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Alpha-Mangostin Suppresses LPS-Induced Inflammation in Human Dental Pulp Cells

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Abstract: Xanthones are secondary metabolites isolated from the peel of mangosteen showing medicinal potencies. Alpha-mangostin (α-MG) is the most plentiful xanthone, which has been reported to possess anti-inflammatory, anti-oxidant, and anti-bacterial activities. We aimed to investigate the anti-inflammatory effects of xanthones on LPS-treated hDPCs. Cell viability was determined using the WST-1 assay. The mRNA and protein expression profiles of inflammatory mediators were evaluated using quantitative real-time polymerase chain reaction (qPCR) and Western blot analysis. Anti-inflammatory effects were assessed using the Western blot analysis to examine underlying mechanisms. A one-way analysis of variance followed by Tukey's post hoc test was used to determine statistically significant differences ($p < 0.05$). The study found no significant differences between the cytotoxic effects in the α-MG-treated groups and controls. The mRNA and protein expression levels of inflammatory markers in the α-MG treated groups decreased. α-MG significantly inhibited LPS-induced phosphorylation of proteins associated with the MAPK and NF-κB pathways. This study suggests that α-MG exerts anti-inflammatory effects by suppressing the MAPK and NF-κB signaling pathways in LPS-treated hDPCs.

Keywords: α-mangostin; dental pulp; inflammation; lipopolysaccharide; xanthones

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1. Introduction

External stimuli, such as microorganisms and chemicals, can trigger inflammation in the human body [1]. Inflammatory responses activate host defense mechanisms, followed by the synthesis of local inflammatory mediators or reactive oxygen species and an increase in vascular permeability and tissue destruction via leukocyte migration [2]. The secretion of the inflammatory mediators, such as tumor necrosis factor (TNF)-α, nitric oxide (NO), interleukin (IL)-1β, IL-6, and IL-10, is the primary response to inflammation [3].

Natural extracts, especially those isolated from tropical plants, have contributed to developing therapeutic drugs against various human diseases [4]. *Garcinia mangostana* L. (mangosteen) has been used in therapeutics for several hundred years worldwide, especially in Southeast Asia [5]. Many studies have shown that mangosteen extracts contain secondary metabolites, such as xanthones. Xanthones, the major bioactive secondary metabolites, possess anti-inflammatory, anti-oxidant, cytotoxic, anti-histamine, and anti-microbial activities [3,5,6].

Alpha-mangostin (α-MG), the dominant xanthone found in the peel of mangosteen, has been reported to have anti-inflammatory effects due to its ability to decrease the

expression of pro-inflammatory mediators. A previous study demonstrated that α-MG prevented lipopolysaccharide (LPS)-induced inflammation by inhibiting nuclear factor kappa B (NF-κB) signaling in RAW 264.7 macrophages in vitro and inhibited carrageenan-induced peritonitis in vivo [7]. Studies also showed that α-MG exerted inhibitory effects on tumor cells by downregulating the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways [8–10]. However, the effect of α-MG on LPS-induced human dental pulp cells (hDPCs) has not been studied.

Several mechanisms have been proposed to explain the anti-inflammatory effects of α-MG: (1) anti-oxidant and radical scavenging activity; (2) modulation of inflammation-related cellular activities; (3) modulation of the activities of pro-inflammatory enzymes; (4) modulation of the production of other pro-inflammatory molecules; and (5) modulation of pro-inflammatory gene expression [11].

This study evaluated the potential of α-MG to control the levels of inflammatory mediators, such as IL-1β, IL-6intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in LPS-treated hDPCs and aimed to investigate the underlying anti-inflammatory mechanisms.

2. Materials and Methods

2.1. Reagents

α-MG (purity ≥ 98%) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). α-MG was dissolved in DMSO to prepare a 1 mg/mL stock solution and stored at −20 °C. All subsequent dilutions were performed using the cell culture medium.

2.2. Cell Culture

The experimental protocol of this study was approved by the Institutional Review Board of Chonnam National University Dental Hospital (CNUDH-2016-0016). HDPCs were isolated from freshly extracted caries-free supernumerary teeth (n = 10, 6–12 years old). All procedures were performed with the informed consent of each patient. The dental pulp tissues were immediately separated from the teeth under aseptic conditions after tooth extraction and washing with Dulbecco's phosphate-buffered saline solution (DPBS, Welgene, Daegu, Korea). The pulp tissues were minced and placed in cell culture dishes. We cultured the tissue explants in alpha minimum essential medium (α-MEM (1X), Gibco Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin, Gibco Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. We used the cells between passage numbers three to six.

2.3. Cytotoxicity Assay

Cells were cultured in 96-well plates at a density of 1×10^4 cells per well and treated with various concentrations (1.5, 3, and 6 μg/mL) of α-MG for 24 h. Cell viability was analyzed using the WST-1 assay (EZ-Cytox assay kit, Daeil Lab Service, Seoul, Republic of Korea) according to the manufacturer's recommendations. Briefly, 10 μL Ez-Cytox reagent was added to the medium. After cells were incubated at 37 °C for 3 h, absorbance was measured at 450 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, USA). The relative cell viability was calculated as a percentage of the mean of the control.

2.4. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Cells were cultured in 6-well plates at a density of 2×10^5 cells per well and pre-incubated in α-MEM containing 10% FBS and antibiotics for 24 h. The LPS from *Escherichia coli* was used to induce the inflammation. Cells were pretreated with 1.5 and 3 μg/mL of α-MG for 1 h before stimulation with 1 μg/mL LPS for 24 h. Total RNA from each group was extracted using the TRIzol reagent (Gibco Invitrogen). Complementary DNA (cDNA) was synthesized using an AccessQuickTM RT-PCR system (Promega, Madison, WI, USA)

and qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) in triplicates, and Rotor-Gene 6000 (Corbett Research, Sydney, Australia). All quantified values were normalized to the endogenous control β-actin. Relative gene expression values were analyzed using the ΔΔCt method (as described previously) [12]. The primer sequences are detailed in Table 1.

Table 1. Primer sequences used for real-time PCR.

	Primer Sequences (5′-3′)
IL-1β	Forward: AGCAGCACATCAACAAGAGC Reverse: GGTGTGCCGTCTTTCATTAC
IL-6	Forward: GAGACTTCCATCCAGTTGCC Reverse: TACTCCAGAAGACCAGAGG
ICAM-1	Forward: CTCCAATGTGCCAGGCTTG Reverse: CAGTGGGAAAGTGCCATCCT
VCAM-1	Forward: TTCCCTAGAGATCCAGAAATCGAG Reverse: CCTGCAGCTTACAGTGACAGAGC
β-actin	Forward: TGGATGGCTACGTACATGGCTGGG Reverse: TTCTTTGCAGCTCCTTCGTTGCCG

2.5. Western Blot Analysis

Protein expression levels of inflammatory markers and activation of MAPK or NF-κB pathway were analyzed by Western blotting. hDPCs were cultured at a density of 3×10^5 cells per dish on 60-mm cell culture plates and pretreated with 1.5 and 3 μg/mL α-MG for 1 h before stimulation with 1 μg/mL LPS for 24 h. Cells were washed twice with DPBS. After cell treatment, cells were lysed in protein lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Protein concentrations were determined using the Lowry protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed using polyvinylidene difluoride membranes. After blocking with blocking solution (5% nonfat dried skimmed milk, 0.01 mol/L DPBS, and 0.1% Tween 20 (PBST) [Biosesang, Sungnam, Republic of Korea]) at room temperature for 1 h, membranes were incubated with the following primary antibodies: anti-IL-1β (Thermo Fisher Scientific, Rockford, IL, USA), anti-IL-6 (Abcam, Cambridge, MA, UK), anti-ICAM (Abcam), anti-VCAM (Thermo Fisher Scientific), anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, anti-AKT, and anti-phospho-Akt (Cell Signaling Technology) at 4 °C overnight. After washing membranes with PBST, membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, Sigma-Aldrich) at room temperature for 1 h. After washing with DPBS, a chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) was used for detection, and protein signals were visualized with a chemiluminescence imaging system (Ez-capture; Atto, Tokyo, Japan).

2.6. Alkaline Phosphatase Staining

Cells were cultured at a density of 2×10^4 cells per well on 48-well plates and treated with 1 μg/mL LPS for 1 h. Next, the cells were incubated in an induction medium, with or without 1.5 and 3 μg/mL α-MG for 7 days. Fresh medium containing LPS (with or without α-MG) was replaced every 2 days. After 7 days of exposure, cells were washed with DPBS, fixed with 70% ethanol for 30 min, and rinsed with distilled water. Fixed cells were treated with 300 μL staining reagent per well (BCIP®/NBT Liquid substrate System; Sigma-Aldrich). Samples were photographed using a scanner (Epson, Seoul, Republic of Korea). For quantitative evaluation, the stains were treated with 10% (*w*/*v*) cetylpyridinium chloride (pH = 7.0) for 30 min. Absorbance was measured at a wavelength of 562 nm using the spectrophotometer.

2.7. Statistical Analysis

Each experiment was performed at least twice and consisted of triplicate independent tests. A one-way analysis of variance was performed, and Tukey's post hoc test was used to determine any statistically significant differences. The SPSS 18.0 software program was used for analysis (SPSS, Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effects of LPS and α-MG on Cell Viability

The WST-1 assay was performed to evaluate the cytotoxic effects of various concentrations of LPS and α-MG on hDPCs (Figure 1). We observed that treatment with 1 μg/mL LPS did not affect cell viability. Additionally, no cytotoxicity was observed for 0 to 3 μg/mL α-MG. However, α-MG concentrations of 6 μg/mL or more led to significant cell damage ($p < 0.05$). Consequently, α-MG concentrations of 1.5 and 3 μg/mL were applied for all subsequent experiments.

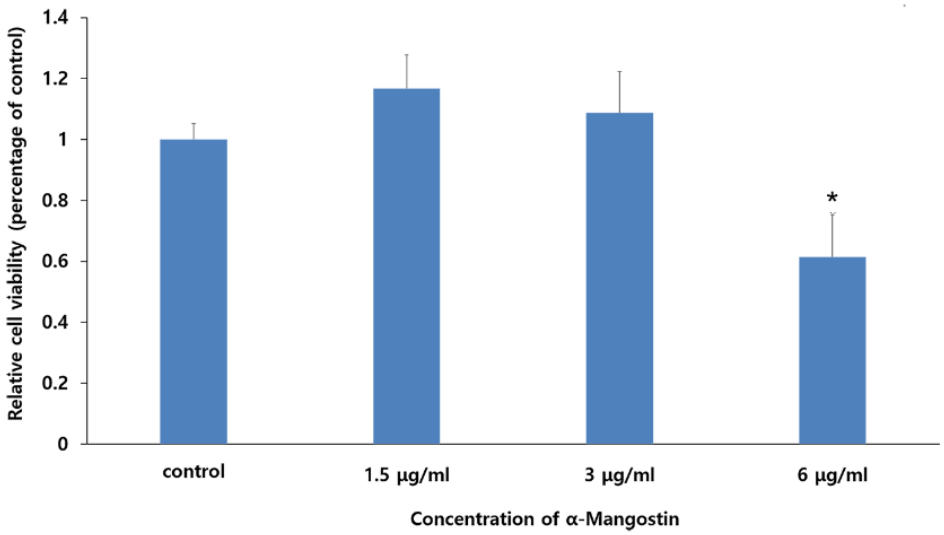


Figure 1. The effect of α-MG on the viability of hDPCs. Results are expressed as relative cell viabilities depending on the different concentrations of α-MG. Cell viability significantly decreased in the 6 μg/mL α-MG treated group (* $p < 0.05$ compared to the control group).

3.2. Effects of α-MG on LPS-Induced mRNA and Protein Expression of Inflammatory Mediators in hDPCs

The mRNA expression of inflammatory mediators was measured by qPCR to evaluate the effects of α-MG on the inflammatory response. As shown in Figure 2, the mRNA expression of all inflammatory mediators increased significantly upon stimulation with 1 μg/mL LPS. However, pretreatment with 3 μg/mL α-MG resulted in significant suppression of the increase in IL-6 and ICAM-1 ($p < 0.05$) (Figure 2B,C). α-MG pretreatment also attenuated IL-1β and VCAM-1 in a concentration-dependent manner, but there were no significant differences ($p > 0.05$) (Figure 2A,D).

Consistent with the results of mRNA expression, the protein expression levels of inflammatory mediators significantly increased by LPS; however, α-MG pretreatment attenuated this increase in a dose-dependent manner ($p < 0.05$) (Figure 3).

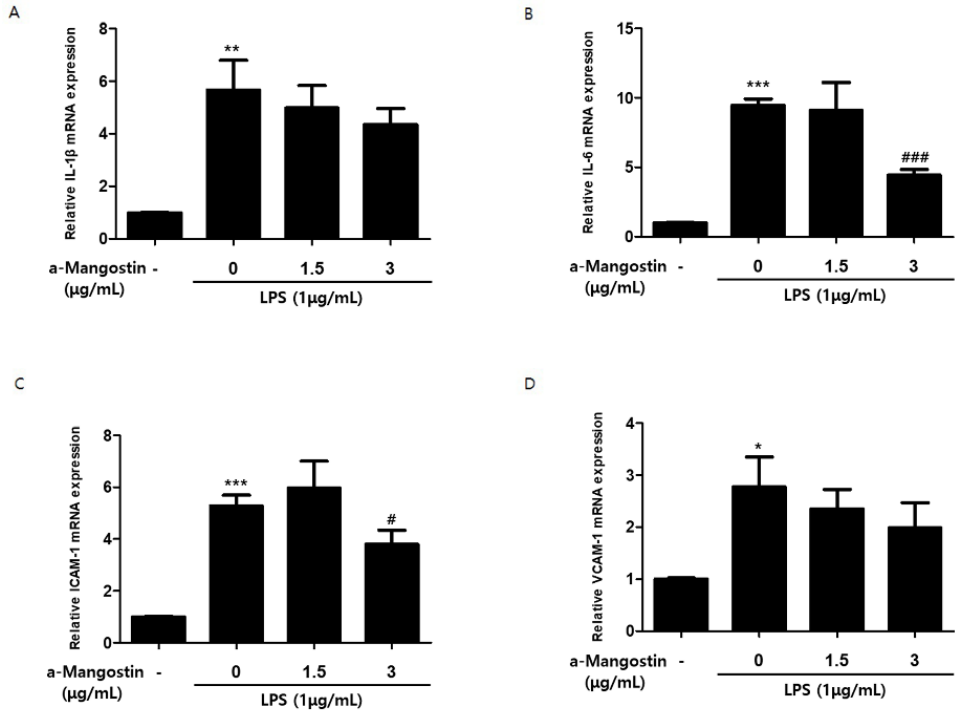


Figure 2. The effect of α-MG on the mRNA expression of LPS-induced inflammatory mediators analyzed by qPCR. Relative mRNA expression levels of (**A**) IL-1β, (**B**) IL-6, (**C**) ICAM-1, and (**D**) VCAM-1 were normalized by β-actin (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group, $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ compared to the LPS-only treated group).

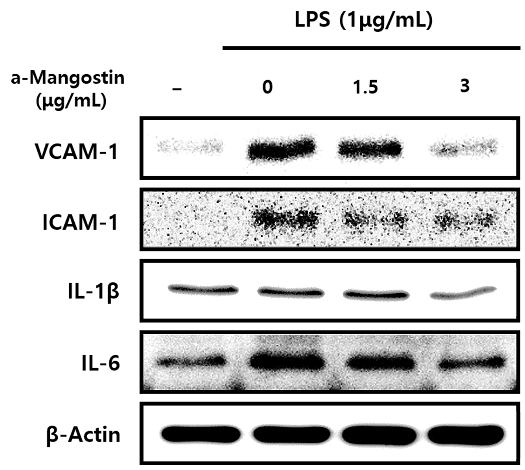


Figure 3. The effect of α-MG on protein expressions of inflammatory mediators after LPS treatment using Western blot analysis. The expression of IL-1β, IL-6, ICAM-1, and VCAM-1 proteins markedly decreased after pretreatment with 3 μg/mL α-MG.

3.3. Effects of α-MG on ALP Activity

ALP activity was examined by ALP staining (Figure 4) to ascertain if α-MG could affect the odontoblastic differentiation of hDPCs. LPS significantly downregulated ALP activity compared to the non-LPS treated control ($p < 0.05$). This result was reversed when the cells were treated with either 1.5 or 3 μg/mL α-MG. The α-MG treated cells showed higher ALP activity than the LPS-only treated group ($p < 0.05$).

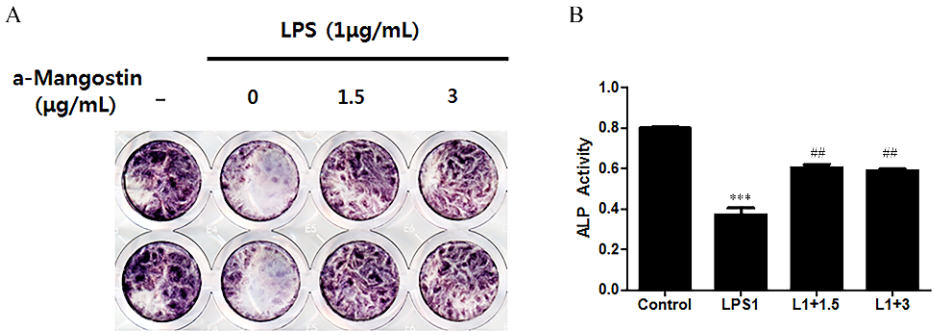


Figure 4. The effect of α-MG on the expression of ALP using ALP staining. (**A**) Image of ALP staining and (**B**) Quantification of ALP staining. α-MG increased the ALP activity at concentrations of 1.5 and 3 μg/mL compared to the LPS-only treated group (*** $p < 0.001$ compared to the control group, ## $p < 0.01$ compared to the LPS-only treated group).

3.4. Effects of α-MG on MAPK and NF-κB Signaling Pathways in hDPCs

Western blot analysis was performed, and phosphorylation of proteins related to the MAPK and NF-kB signaling pathways was examined to elucidate the molecular mechanisms by which α-MG inhibits LPS-induced inflammatory responses in hDPCs. The expression levels of phosphorylated ERK, JNK, and p38 increased in the LPS-treated group compared to untreated controls. However, treatment with 3 μg/mL α-MG inhibited the increased p-ERK, p-JNK, and p-p38 levels in LPS-treated cells (Figure 5A). Furthermore, the upregulation of phosphorylated p65, induced by LPS, was notably suppressed after treatment with 3 μg/mL α-MG (Figure 5B). These results suggest that α-MG may affect the MAPK and NF-kB signaling pathways by suppressing the phosphorylation of ERK, JNK, p38, and p65.

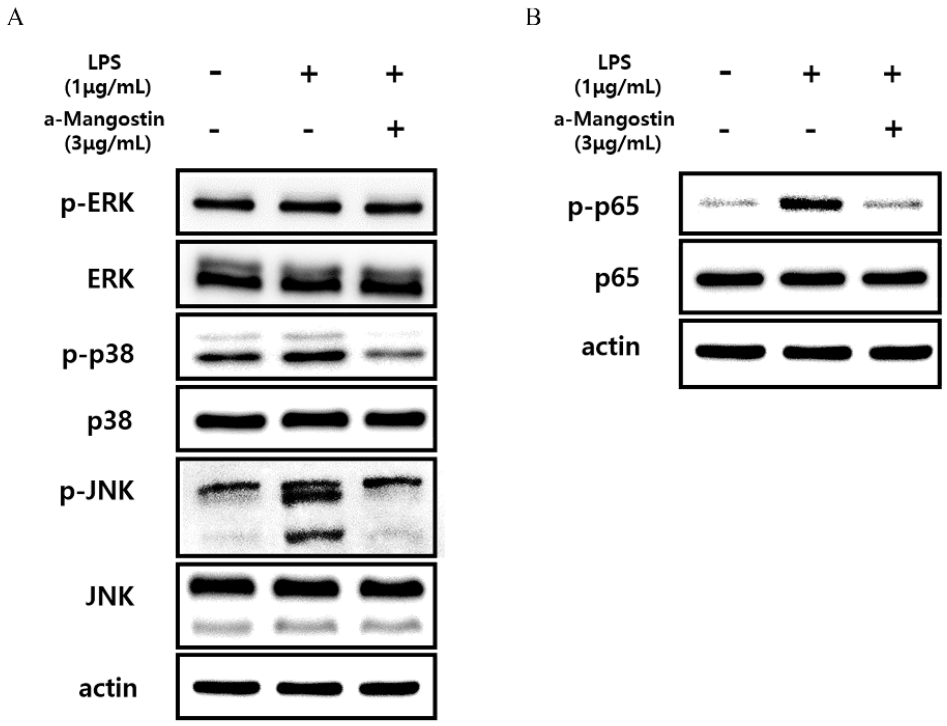


Figure 5. Western blot analysis monitoring the effect of α-MG on the activation of the MAPK and NF-kB signaling pathways in LPS-treated cells. The expression levels of (**A**) p-ERK, p-p38, p-JNK, and (**B**) p-p65 were evaluated.

4. Discussion

α-MG is known to possess diverse biological properties. Many studies have revealed the inhibitory effects of α-MG on inflammation using human cell lines, such as adipocytes [13], macrophage-like U397 cells [14,15], and macrophage-like THP-1 cells [16]. To the best of our knowledge, there are no studies investigating the inhibitory effects of α-MG on LPS-treated hDPCs. Therefore, we hypothesized that α-MG could have similar anti-inflammatory effects on hDPCs.

We also examined the cytotoxic effects of LPS and α-MG on hDPCs. The results showed that non-cytotoxic concentrations of LPS increased mRNA and protein expressions of IL-1β and IL-6. However, pretreating cells with non-cytotoxic concentrations of α-MG (especially with 3 μg/mL) caused a decrease in the upregulation of mRNA and protein expression in hDPCs stimulated with LPS.

Consistent with our results, a previous study showed that α-MG suppressed LPS-induced IL-6 gene expression in macrophage-like cells [13] and adipocytes [17]. Likewise, α-MG also blocked the activation of LPS-induced RAW 264.7 cells and inhibited the secretions of inflammatory mediators, such as NO, COX-2, IL-1β andIL-6 [18].

Meanwhile, cell adhesion molecules involved in cell–cell or cell–extracellular matrix binding have been associated with immune cell activation [19], leukocyte migration [20], and cellular mechanisms of growth or apoptosis. Of the CAMs, ICAM-1 and VCAM-1 attract mononuclear cells and neutrophils [21] to inflamed sites to induce inflammatory responses. This study also examined whether α-MG suppressed the expressions of ICAM-1 and VCAM-1 in hDPCs after LPS treatment. The results of the present study show that LPS stimulation led to an increase in the mRNA and protein expression of ICAM-1 and VCAM-1. Furthermore, pretreatment with α-MG (especially with 3 μg/mL α-MG) prevented this upregulation in ICAM-1 and VCAM-1 expression. These results indicate that α-MG could attenuate the gene expression and secretion of inflammatory mediators, such as IL-1β, IL-6, ICAM-1, and VCAM-1 in hDPCs, and the most effective and non-cytotoxic concentration was 3 μg/mL.

Previous studies reported that α-MG inhibited LPS-induced inflammatory responses via several intracellular signaling pathways, such as the MAPK [13–15] and NF-κB [22,23] pathways. This study found that treating LPS-stimulated hDPCs with 3 μg/mL α-MG inhibited the phosphorylation of MAPK-related proteins, such as ERK, JNK, and p38, implying that α-MG could exert its anti-inflammatory effects by suppressing the MAPK pathway. Moreover, there was a decrease in the phosphorylated p65 levels after α-MG treatment, indicating that α-MG could also partially influence the NF-κB pathway. Further studies are needed to clarify underlying molecular mechanisms.

The results from the present study also highlight the positive effects of α-MG on ALP activity. Increased ALP levels are relevant to the odontoblastic differentiation of hDPCs [24]. Consequently, α-MG may affect the differentiating hDPCs and possibly promote mineralization. Follow-up experiments and in vivo studies are needed to support the use of α-MG for treating inflammatory conditions in the human dental pulp.

5. Conclusions

In conclusion, α-MG attenuates the LPS-induced release of proinflammatory factors, such as IL-1β, IL-6, ICAM-1, and VCAM-1 in hDPCs by suppressing the activation of MAPK completely and NF-kB signaling pathways partially. Therefore, α-MG could be considered a therapeutic method for vital pulp therapy and regenerative endodontics.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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