a**). Subsequent to treatment with C1 and K1, the LPS-driven protein expression of both iNOS and COX-2 was significantly diminished in a concentration-dependent fashion. Furthermore, Western blotting confirmed the modulatory impact of C1 and K1 on the activation of MAPK, a critical signaling cascade involved in the regulation and suppression of inflammatory responses, at the protein level (**Figure 4b**). The profound phosphorylation of p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) triggered by LPS exposure in BV2 cells was substantially reduced following treatment with C1 and K1.

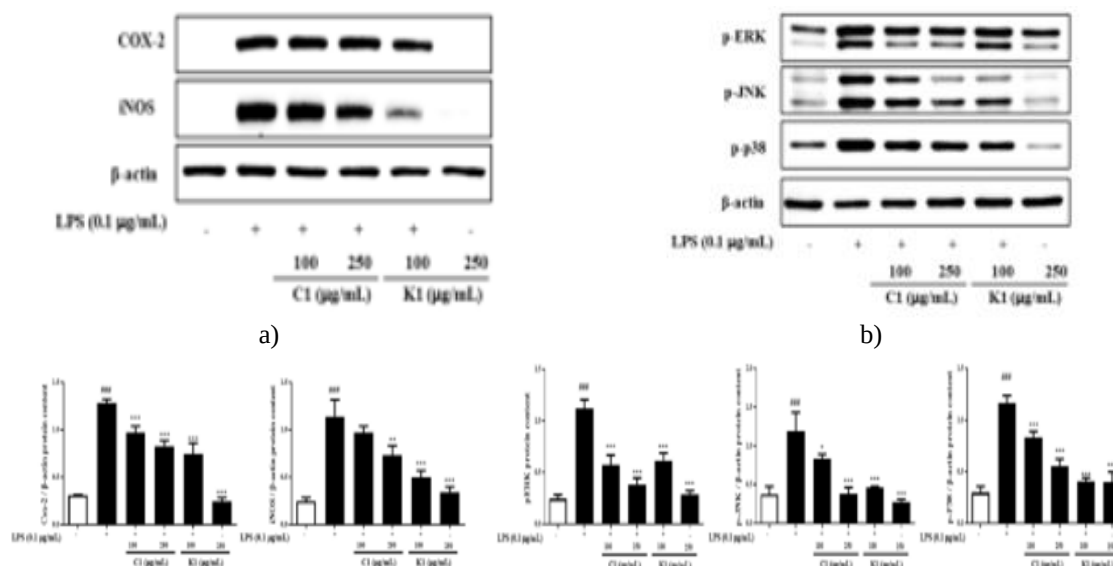


Figure 4. Impact of C1 and K1 on LPS-stimulated iNOS and COX-2 protein expression (a) and MAPK phosphorylation (b) in BV2 microglial cells. BV2 cells were seeded in 6-well plates at a density of 3×10^6 cells/well. Following a 24-h incubation, the cells underwent a 1-h pretreatment with C1 and K1 prior to stimulation with LPS (0.1 $\mu\text{g/mL}$). The protein expression levels of iNOS and COX-2, alongside the phosphorylation status of mitogen-activated protein kinases (MAPK)—specifically ERK, JNK, and p38—were determined via Western blot analysis. Results are presented as the mean $\pm$ SD derived from three independent trials. Data were deemed statistically significant at the indicated thresholds. ### $p < 0.001$ denotes a significant variance relative to the control group; * $p < 0.05$, $p < 0.01$, and *** $p < 0.001$ denote significant variances relative to the LPS-treated group.

Impact of C1 and K1 on NF- κ B activation in LPS-stimulated BV2 microglial cells

Immunofluorescence microscopy was utilized to evaluate and contrast the cellular localization and expression levels of NF- κ B at both the transcriptional and translational stages. Initially, immunofluorescence visualization revealed that in BV2 cells maintained under standard conditions (lacking LPS), NF- κ B was predominantly localized within the cytosol; conversely, in LPS-stimulated BV2 cells, nuclear accumulation of the NF- κ B p65 subunit was augmented (**Figure 5a**). The administration of C1 and K1 profoundly inhibited this LPS-driven nuclear translocation of NF- κ B. Following LPS exposure, the levels of the NF- κ B p65 subunit dropped considerably in the cytosol while simultaneously rising in the nucleus (**Figure 5b**). Concurrently, LPS administration led to a reduction in cytosolic I- κ B α expression.

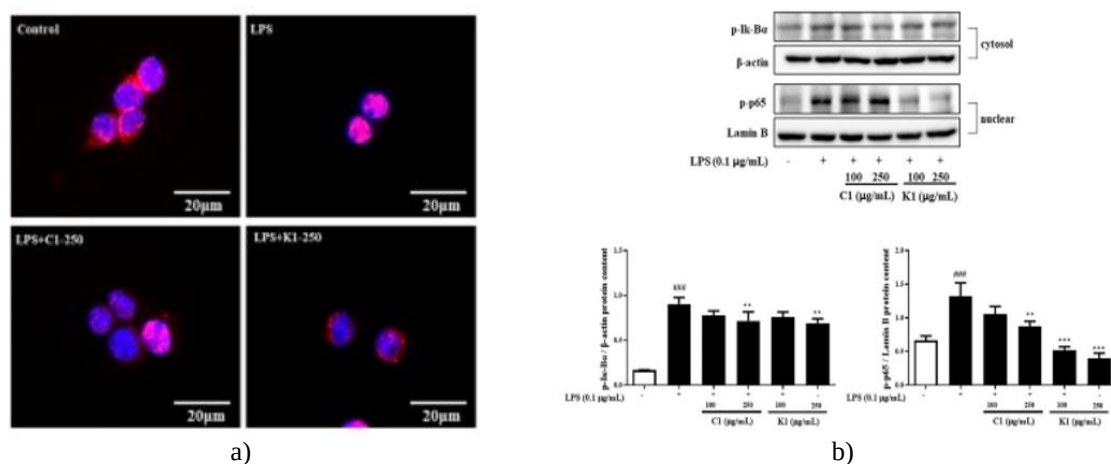
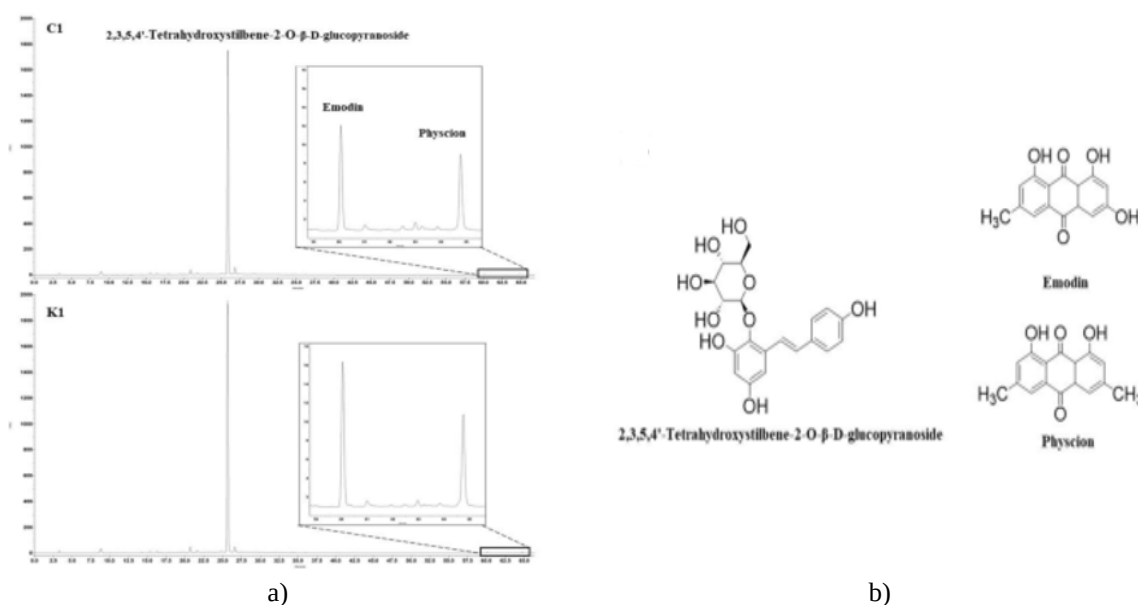


Figure 5. Impact of C1 and K1 on LPS-stimulated NF-κB expression (a) and signaling pathway alterations (b) in BV2 cells. Cells were seeded into 6-well plates at a density of 3×10^6 cells/well. Following a 24-h incubation, a 1-h pretreatment with C1 and K1 was administered, followed by a 20-min stimulation with LPS ($0.1 \mu\text{g/mL}$). Post-treatment, the localization of p65 in the LPS-activated microglia was visualized via immunofluorescence (a), and cytosolic and nuclear fractions were extracted for Western blot evaluation (b). Results are depicted as the mean $\pm$ SD of three independent experiments. Statistical significance is indicated by the following values: ### $p < 0.001$ represents a significant difference from the control; $p < 0.01$ and *** $p < 0.001$ represent significant differences compared with the LPS-treated cohort.

Quantitative HPLC evaluation

The findings from the HPLC evaluation are depicted in **Figure 6**. For the quantitative assessment, the extraction yields were determined to be 15.5% for C1 and 23.7% for K1. The chromatographic profiles revealed that the primary constituent concentrations were greater in K1 compared to C1. Quantitative measurements derived from calibration curves confirmed that K1 possessed elevated quantities of THSG ($30.95 \pm 0.46 \mu\text{g/mg}$ versus $16.83 \pm 0.79 \mu\text{g/mg}$), emodin ($0.55 \pm 0.01 \mu\text{g/mg}$ versus $0.26 \pm 0.01 \mu\text{g/mg}$), and physcion ($0.53 \pm 0.02 \mu\text{g/mg}$ versus $0.27 \pm 0.01 \mu\text{g/mg}$) relative to C1. Attributable to processing techniques aimed at maximizing root tuber yields via refined cultivation standards [8, 9], K1 outperformed C1, demonstrating 1.8-fold, 2.16-fold, and 1.8-fold higher contents of THSG, emodin, and physcion, respectively.



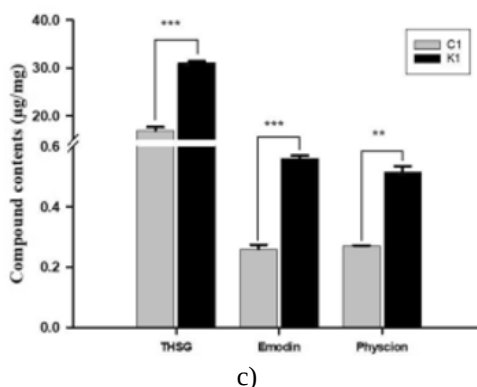


Figure 6. HPLC chromatograms of extracts C1 and K1 (a), structural representations of the primary compounds (b), and a comparative analysis of the principal compound concentrations (c). The dilution concentrations employed for the HPLC evaluation were 10 mg/mL for 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucopyranoside and 20 mg/mL for both emodin and physcion. The ultimate concentrations of these principal compounds were determined by factoring in the extraction yields, which were 15.5% for C1 and 23.7% for K1. Data are expressed as the mean ± SD across three independent experiments. Statistical significance is indicated as follows: $p < 0.01$ and $***p < 0.001$ when comparing extract C1 to K1.

Microglia traditionally function as the phagocytic cells of the central nervous system (CNS), modulating initial immune reactions, reactive oxygen species (ROS), and inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and eicosanoids [12]. The overproduction of ROS, nitric oxide (NO), and superoxide anions plays a role in the pathogenesis of degenerative conditions. While initial neuroinflammation serves as a neuroprotective mechanism, an exaggerated inflammatory response can be detrimental, hindering nerve regeneration and precipitating neurodegenerative and neurological disorders [13]. Following acute or prolonged exposure to pathogenic proteins, environmental neurotoxins, cytokines, or neuronal injury, microglia—the primary regulators of neuroinflammation—become persistently activated. This sustained activation results in the continuous release of cytokines and ROS, driving chronic neuroinflammation and progressive neuronal loss characteristic of neurodegenerative diseases [14, 15]. Lipopolysaccharide (LPS), a bacterial endotoxin, is a potent inflammatory trigger that stimulates microglia to release NO, prostaglandin E2 (PGE2), TNF-α, IL-1β, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1. Furthermore, LPS induces the detrimental hypersecretion of IL-16, IL-18, and macrophage colony-stimulating factor, which exert cytotoxic effects, exacerbate the inflammatory cascade, and ultimately promote the onset of chronic diseases [16–18].

Consequently, monitoring the generation of inflammatory mediators and the transcription of associated genes in activated BV2 cells is considered a valuable approach for developing treatments and preventive strategies for various inflammatory and systemic diseases. This study sought to evaluate and compare the anti-inflammatory properties of conventional *P. multiflorum* (C1) against the KIOM-patented, in vitro-propagated *P. multiflorum* (K1)—which features elevated biomass and active compound concentrations—under conditions of LPS-stimulated neuroinflammation. Specifically, we examined the release of diverse inflammatory mediators and the transcriptional alterations of their corresponding genes at concentrations that did not induce cytotoxicity.

DPPH is a water-soluble compound harboring a chemically stable free radical. It undergoes reduction when exposed to polyhydroxy aromatic compounds, tocopherol, or ascorbic acid, a process visually indicated by the fading of its intense purple hue [19]. This assay is a standard technique for evaluating the antioxidant capacity of diverse natural products. Physiologically, intrinsic defense systems neutralize the majority of generated ROS; however, unquenched ROS rapidly interact with biomolecules, precipitating DNA damage, protein denaturation, and the lipid peroxidation of cell membranes. The resulting lipid peroxides can permeate cells or circulate systemically, propagating further radical chain reactions and instigating various pathologies. In the current investigation, assessments of DPPH radical scavenging activity revealed that K1 possessed a greater antioxidant capacity than C1 at reduced concentrations. Additionally, neither extract exhibited cytotoxic effects at a dose of 1.0 mg/mL.

Nitric oxide (NO), a low-molecular-weight, toxic reactive nitrogen species, plays a physiological role in immune defense mechanisms [20, 21]. Macrophages synthesize NO from L-arginine via nitric oxide synthases (NOS), which include both constitutive isoforms and inducible NOS (iNOS). Microglia upregulate iNOS following

exposure to pro-inflammatory triggers like LPS, TNF- α , interferon-gamma (IFN- γ), or IL-1 β . In vivo, the overproduction of NO induces cellular toxicity, exacerbates inflammatory cascades, drives neuroinflammation in the CNS, and participates in the pathogenesis of neurodegenerative conditions [22, 23]. Concurrently, specific prostaglandin classes progressively amplify inflammatory reactions. Enzymes such as cyclooxygenase (COX), lipoxygenase, or cytochrome P-450 monooxygenase catalyze the conversion of arachidonic acid into the five primary synthesized prostaglandins: PGE₂, PGD₂, PGF_{2 α} , PGI₂, and thromboxane A₂ [24]. Much like NO, PGE₂ serves as a prominent inflammatory mediator implicated in the progression of numerous inflammatory disorders [25]. Our findings indicate that both C1 and K1 markedly diminished LPS-stimulated NO and PGE₂ synthesis in BV2 cells. Furthermore, K1 demonstrated greater efficacy than C1 in mitigating neuroinflammation, evidenced by a more robust, concentration-dependent suppression of iNOS and COX-2 protein expression.

Cytokines are critical modulators of immunity, hematopoiesis, cell proliferation, tissue repair, and host defense during infections, mediating both physiological and pathological responses across various conditions. Upon activation, microglia release cytokines—including IL-1 β , IL-6, IL-10, transforming growth factor-beta (TGF- β), and TNF- α . While maintained at trace levels under basal conditions, these molecules are markedly upregulated following severe injury, functioning as pro-inflammatory drivers. TNF- α , IL-1 β , and IL-6 are primary cytokines that directly or indirectly precipitate neurodegeneration, inflict neuronal damage in neurodegenerative disorders, and contribute to increased mortality rates [26, 27]. In the present investigation, both C1 and K1 substantially inhibited the synthesis and transcriptional expression of IL-1 β and IL-6 in LPS-stimulated BV2 cells. However, a concentration-dependent attenuation of TNF- α production and gene expression was uniquely observed with K1 treatment. These findings indicate that K1 has a distinct capacity to inhibit TNF- α generation, thereby offering a highly effective means of ameliorating symptoms and pathologies associated with neuroinflammatory brain diseases.

Signaling cascades involving the MAPK subfamily—comprising JNK, ERK, and p38—drive the production of pro-inflammatory mediators. Inflammatory cascades triggered by pathogenic infections releasing LPS lead to the phosphorylation of MAPKs and the subsequent activation of transcription factors like activator protein-1 and NF- κ B, which in turn promote the generation of free radicals such as superoxide anions and NO [28, 29]. MAPKs are integral to the synthesis of neuroinflammatory mediators and govern essential cellular processes, including inflammation, apoptosis, and survival [30]. Our study validated the anti-neuroinflammatory properties of C1 and K1, evidenced by their potent suppression of MAPK phosphorylation in LPS-challenged BV2 cells. Notably, K1 exhibited a superior, concentration-dependent ability to inhibit p38 MAPK phosphorylation compared to C1. The therapeutic relevance of p38 inhibitors has gained recent prominence due to evidence linking MAPK-driven microglial neuroinflammation to the exacerbation of neurodegenerative diseases [31]. The capacity of K1 to block MAPK phosphorylation underscores its strong potential as a therapeutic agent against neuroinflammation. Furthermore, both C1 and K1 prevented the nuclear translocation of the NF- κ B p65 subunit. Consequently, the dampening of MAPK and NF- κ B signaling by C1 and K1 appears directly responsible for the observed reduction in inflammatory cytokine expression.

In mice subjected to chronic restraint stress (CRS), the primary bioactive compound THSG reportedly mitigates oxidative stress across both peripheral and central nervous systems. It has also demonstrated anti-inflammatory efficacy by reversing the upregulated expression of pro-inflammatory markers (TNF- α , IL-1 β , and IL-6) in the prefrontal cortex and hippocampus [32]. Additionally, THSG reduces ROS production, providing robust neuroprotection against glutamate-triggered damage in the hippocampus [33]. Emodin, another principal bioactive constituent, reportedly prevents neuronal apoptosis via antioxidant mechanisms, curtails neuroinflammation and amyloid-beta (A β) plaque accumulation in APP/PS1 transgenic mice, upregulates antioxidant enzyme expression, and preserves mitochondrial integrity, ultimately preventing neuronal death [34]. High-performance liquid chromatography (HPLC) analysis verified that K1—developed via vegetative propagation optimized under refined cultivation parameters—yielded a greater biomass and possessed higher concentrations of these three critical active compounds: 2,3,5,4'-tetrahydroxystilbene-2-O- β -D-glucopyranoside (THSG), emodin, and physcion, when compared to C1.

Conclusion

In summary, this study established that K1 possesses anti-inflammatory properties superior to those of C1 in LPS-stimulated BV2 cells. Mechanistically, this efficacy is mediated through the blockade of the MAPK and NF- κ B

signaling cascades, which subsequently represses the secretion of inflammatory cytokines and mediators. As a result, K1 demonstrates greater therapeutic potential than C1, suggesting its broader applicability in the formulation of therapeutic and prophylactic agents targeting a spectrum of inflammation-driven neurodegenerative diseases.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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