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Anti-Inflammatory Potential of Umckalin Through the Inhibition of iNOS, COX-2, Pro-Inflammatory Cytokines, and MAPK Signaling in LPS-Stimulated RAW 264.7 Cells

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Abstract: Background/Objectives: Umckalin, a coumarin derivative abundantly present in the root extract of Pelargonium sidoides, is a key bioactive compound known for its antimicrobial, antiviral, antitubercular, and immunomodulatory properties. Its therapeutic potential has been extensively studied, particularly in the context of respiratory diseases. This study aimed to evaluate the potential of umckalin as a therapeutic agent for chronic inflammatory diseases and to elucidate its underlying mechanisms of action. **Methods**: Using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages as an experimental model, we investigated the inhibitory effects of umckalin on inflammatory mediators and cytokine production. We measured levels of nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), and assessed the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Additionally, the regulation of MAPK signaling pathways, including JNK, p38 MAPK, and ERK, was analyzed. **Results**: The results demonstrated that umckalin significantly reduced the levels of NO, PGE_2, TNF-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells. Umckalin also suppressed the expression of iNOS and COX-2, leading to decreased NO and PGE_2 production. Furthermore, umckalin effectively regulated inflammatory responses by reducing the phosphorylation of MAPK signaling pathways, including JNK, p38 MAPK, and ERK. **Conclusions**: These findings indicate that umckalin inhibits the production of TNF-α, IL-6, IL-1β, and NO, while regulating MAPK signaling pathways, thereby suppressing the expression of iNOS and COX-2. This study highlights the potent anti-inflammatory effects of umckalin and suggests its potential as a promising candidate for the treatment of chronic inflammatory diseases.

Keywords: anti-inflammation; coumarin; RAW 264.7 macrophages; *Pelargonium sidoides*; signaling pathway; umckalin

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1. Introduction

Umckalin, with the chemical formula $C_{11}H_{10}O_5$, is a coumarin derivative structurally defined as 7-hydroxy-5,6-dimethoxycoumarin. It features a benzopyran backbone substituted with methoxy (-OCH_3) and hydroxy (-OH) functional groups, granting it unique chemical properties that have made it a subject of interest in bioactivity research (Figure 1). This compound is predominantly found in plants of the *Pelargonium* genus, particularly in the root extracts of *Pelargonium sidoides* and *Pelargonium reniforme* [1,2]. Traditionally, *P. sidoides* has been widely used in South African ethnomedicine by communities such as the Xhosa, Zulu, and Sotho to treat respiratory ailments including coughs, colds, and

bronchitis [3]. Additionally, it has been utilized to alleviate gastrointestinal issues like diarrhea, promote wound healing, enhance liver function, and strengthen the immune system [2,4]. Umckaloabo is the Zulu name for the native *P. sidoides* root, traditionally used by tribal healers for centuries to treat colds and respiratory infections. To make the name more accessible, the company that brought this product to the U.S. market simplified it to Umcka [5].

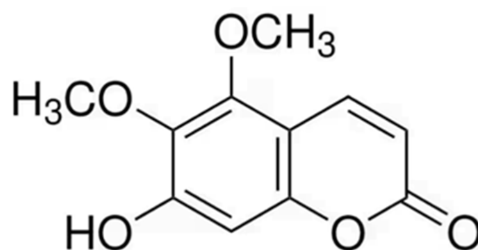

Figure 1. The chemical structure of umckalin. Umckalin, a coumarin derivative with the molecular formula $C_{11}H_{10}O_5$, is characterized by a benzopyran backbone substituted with methoxy (-OCH_3) and hydroxy (-OH) groups, defining its unique chemical properties.

Modern studies have validated the antimicrobial, antiviral, and immunomodulatory effects of *P. sidoides* extracts, leading to the development of commercial formulations such as Umckaloabo®, Kaloba®, and Eps 7630 [6]. These formulations have been recognized for their effectiveness in alleviating symptoms of infectious diseases like colds, bronchitis, and pharyngitis, with minimal side effects and long-term safety [7,8]. Among the key active compounds in *P. sidoides* extracts are umckalin and scopoletin, both of which play pivotal roles in antimicrobial and antiviral activity as well as immune system enhancement. Recent findings have also suggested that umckalin exhibits mild antiviral activity against SARS-CoV-2, expanding its potential beyond traditional uses to include modern therapeutic applications [9–11].

Inflammation is a critical physiological response that protects the body from tissue damage and pathogens. However, excessive or chronic inflammation can lead to severe health issues, including tissue damage, loss of function, and the progression of chronic diseases. Chronic inflammation is closely associated with various disorders such as arthritis, inflammatory bowel disease, and cardiovascular diseases, underscoring the need for effective therapeutic strategies [12–14]. In this context, natural compounds with minimal side effects and the ability to target molecular mechanisms of inflammation hold significant promise as novel anti-inflammatory agents.

This study aims to evaluate the anti-inflammatory effects and mechanisms of action of umckalin using an LPS-induced RAW 264.7 macrophage model. By investigating its ability to inhibit inflammatory mediators and cytokines, this research seeks to establish umckalin's potential as a therapeutic agent not only for respiratory and infectious diseases but also for inflammation-related conditions. The findings are expected to provide foundational insights into the development of umckalin as an anti-inflammatory agent, contributing to new therapeutic strategies for managing inflammatory diseases.

2. Materials and Methods

2.1. Chemicals and Reagents

Umckalin, lipopolysaccharide (LPS), and the Griess Reagent Kit used for measuring nitrite levels were sourced from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were acquired from Gibco (Grand Island, NY, USA). The bicinchoninic acid (BCA) protein assay kit for protein assay was obtained from Thermo Fisher Scientific (Waltham,

MA, USA). Mouse PGE_2 and IL-1β ELISA kits were supplied by R&D Systems (St. Louis, MO, USA), while the mouse IL-6 and TNF-α ELISA kits, along with the primary COX-2 antibody, were procured from BD Biosciences (San Diego, CA, USA). The antibodies for P-ERK, P-JNK, P-p38, T-ERK, T-JNK, T-p38, β-actin, as well as secondary antibodies and the protease/phosphatase inhibitor cocktail, were sourced from Cell Signaling Technology (Beverly, MA, USA). The anti-iNOS antibody was purchased from Merck Millipore (Burlington, MA, USA). Phosphate-buffered saline (PBS), MTT reagent, dimethyl sulfoxide (DMSO), RIPA buffer, Tris-buffered saline (TBS), and enhanced chemiluminescence (ECL) kits were obtained from Biosesang (Sungnam, Gyeonggi-do, Republic of Korea). The 4× sample buffer was acquired from Invitrogen (Waltham, MA, USA).

2.2. Cell Culture

The RAW 264.7 macrophage cell line (KCLB No. 40071) was purchased from the Korea Cell Line Bank (Seoul, Republic of Korea). The cells were maintained in DMEM containing 10% FBS and 1% P/S at 37 °C under a 5% CO_2 environment. The culture medium was refreshed every 48 h to support optimal cell growth.

2.3. Cell Viability Assay

RAW 264.7 cells were plated in 24-well plates at a concentration of 1.5×10^5 cells per well and incubated for 24 h. The cells were subsequently exposed to umckalin at concentrations of 75, 150, 300, and 500 µM along with LPS (1 µg/mL) for another 24 h. Following treatment, the supernatant was removed, and 500 µL of MTT solution (0.4 mg/mL) was added to each well and incubated for 4 h. After incubation, the MTT solution was discarded, and DMSO was used to solubilize the formazan crystals. The absorbance was recorded at a wavelength of 570 nm using a microplate spectrophotometer (Tecan, Salzburg, Austria).

2.4. NO Production

The amount of NO in the culture medium of LPS-stimulated RAW 264.7 cells, where an inflammatory response was induced, was indirectly measured using the Griess reagent as follows. RAW 264.7 cells were plated in 24-well plates at a density of 1.5×10^5 cells per well and allowed to incubate for 24 h. The cells were then exposed to umckalin at concentrations of 75, 150, and 300 µM along with LPS (1 µg/mL) for an additional 24 h. The culture supernatant (100 µL) was transferred to a 96-well plate and mixed with an equal volume of Griess reagent, followed by a 15 min incubation. The absorbance was read at 540 nm wavelength using a microplate spectrophotometer.

2.5. Measurement of Inflammatory Cytokines

The cells were exposed to the same treatment conditions used for NO production. After incubation, the supernatant was harvested, and the concentrations of PGE_2, IL-6, IL-1β, and TNF-α were quantified using ELISA kits following the manufacturer's instructions. The optical density was measured with a microplate reader.

2.6. Western Blotting Assay

Protein analysis using the Western blotting assay was performed with slight modifications based on the previously published studies [15,16]. RAW 264.7 cells were plated in 6-well plates at a density of 6×10^5 cells per well and incubated for 24 h. The cells were then treated with umckalin (75, 150, and 300 µM) and LPS (1 µg/mL) for the specified time periods. After treatment, the cells were lysed using RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with 1% protease/phosphatase inhibitor cocktail. The lysates were centrifuged at 14,000 rpm for 30 min at 4 °C, and the protein concentrations

were determined using the BCA protein assay kit. The samples were mixed with 4× sample buffer and heated at 100 °C for 5 min. Proteins were separated using 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA for 1 h and washed four times with TBS-T (TBS containing 0.1% Tween 20) for 20 min. Primary antibodies (1:1000 dilution) were applied to the membranes and incubated overnight at 4 °C, followed by another TBS-T wash. Secondary antibodies (1:1000 dilution) were added and incubated at room temperature for 1.5 h. Protein bands were detected using an ECL kit for visualization.

2.7. Statistical Analysis

All data are presented as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way ANOVA in IBM SPSS (v. 20, SPSS Inc., Armonk, NY, USA), with a p-value of <0.05 indicating statistical significance.

3. Results and Discussion

3.1. Effects of Umckalin on the Viability in RAW 264.7 Cells

In inflammation research, RAW 264.7 macrophages are widely utilized as a representative in vitro model system. Derived from mouse macrophages, these cells respond to external stimuli, such as lipopolysaccharide (LPS), by producing pro-inflammatory cytokines (e.g., TNF-α, IL-6) and inflammatory mediators (e.g., NO, PGE_2). These characteristics make RAW 264.7 cells an invaluable tool for elucidating the molecular mechanisms of inflammation and evaluating the efficacy of anti-inflammatory candidates. Additionally, RAW 264.7 cells retain the primary functions of macrophages, including phagocytosis and the production of inflammatory mediators [17–19]. These properties, combined with their ease of culture, high reproducibility, and sensitivity to experimental conditions, render them an ideal model for immune and inflammation-related studies. Based on these advantages, the current study employed RAW 264.7 cells to investigate the anti-inflammatory effects of umckalin.

A critical preliminary step in assessing the anti-inflammatory potential of any candidate compound is evaluating its cytotoxicity. This step ensures the compound's safety, efficacy, and validity in subsequent experiments. Cytotoxic compounds can damage the cell membrane or trigger abnormal release of inflammatory mediators, thereby distorting the assessment of anti-inflammatory activity. Furthermore, changes in inflammatory mediator levels due to cell damage or death may obscure whether the observed effects are caused by cytotoxicity or the actual anti-inflammatory properties of the compound [18,19].

To address these concerns, cytotoxicity assays are essential to determine the maximum viable concentration of a compound for macrophages and to establish appropriate experimental concentrations. In this study, the cytotoxic effects of umckalin on RAW 264.7 cells were assessed using the MTT assay, a standard method for evaluating cell viability. The MTT assay measures the ability of living cells' mitochondria to reduce MTT, a yellow water-soluble compound, into insoluble purple formazan crystals, providing a reliable indicator of cell viability [20–24]. RAW 264.7 cells were treated with varying concentrations of umckalin (72.5, 125, 300, and 500 μM) in the presence of LPS (1 μg/mL) for 24 h. The results demonstrated that umckalin did not exhibit significant cytotoxicity at concentrations up to 300 μM (Figure 2a). Based on these findings, subsequent experiments were conducted using umckalin at concentrations of 72.5, 125, and 300 μM.

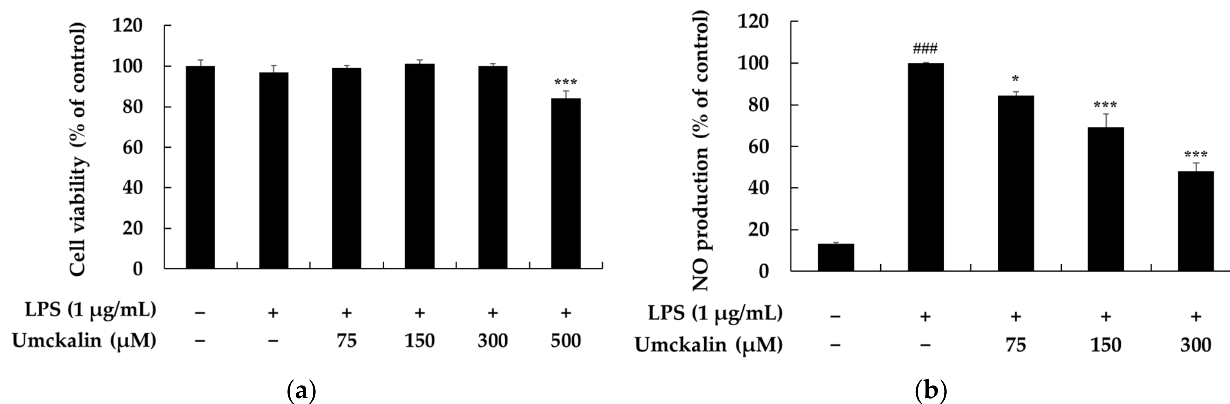

Figure 2. Effects of umckalin on cell viability (**a**) and NO production (**b**) in LPS-induced RAW 264.7 cells. For cell viability, the cells were treated with umckalin at concentrations of 75, 150, 300, and 500 μM along with LPS (1 μg/mL) for 24 h. Viability was assessed using the MTT assay and expressed as percentages relative to untreated control cells. For NO production, the cells were treated with umckalin at concentrations of 75, 150, and 300 μM along with LPS (1 μg/mL) for 24 h. NO levels were measured using the Griess assay. Both cell viability and NO production data were presented as mean ± standard deviation (SD) from three independent experiments (n = 3). ### $p < 0.001$ compared to the untreated control group and * $p < 0.05$, *** $p < 0.001$ compared to the α-MSH-treated group.

NO plays a pivotal role as a mediator in the inflammatory process, where excessive NO production exacerbates inflammation. Therefore, the ability to inhibit NO production is widely utilized as a strategy to evaluate the anti-inflammatory potential of compounds. In this context, NO levels in the supernatant of LPS-stimulated RAW 264.7 macrophages are commonly measured using the Griess assay. The Griess assay quantifies nitrite (NO_2^-), a stable metabolite of NO, through a colorimetric method that is simple, sensitive, and widely applied to detect NO in biological samples. In this method, nitrite reacts with sulfanilamide and N-(1-naphthyl)ethylenediamine to form a pink azo compound, whose intensity is measured spectrophotometrically at a 540 nm wavelength [25,26]. As shown in Figure 2b, LPS-treated RAW 264.7 cells exhibited a significant increase in NO production compared to the untreated control group. However, treatment with umckalin dose-dependently suppressed LPS-induced NO synthesis. These findings support the potential of umckalin to exert anti-inflammatory effects by inhibiting NO production.

3.2. Effects of Umckalin on Inflammatory Mediators and Cytokines in RAW 264.7 Cells

Next, to further evaluate the anti-inflammatory effects of umckalin, its impact on the production of PGE_2 and pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) was analyzed. PGE_2, a key inflammatory mediator produced via the COX-2 pathway of arachidonic acid metabolism, plays a crucial role in amplifying inflammatory responses. PGE_2 promotes vascular dilation, edema formation, and pain signaling, while enhancing the activation of immune cells and stimulating the secretion of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. As such, the inhibition of PGE_2 production serves as an important indicator of a compound's anti-inflammatory efficacy [27,28]. Pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, further amplify inflammatory responses. IL-6 regulates acute inflammatory responses and promotes immune cell differentiation. IL-1β enhances inflammatory signaling, induces tissue damage, and modulates febrile responses, while TNF-α acts as an early signal in inflammation, stimulating the secretion of other cytokines. Thus, suppressing the expression or secretion of these cytokines is a key criterion for evaluating the potential of anti-inflammatory agents [29,30]. The results demonstrated that umckalin dose-dependently and significantly reduced the production of PGE_2, IL-6, IL-1β, and TNF-α (Figure 3). These findings confirm that umckalin effectively inhibits the

production of key inflammatory mediators and cytokines, suggesting its potential to exert strong anti-inflammatory effects. In conclusion, this study highlights the ability of umckalin to suppress the production of inflammatory mediators, including NO and PGE_2, as well as pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. These results indicate that umckalin has significant potential as a natural anti-inflammatory agent for the treatment of inflammatory diseases.

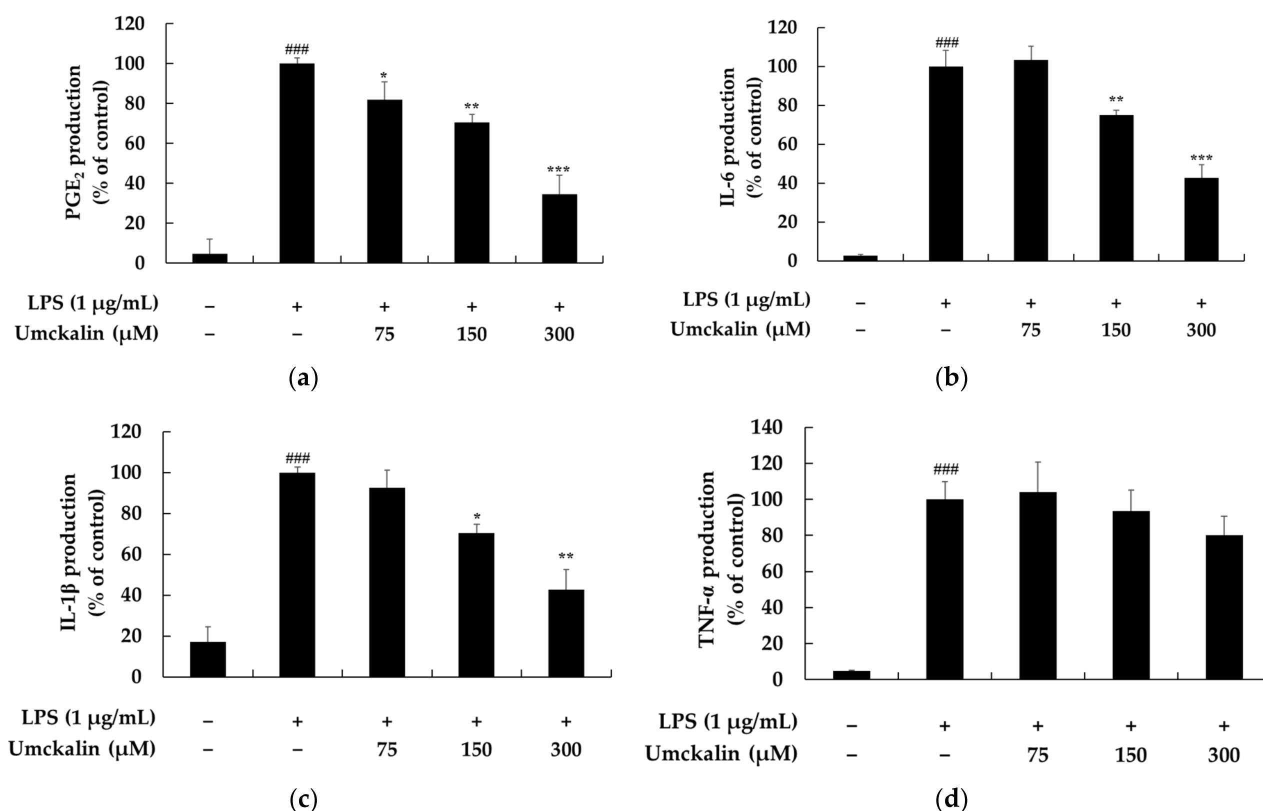

Figure 3. Effects of umckalin on PGE_2 and pro-inflammatory cytokine production in LPS-induced RAW 264.7 Cells. RAW 264.7 cells were treated with umckalin (75, 150, and 300 μM) and LPS (1 μg/mL) for 24 h. The production of PGE_2 (**a**), IL-6 (**b**), IL-1β (**c**), and TNF-α (**d**) was quantified using ELISA. Data are expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical significance is indicated as ### $p < 0.001$ vs. no-treatment control group and * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ vs. LPS-induced control group.

In the inflammatory response, iNOS and COX-2 play key roles in regulating the production of NO and PGE_2, respectively. iNOS is induced by inflammatory stimuli, such as LPS and cytokines, to generate NO, while COX-2 metabolizes arachidonic acid to produce prostaglandins, including PGE_2. These enzymes contribute to the amplification of the inflammatory response by promoting the excessive production of NO and PGE_2, which are major drivers of chronic inflammatory diseases. To investigate the anti-inflammatory mechanism of umckalin, the expression levels of iNOS and COX-2 proteins were evaluated using Western blot analysis in RAW 264.7 macrophages. As shown in Figure 4, umckalin significantly reduced the expression of iNOS and COX-2 in a dose-dependent manner at concentrations of 72.5, 150, and 300 μM in LPS-stimulated cells. These findings demonstrate that umckalin effectively inhibits the production of NO and PGE_2 by suppressing iNOS and COX-2 expression.

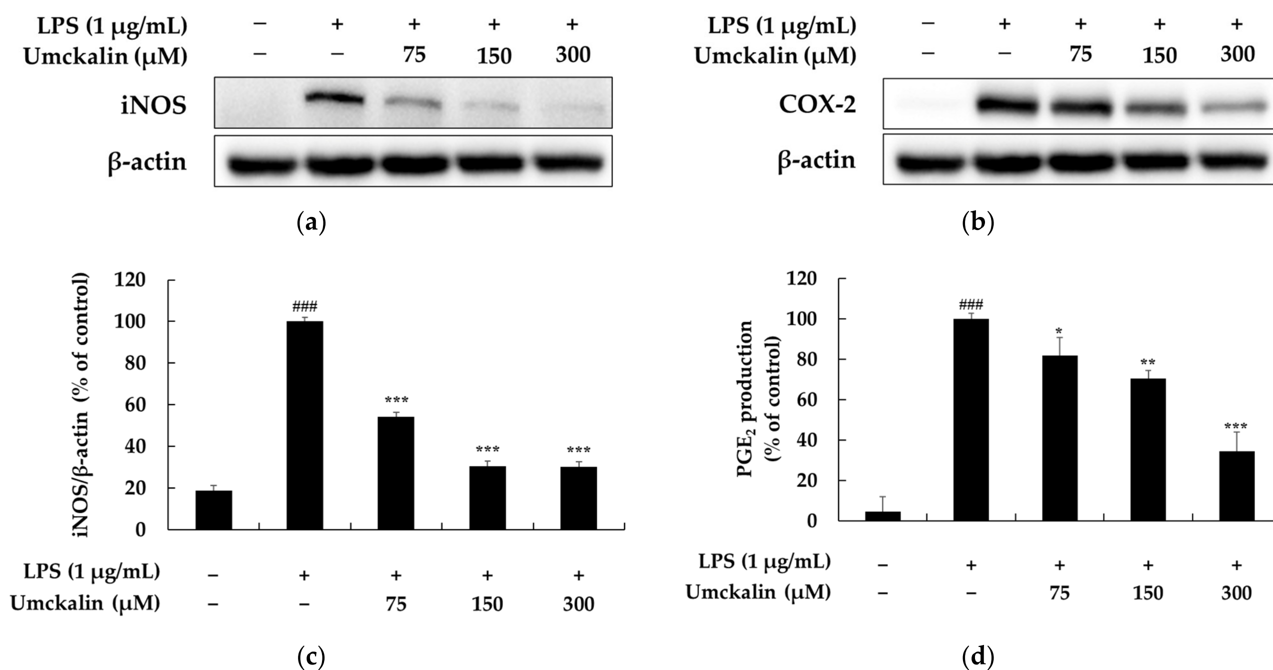

Figure 4. Effects of umckalin on iNOS and COX-2 protein expression in LPS-induced RAW 264.7 cells. RAW 264.7 cells were treated with umckalin (75, 150, and 300 μM) and LPS (1 μg/mL) for 24 h. iNOS (**a**) and COX-2 (**b**) protein expression levels were evaluated using Western blot analysis, while (**c**,**d**) represent the corresponding densitogram results. Data were collected from three independent experiments and analyzed using ImageJ software version 1.54 K (NIH, Bethesda, MD, USA). The original Western blot data are provided in Supplementary Figure S1. Results are expressed as mean ± standard deviation (SD). Statistical significance is indicated as ### $p < 0.001$ (vs. untreated control group), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (vs. LPS-treated control group).

3.3. Effects of Umckalin on the Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

The MAPK signaling pathway is a critical intracellular signaling system that plays a central role in the inflammatory response and is closely associated with the development of anti-inflammatory agents. MAPK pathways respond to external stimuli, such as LPS and cytokines, or stress signals, transmitting signals intracellularly to regulate the expression of inflammatory mediators and cytokines. The MAPK pathway comprises three main components: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, each of which is involved in distinct cellular functions, including cell survival, amplification of inflammatory responses, and immune regulation [31,32]. This pathway mediates the activation of NF-κB, a key transcription factor in the inflammatory response, thereby inducing the expression of iNOS and COX-2, promoting the production of NO and PGE_2, and facilitating the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Overactivation of the MAPK pathway contributes to the progression of inflammatory diseases, making it a key therapeutic target for mitigating inflammation [33,34]. Compounds that inhibit the MAPK pathway suppress the production of inflammatory mediators and cytokines, exhibiting significant anti-inflammatory effects. Natural anti-inflammatory substances, in particular, have been shown to inhibit MAPK pathways, providing a safe and effective approach for treating inflammatory diseases. For instance, tiliroside and 4-methoxyhonokiol have been reported to suppress p38 MAPK and JNK pathways, reducing the expression of COX-2 and iNOS [35,36], while dihydrofisetin inhibits ERK1/2 and p38 MAPK, effectively decreasing NO production and cytokine expression [37]. In conclusion, the MAPK signaling pathway is pivotal in regulating the production of inflammatory mediators and cytokines. Compounds that target this pathway represent a core strategy in the development of anti-inflammatory agents. Research on MAPK-targeted therapeutics provides a

promising approach for managing chronic inflammation and inflammatory diseases, serving as a foundational basis for developing natural product-based anti-inflammatory treatments.

To examine the connection between umckalin and the MAPK pathway, we measured the phosphorylation levels of ERK, JNK, and p38 in LPS-stimulated macrophages. As illustrated in Figure 5, LPS treatment markedly increased the phosphorylation of ERK, JNK, and p38 in the vehicle + LPS group compared to the untreated control group. In contrast, umckalin treatment significantly reduced the phosphorylation levels of these MAPK proteins in LPS-stimulated RAW 264.7 cells relative to the vehicle + LPS group. Notably, umckalin treatment at concentrations of 75 μM and 150 μM markedly reduced JNK phosphorylation relative to the LPS-treated group. These findings collectively suggest that umckalin effectively suppresses LPS-induced inflammatory responses by regulating iNOS-mediated COX-2 induction, pro-inflammatory cytokine transcription, and the MAPK signaling pathway.

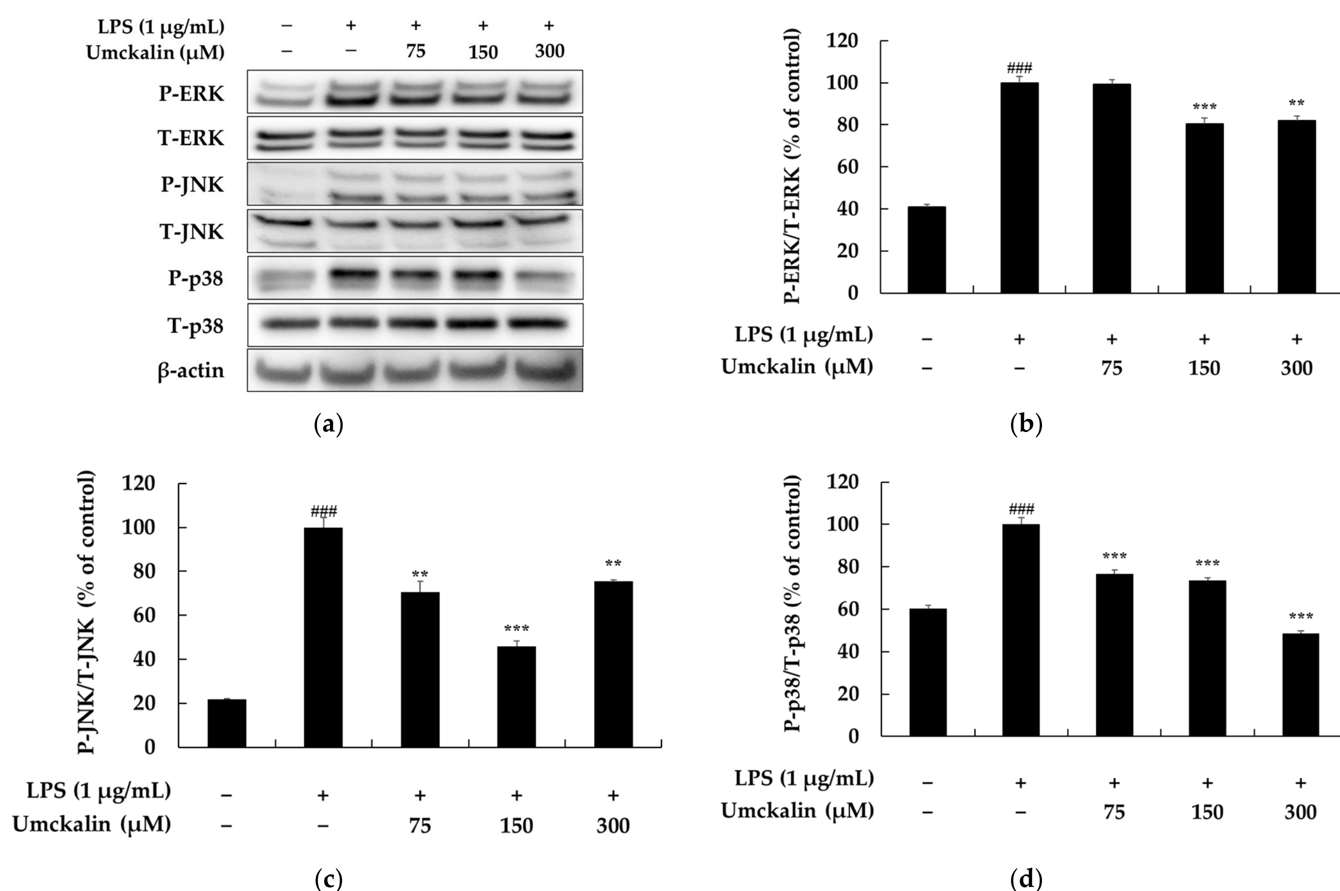

Figure 5. Effects of umckalin on MAPK protein expression in LPS-induced RAW 264.7 cells. RAW 264.7 cells were treated with umckalin (75, 150, and 300 μM) and LPS (1 μg/mL) for 30 min. Western blot analysis was performed to assess protein expression levels of P-ERK (**b**), P-JNK (**c**), and P-p38 (**d**), with representative blot images shown in (**a**). Data were obtained from three independent experiments and analyzed using ImageJ software. The original Western blot data are provided in Supplementary Figure S2. Results are expressed as mean ± standard deviation (SD). Statistical significance is indicated as ### $p < 0.001$ vs. no-treatment control group and ** $p < 0.01$ or *** $p < 0.001$ vs. LPS-induced control group.

4. Conclusions

This study demonstrated that umckalin, a coumarin derivative, exhibits significant anti-inflammatory effects by modulating key molecular pathways and holds potential as a therapeutic agent for chronic inflammatory diseases. Using LPS-stimulated RAW

264.7 macrophages, umckalin effectively suppressed the production of inflammatory mediators such as NO and PGE_2, as well as pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β. These effects were achieved through the inhibition of iNOS and COX-2 expression and the downregulation of MAPK signaling pathways, specifically the phosphorylation of ERK, JNK, and p38. Umckalin was shown to exert anti-inflammatory activity by targeting multiple inflammatory signaling pathways, reducing the transcription of inflammatory genes, and decreasing the release of mediators. Notably, the dose-dependent inhibition of iNOS and COX-2 protein expression and MAPK phosphorylation demonstrates umckalin's ability to effectively regulate inflammation at both upstream and downstream levels. Collectively, this study supports the therapeutic potential of umckalin as a natural anti-inflammatory agent for the treatment of chronic inflammatory diseases and offers foundational insights for the development of new anti-inflammatory therapies. However, to further strengthen these findings, additional studies are necessary to address the current limitations. Specifically, investigations into umckalin's molecular targets and inhibitory mechanisms are essential to clarify its mode of action. Moreover, preclinical experiments using animal models are crucial to comprehensively evaluate its safety and efficacy. These future studies will not only reinforce the clinical relevance of umckalin but also provide a robust scientific basis for the development of novel anti-inflammatory therapeutics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/futurepharmacol5010006/s1, Figure S1: Western blot data of iNOS and COX-2 protein expression in LPS-induced RAW 264.7 cells, Figure S2: Western blot data of MAPK protein expression in LPS-induced RAW 264.7 cells.

Author Contributions: Conceptualization, C.-G.H.; methodology, S.-Y.O.; software, S.-Y.O.; formal analysis, S.-Y.O.; investigation, S.-Y.O.; data curation, C.-G.H.; writing—original draft preparation, C.-G.H.; writing—review and editing, C.-G.H.; supervision, C.-G.H.; project administration, C.-G.H.; funding acquisition, C.-G.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

COX-2: cyclooxygenase-2, ERK: extracellular signal-regulated kinases, IL-1β: interleukin-1 beta, IL-6: interleukin-6, iNOS, nitric oxide synthase, JNK: c-Jun NH2-terminal kinase, LPS: lipopolysaccharide, MAPK: mitogen-activated protein kinase, PGE_2: prostaglandin E_2, p-ERK: phosphorylated ERK, p-JNK: phosphorylated JNK, p-38: phospho-p38 MAPK, TNF-α: tumor necrosis factor-alpha.

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