Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (3): 367-375, 2025
DOI: 10.9775/kvfd.2024.33592

RESEARCH ARTICLE

Dendrobium officinale Polysaccharide Regulates the Immune Function of RAW264.7 Cells Via the NF-κB and MAPK Signaling Pathways

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How to cite this article?

Xu G, He Y, Jiang P, Yang L, Cao J, Sun C: Dendrobium officinale polysaccharide regulates the immune function of RAW264.7 cells via the NF-κB and MAPK signaling pathways. *Kafkas Univ Vet Fak Derg*, 31 (3): 367-375, 2025.
DOI: 10.9775/kvfd.2024.33592

Article ID: KVFD-2024-33592
Received: 26.12.2024
Accepted: 28.04.2025
Published Online: 16.05.2025

Abstract

To investigate the immunomodulatory effects of *Dendrobium officinale* polysaccharide (DOP) on RAW264.7 macrophages. DOP was purified from *Dendrobium officinale* using water extraction-alcohol precipitation and gel column chromatography. The molecular weight distribution of the polysaccharide was analyzed by high-performance gel permeation chromatography (HPGPC). The effects of DOP on RAW264.7 cells were evaluated as follows: cell viability was assessed via MTT assay to screen appropriate treatment concentrations; ELISA was used to detect the secretion of inflammatory cytokines (IL-10, IL-12 p40, and IL-6); RT-qPCR was employed to measure the expression of inflammatory genes *(IL-1β, IL-10, IL-12 p40*, and *IL-6);* and Western blotting was performed to analyze the expression of P65, phosphorylated P65 (p-P65), JNK, phosphorylated JNK (p-JNK), ERK, and phosphorylated ERK (p-ERK). The purified DOP exhibited a yield of 8.24% and a total sugar content of 95.33±3.11%. HPGPC analysis revealed three peaks corresponding to molecular weights of 1.89 x 10^5 Da, 549 Da, and 211 Da. DOP showed no cytotoxicity toward RAW264.7 cells at concentrations ranging from 3.125 to 400 μg/mL. All tested concentrations of DOP significantly enhanced the mRNA expression of *IL-1β, IL-10, IL-12 p40*, and *IL-6* ($P<0.05$, $P<0.01$, $P<0.001$) and promoted the secretion of IL-10, IL-12 p40, and IL-6 in a dose-dependent manner. Regarding the NF-κB and MAPK signaling pathways, DOP markedly increased the expression of p-JNK, p-ERK, and p-P65 proteins ($P<0.001$), with sustained effects observed for up to 120 minutes ($P<0.001$). DOP activates RAW264.7 cells by promoting inflammatory gene expression, stimulating cytokine secretion, and triggering the NF-κB and MAPK pathways, thereby exerting immunomodulatory effects.

Keywords: *Dendrobium officinale* polysaccharide, RAW264.7 Macrophages, Cytokines, Immunomodulation

INTRODUCTION

Dendrobium officinale, a perennial herb of the Orchidaceae family, is revered as the foremost among the "Nine Sacred Herbs" in traditional Chinese medicine and is listed in the Pharmacopoeia of the People's Republic of China (2020 edition). It is renowned for its pharmacological effects, including assisting in clearing internal heat, regulating gastric function, and enhancing digestive absorption capacity [1]. Modern pharmacological studies have identified diverse bioactive components in *Dendrobium officinale*, such as polysaccharides, alkaloids, flavonoids, phenylpropanoids, and lignans, with polysaccharides being the most abundant [2]. Research on polysaccharides in China began relatively late, starting in the 1970s, but has rapidly advanced, emerging as a focal point in modern pharmacological investigations. Recent studies have confirmed that polysaccharides exhibit multifaceted biological functions, including antibacterial, antioxidant, gut microbiota-modulating, and immunomodulatory activities [3,4], positioning them as a valuable resource for novel drug development.

Macrophages, extensively distributed across various tissues in organisms, play a dual role in both phagocytosing foreign pathogens/tumor cells and mediating the immune response via cytokine release (e.g., tumor necrosis factor and interleukins), thereby serving as primary agents of immune functionality [5]. Research demonstrated that polysaccharide components derived from traditional Chinese medicine can stimulate macrophages, enhancing secretion of cytokines such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) [6].

This study involved the extraction of crude polysaccharides from *Dendrobium officinale* via ethanol precipitation, followed by purification and separation through gel column chromatography, yielding *Dendrobium officinale* polysaccharide (DOP) with a total polysaccharide content exceeding 90%. The aim was to analyze the immunomodulatory effects of these DOP on RAW264.7 cells, providing a scientific foundation for further exploration and development of the active components within DOP.

Material and Methods

Ethical Statement

This study does not require ethical permission.

Materials and Chemicals

Dendrobium officinale was collected from the Dendrobium base of West Anhui University and identified by Professor LU Baowei of West Anhui University. RAW264.7 mouse macrophage cells were procured from the Shanghai Institute of Cell Research. The required fetal bovine serum, high-glucose DMEM culture medium, 0.25% trypsin with EDTA, and PBS phosphate-buffered saline were purchased from Shanghai Pufei Biotech Co., Ltd. Lipopolysaccharide (LPS) and MTT assay reagents were acquired from Sigma, USA. Sinopharm Chemical Reagent Co., Ltd supplied anhydrous ethanol, chloroform, and isopropanol. Mouse IL-10, IL-12p40, IL-6 ELISA kits, along with antibodies for GAPDH, NF-κB p65, Phospho-NF-κB p65, c-Jun N-terminal kinase (JNK), p-JNK, ERK, and p-ERK, were obtained from Wuhan Boster Biological Technology, Ltd. TRIzol reagent for RNA isolation was sourced from Shandong Seko Biotech Co., Ltd. For cDNA synthesis and qPCR analysis, NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge), and NovoScript® SYBR qPCR SuperMix Plus were acquired from Coastal Biotech Co., Ltd. The antibodies targeting GAPDH, NF-κB p65, Phospho-NF-κB p65, JNK, p-JNK, ERK, and p-ERK were also sourced from Wuhan Boster Biological Technology, Ltd.

Preparation of DOP

In this study, 300 g of fresh *Dendrobium officinale* stems were initially blended with 200 mL of water, then mixed with an additional 400 mL of water for heat reflux extraction (2 x 2 h). The extract was filtered through filter paper, and the filtrate was subsequently concentrated under reduced pressure to a volume of 600 mL, achieving a relative density of 1.07. During stirring, 95% ethanol was added to achieve an alcohol concentration of 80%, and the mixture was allowed to stand at 4°C for 24 h. The resultant precipitate was collected through vacuum filtration, redissolved in water, and subjected to alcohol precipitation three times by the same method to yield crude DOP. The polysaccharides were then defatted with ethanol, acetone, and petroleum ether, followed by deproteinization using the Sevage method [7]. For purification, the DOP underwent DEAE Sephadex A50 gel column chromatography, eluting in a sequence with distilled water and escalating concentrations of NaCl solutions (0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 M) at a flow rate of 0.5 mL/min. An automatic fraction collector gathered 7.5 mL samples every 15 min. The phenol-sulfuric acid assay was utilized to determine the absorbance at 490 nm of each eluate, with tube numbers plotted against absorbance values to establish an elution curve. Sampling followed an alternating tube sequence (1, 3, 5, 7, 9, 11, 13, etc.), with positive peaks merged based on the elution curve. These combined eluates were then concentrated to a smaller volume under reduced pressure at 45°C using a rotary evaporator and dialyzed in a dialysis bag with a molecular weight cutoff of 7.000 Da against tap water for 48 h, refreshing the water every 6 h, before further dialysis against distilled water for 48 h The dialysate was freeze-dried to obtain purified DOP.

Determination of Total Polysaccharide Content of *Dendrobium polysaccharides*

Using phenol-sulfuric acid method [8] to detect the total sugar content of DOP. Weigh 10.1 mg of anhydrous glucose and dissolve it in double-distilled water, adjusting the volume to 100 mL to prepare a glucose standard solution with a concentration of 0.101 mg/mL. Sequentially transfer 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of this solution into 10 mL test tubes with stoppers, and dilute each to 2.0 mL with double-distilled water. To each tube, add 1.0 mL of 6% phenol solution, followed by 5 mL of concentrated sulfuric acid. Thoroughly mix by vortexing and allow the mixture to stand for 20 minutes for color development before measuring the absorbance at 490 nm. Construct a standard curve plotting glucose concentration on the x-axis against absorbance on the y-axis. For the polysaccharide sample analysis, weigh 5 mg of the sample and dissolve it in water, adjusting the total volume to 25 mL. Transfer 1 mL of this solution into a 10 mL stoppered test tube. Measure the absorbance of this solution using the established method.

Analysis of Molecular Weight Distribution of DOP

An Agilent 1260 HPLC system equipped with a Shodex KS805 column (8.0 x 300 mm) was utilized for the analysis. The mobile phase was ultrapure water, with detection conducted via a differential refractive index detector. The settings included an injection volume of 50 µL, a column temperature of 35°C, and a flow rate of 1.0 mL/min. Glucan standards, with molecular weights of 10, 40, 70, and 500 kDa, were individually prepared in ultrapure water to a concentration of 1 mg/mL. A linear

regression analysis was conducted with the retention time on the x-axis and the logarithm of molecular weight (log MW) on the y-axis, using the glucan standards to establish a linear regression equation. The samples were prepared at a concentration of 1 mg/mL in ultrapure water and filtered through a 0.22 μm microfiltration membrane prior to injection for chromatographic analysis.

MTT Analysis of the Effect of DOP on RAW264.7 Cells Proliferation

MTT assay was used to detect the cell proliferation of RAW264.7 cells [9]. RAW264.7 cells were plated at a density of 1.5×10^5 cells per well and underwent a 24-h culture period to ensure proper adhesion. Subsequently, 100 μL of varying concentrations of DOP solution were introduced to the cells, which were then incubated at 37°C in a CO_2 incubator for 20 h. Following this, MTT solution was administered, and the cells were incubated for an additional 4 h. Post-incubation, the 96-well plate was centrifuged at 2.000 rpm for 5 min, the supernatant was removed, and 150 μL of acidic DMSO was added to each well. The plate was then subjected to shaking on a micro-vibrator for 15 min, after which the optical density (OD) at a wavelength of 490 nm was recorded.

ELISA Detection of Inflammatory Cytokine Secretion in RAW264.7 Cells

In a separate set of experiments, RAW264.7 cells were seeded at a concentration of 2×10^5 cells per well in a 24-well plate and cultured for 24 h to promote cell adhesion. Following this, the cells were treated with equal volumes of DOP solution at various concentrations and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. After incubation, the supernatants from the cell cultures were harvested, and the concentrations of IL-10, IL-12p40, and IL-6 were quantified using an ELISA, in accordance with the instructions provided in the kit's manual.

RT-qPCR Detection of Inflammatory Gene Expression

RAW264.7 cells were cultured in 24-well plates at a density of 1.5 × 10^5 cells per well and treated with varying concentrations of DOP solution. Incubation was carried out at 37°C in a 5% CO_2 incubator for durations of 1, 2, and 4 h. Subsequent to the removal of the cell culture supernatant, each well was treated with 1 mL of TRIzol for cell sample collection. The total RNA was extracted from these samples using the chloroform-isopropanol extraction technique [7]. Following RNA extraction, cDNA synthesis was performed using a reverse transcription kit. The RT-qPCR was then conducted by adding 1 μL of cDNA template, specific primers (detailed in *Table 1*), and the reaction mixture under the following amplification conditions: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with a final extension step at 95°C for 5 sec and 65°C for 5 sec. Primer specificity for each gene was validated through dissociation curve analysis and agarose gel electrophoresis. GAPDH served as the internal control. The relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, with primer sequences listed in *Table 1*.

Western Blot Analysis of the Expression of Relevant Proteins

RAW264.7 cells were cultured at a concentration of 2×10^5 cells per well in 24-well plates and incubated for 24 h prior to treatment with DOP (400 μg/mL) at five designated time points: 0, 15, 30, 60, and 120 min. Following treatment, 30 μL of lysis buffer was added to each well. The mixture was thoroughly mixed by pipetting and then transferred to 1.5 mL centrifuge tubes. The bromocresol green with albumin (BCA) assay was used to measure the total protein concentration in each sample. For protein separation, samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (with an initial voltage of 70 V for the stacking gel for 30 min and 110 V for the resolving gel for 60 min). Proteins were then transferred to Polyvinylidene fluoride (PVDF) membranes at 300 mA for 90 min. Post-transfer, membranes were blocked in blocking solution at room temperature for 2 h on a shaker, then washed with TBST. The primary antibody, diluted 1:1000, was applied and

Table 1. *Primer sequence*

Genes	Primer Sequence		Product Size (bp)
GAPDH	F	5'-ATCCTGTAGGCCAGGTGATG-3'	104
	R	5'-TATGCCCGAGGACAATAAGG-3'	
IL-1β	F	5'-TTGACAGTGATGAGAATGACCTG-3'	137
	R	5'-GCTCTTGTTGATGTGCTGCT-3'	
IL-10	F	5'-GCTCTTACTGACTGGCATGAG-3'	105
	R	5'-CGCAGCTCTAGGAGCATGTG-3'	
IL-12p40	F	5'-GGAAGCACGGCAGCAGAATAAAT-3'	180
	R	5'-AACTTGAGGGAGAAGTAGGAATGG-3'	

left to incubate overnight at 4°C. After primary antibody incubation and subsequent washes, the secondary antibody was applied and incubated with shaking at room temperature for 2 h. After this incubation period, membranes were washed and subjected to development. ImageJ software was utilized to quantify the grayscale values of the protein bands.

Statistical Analysis

All data were analyzed using a one-way analysis of variance with LSD test and expressed as the mean ± standard error of the mean (SD). SPSS software (SPSS 22.0, IBM, USA) was used and statistical significance was set at $P<0.05$.

Results

Preparation of DOP

Crude polysaccharides from *Dendrobium officinale* (CDOP) were isolated via water extraction, followed by concentration under reduced pressure, ethanol precipitation, and deproteinization, resulting in an extraction efficiency of 19.22%. The total sugar content was determined to be 67.53±4.32%, using the phenol-sulfuric acid method. Subsequent separation of CDOP employed DEAE Sephadex A50 column chromatography, utilizing varying concentrations of NaCl solution for elution. An elution curve was generated, mapping eluent tube numbers against optical density (OD) values *(Fig. 1)*. Peaks identified as positive from this curve were pooled, concentrated via reduced pressure to a minimal volume, and subjected to desalination by dialysis in a 7000 Da molecular weight cutoff bag. This process was followed by freeze-drying the dialysate to yield DOP, achieving a yield of 8.24% and a total sugar content of 95.33±3.11%.

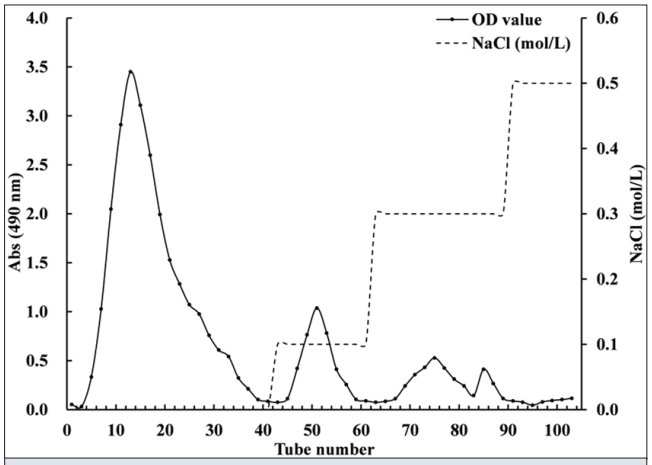

Fig 1. Elution curve of DOP DEAE-A50

The Molecular Weight Distribution of DOP was Analyzed Using HPGPC

A standard curve was constructed by plotting the logarithm of molecular weights of standard polysaccharides against their retention times. Analysis of the sample revealed three distinct peaks: the primary peak (Peak 1) with a molecular weight of 1.89×10^5 Da, and the subsequent peaks, Peak 2 and Peak 3, with molecular weights of 549 Da and 211 Da, respectively *(Fig. 2)*.

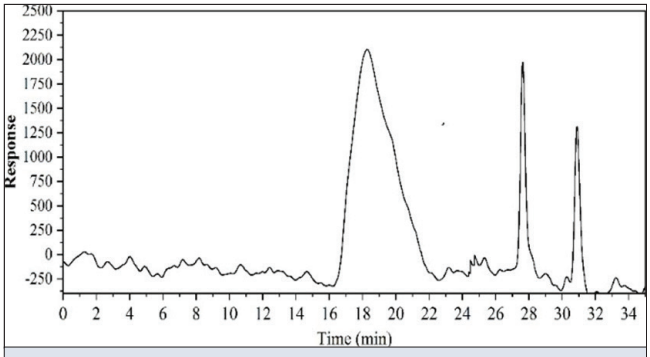

Fig 2. The HPGPC chromatogram of DOP

Impact of DOP on RAW264.7 Cells Proliferation

The *Fig. 3* demonstrates that DOP do not exhibit cytotoxicity towards RAW264.7 cells across a concentration range of 3.125 to 400 μg/mL, showing no significant deviation from the normal control group's results ($P>0.05$). Nonetheless, at a concentration of 600 μg/mL, DOP significantly reduces the proliferation of RAW264.7 cells ($P<0.05$).

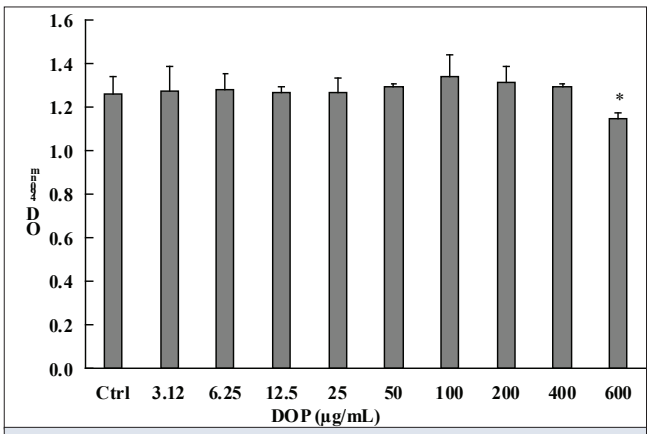

Fig 3. Effect of DOP on the proliferation of RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

Effects of DOP on the Expression of Inflammatory Cytokine Genes in RAW264.7 Cells

As shown in *Fig. 4*, the mRNA expression levels of *IL-1β*, *IL-6*, *IL-10*, and *IL-12 p40* in RAW264.7 cells were analyzed by quantitative real-time PCR after 1, 2, and 4 hours of DOP treatment. All tested concentrations of DOP significantly upregulated the mRNA expression of IL-1β, IL-10, IL-12 p40, and IL-6 in RAW264.7 cells ($P<0.01$). The stimulatory effects of DOP on these cytokines exhibited a clear dose-dependent manner, with progressively

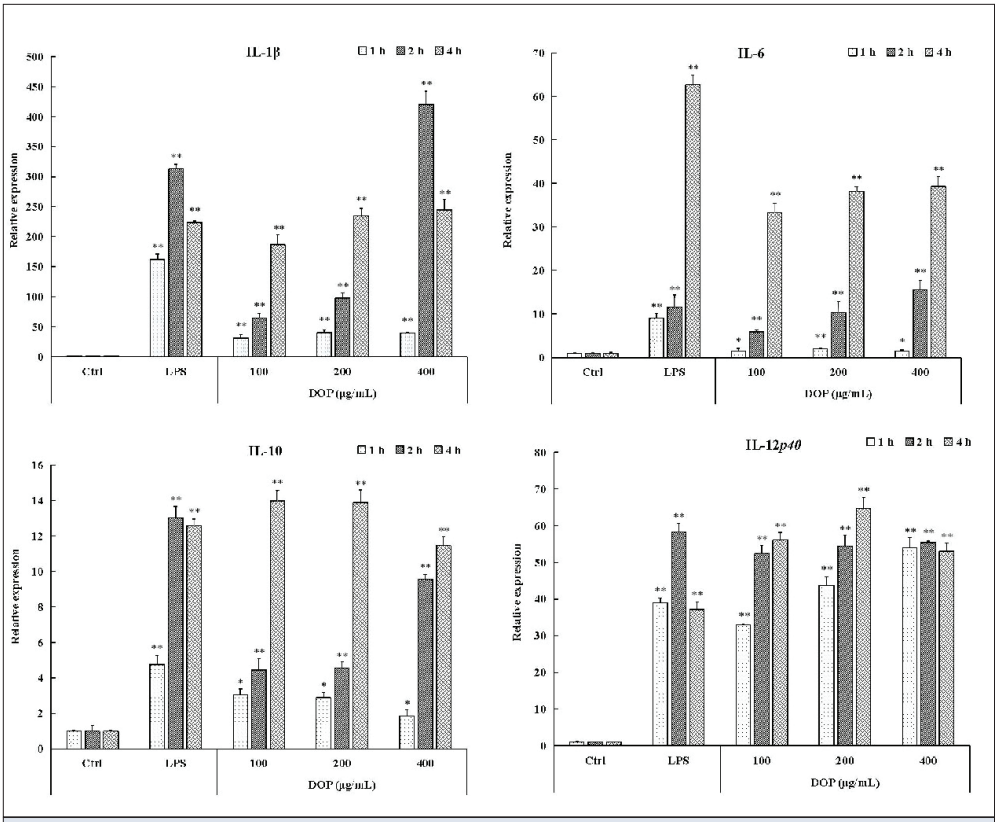

Fig 4. Effect of DOP on the expression of inflammatory cytokine mRNA in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

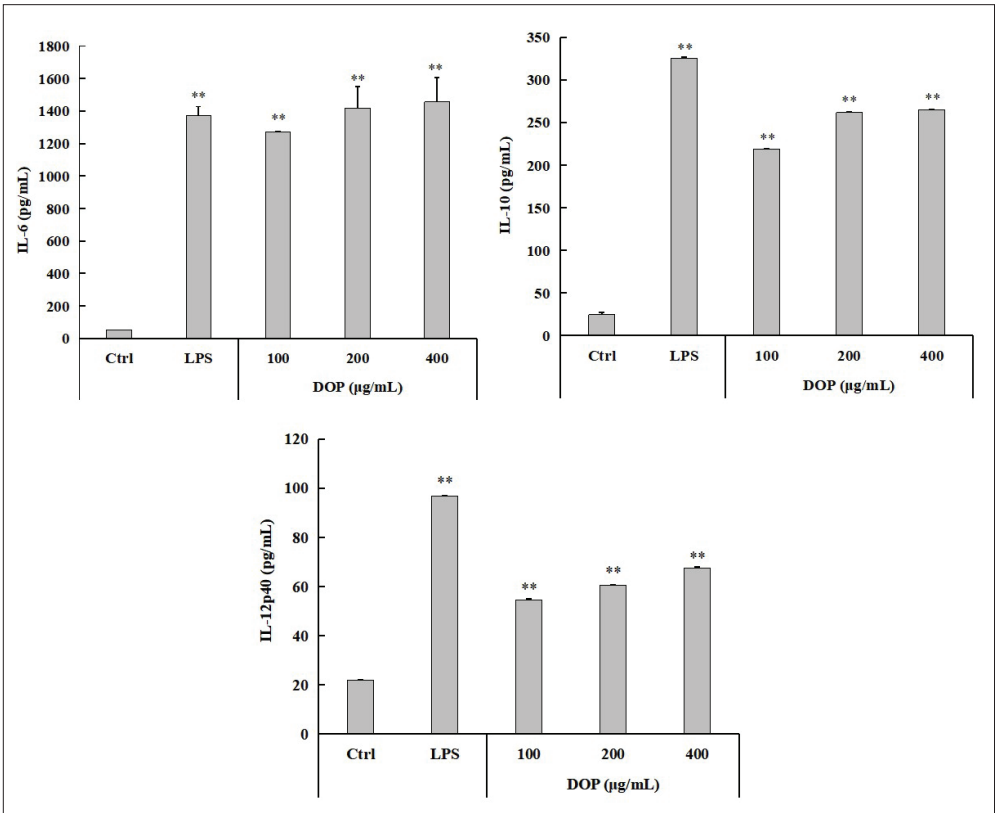

Fig 5. Effect of DOP on the expression of inflammatory cytokine mRNA in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

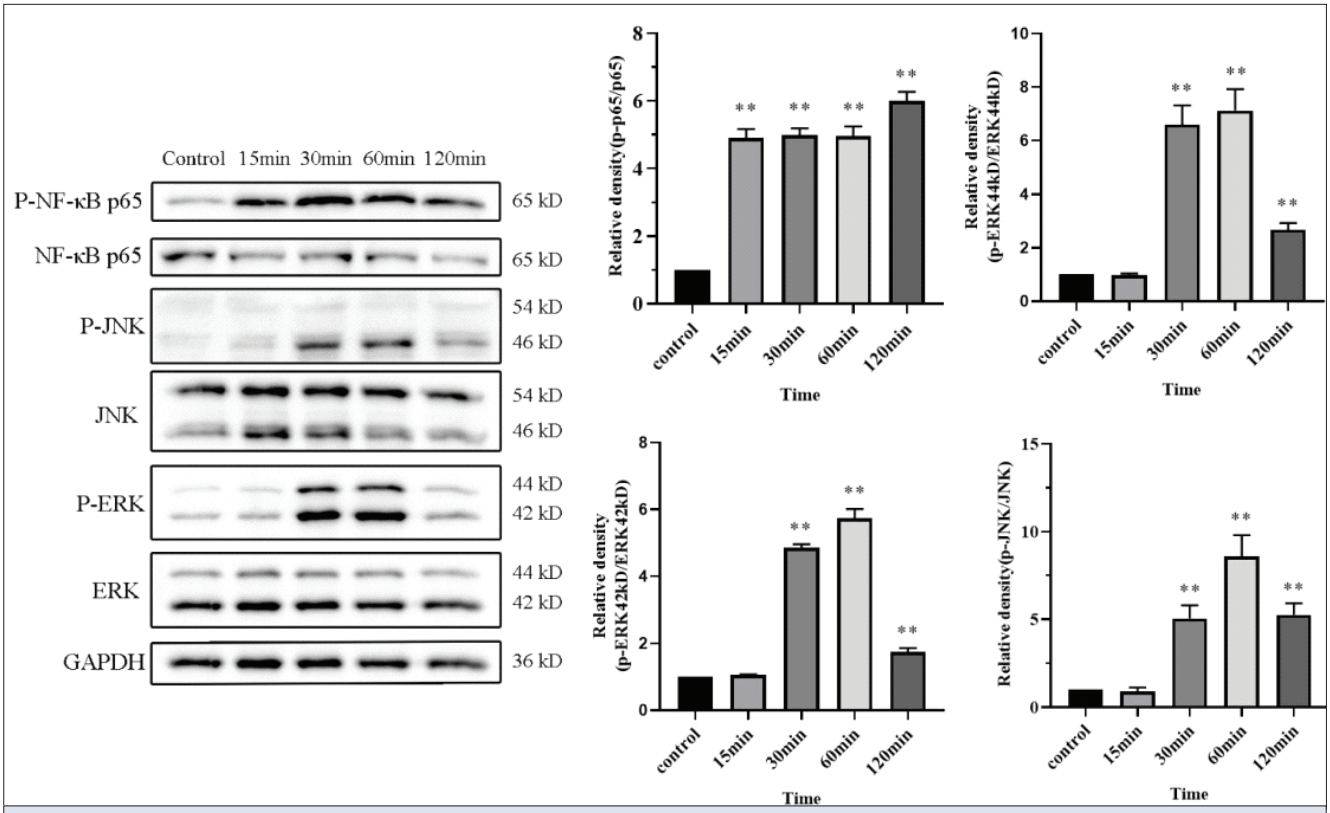

Fig 6. Effects of DOP on the expression of NF-κB/MAPK-related proteins in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

enhanced mRNA upregulation as the DOP concentration increased. Furthermore, prolonged treatment duration (up to 4 h) resulted in a time-dependent elevation of mRNA expression for IL-1β, IL-12 p40, IL-10, and IL-6, with the highest fold increase observed at the 4-h time point (P<0.01).

Influence of DOP on Inflammatory Cytokine Release in RAW264.7 Cells

As shown in *Fig. 5*, the cytokine levels in the culture supernatant of RAW264.7 cells treated with DOP for 24 h were measured using ELISA. The results demonstrated that DOP at various concentrations could significantly promote the secretion of IL-10, IL-12 p40, and IL-6 in RAW264.7 cells (P<0.01). With increasing administration concentrations, the enhancing effect of DOP on the secretion of these cytokines gradually strengthened, showing a favorable dose-dependent relationship.

Impact of DOP on the Expression of Relevant Proteins in RAW264.7 Cells

As shown in *Fig. 6*, compared with the control group, the protein expression levels of p-JNK, p-ERK, and p-p65 were significantly increased in all drug-administered groups (P<0.01). At 15 min after administration, only p65 protein expression showed a significant increase (P<0.01). After 30 and 60 min of drug treatment, the protein expression levels of p-JNK, p-ERK, and p-p65 were all significantly elevated (P<0.01). By 120 min post-administration, while the expression levels of p-JNK and p-ERK decreased compared to those at 1 h, they still remained significantly higher than the control group (P<0.01).

Discussion

Dendrobium officinale, a valued traditional Chinese medicinal herb known as "life-saving fairy grass," has shown significant biological activities in recent pharmacological research [10]. Studies suggest its extracts enhance hemolysin antibody levels and carbon clearance index [11]. However, the exact immune-regulatory mechanisms and target cells of DOP remain unclear. This study investigates DOP's effects on inflammatory factor release and gene expression in RAW264.7 macrophages to clarify its immune-regulatory actions and lay the groundwork for future research.

In this research, DOP were purified using a method that combined water extraction, ethanol precipitation, and gel column chromatography [12], yielding a polysaccharide product with a total sugar content of over 95%. Molecular weight analysis of the purified polysaccharides revealed three distinct peaks, suggesting that, although the total sugar content was high, the molecular weight distribution of the DOP was heterogeneous.

Macrophages, originating from bone marrow hematopoietic

stem cells, differentiate from monocytes in the bloodstream into macrophages in tissues. RAW264.7 cell proliferation aids in understanding immune cell differentiation and roles in immune responses [13]. Macrophages are divided into M1 (classically activated) and M2 (alternatively activated) types, with their balance being critical for immune response outcomes [14]. Investigating the activation process of macrophages and identifying compounds that effectively stimulate this process are key to enhancing immune response. Previous studies, including those by Li et al.[15] and Wei et al.[16] have demonstrated that *Saccharum* polysaccharides and *Astragalus* polysaccharides, respectively, up-regulate the mRNA expression of cytokines such as IL-1β, TNF-α, and IL-6 in RAW264.7 cells. This study's findings reveal that DOP at various concentrations notably enhance the expression of *IL-1β, IL-10, IL-12p40*, and *IL-6* mRNA in RAW264.7 cells, with a dose-dependent increase in the up-regulation of these cytokines' mRNA expression. Furthermore, ELISA results indicate that DOP significantly boosts the secretion of IL-10, IL-12 p40, and IL-6, echoing findings by Meng et al. [17] This demonstrates DOP's potential in modulating macrophage activation and contributing to the immune response.

IL-10, a biomarker for M2 macrophages, suppresses cytokine secretion, including from M1 macrophages, thus reducing inflammation [18]. It downregulates MHC-II and co-stimulatory molecule expression in monocytes/macrophages, indirectly limiting T-cell proliferation and cytokine production [19]. Additionally, IL-10 weakens macrophage antigen presentation and cytokine production, helping to control inflammatory diseases. Wongchana et al. showed that IL-10 inhibits immune complexes and suppresses disorders caused by autoimmune hyperactivity [20]. This study found that DOP markedly increased IL-10 secretion in RAW264.7 cells. IL-12, a heterodimer consisting of p35 and p40 subunits, is predominantly synthesized by macrophages, B lymphocytes, and dendritic cells. It plays a pivotal role in cellular immune responses by activating T cells and NK cells [21]. The release of IL-12 also boosts the cytotoxic actions of NK cells and cytotoxic T lymphocytes (CTLs) against target cells and fosters lymphocyte proliferation, underscoring its significance in orchestrating cellular immunity [22]. Ablimit et al. [23] demonstrated that *Momordica charantia* polysaccharide significantly enhanced the secretion and expression of IL-12 in rats. In parallel, Huang et al. discovered that *Radix Rehmanniae* polysaccharide prompted dendritic cells to secrete a spectrum of cytokines, including IL-12. Hence, it is evident that IL-12 secretion boosts the immune system's function to a certain degree [24].

NF-κB is a pleiotropic transcription factor present in almost all cell types and participates in numerous biological processes, including inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis [25]. NF-κB p65, a member of the NF-κB family, is frequently involved in inflammatory responses [26]. Our experimental results demonstrated that DOP stimulation induced phosphorylation of the p65 protein in RAW264.7 cells, thereby activating the NF-κB signaling pathway. The MAPK signaling pathway serves as a critical hub for cellular responses to external stimuli and regulates cell growth and death. It not only influences proliferation, differentiation, and survival but also plays a pivotal role in pathological processes such as cancer, neurodegenerative diseases, and inflammation [27]. In mammals, MAPK can be categorized into three major subfamilies: ERK (extracellular signal-regulated kinase), JNK (Jun N-terminal kinase), and p38/SAPKs (stress-activated protein kinases) [28]. Among these, ERK is one of the most representative pathways in the MAPK signaling cascade, primarily responding to growth factors and mitogens to induce cell growth and differentiation [29]. The JNK module is crucial for apoptosis, inflammation, cytokine production, and metabolism. In this study, both ERK and JNK protein expressions were upregulated, leading to MAPK pathway activation. Multiple studies have indicated that the MAPK signaling pathway contributes to NF-κB activation [30]. Our findings revealed that DOP significantly promoted the expression of p-JNK, p-ERK, and p-p65 proteins in RAW264.7 cells, effectively activating both the MAPK and NF-κB signaling pathways.

Dendrobium officinale polysaccharides (DOP) exhibit high affinity and binding stability with Toll-like receptor 4 (TLR4), suggesting that they may play a significant role in intestinal immune regulation through the TLR4 signaling pathway. As a key receptor in the innate immune system, TLR4 can recognize pathogen-associated molecular patterns (PAMPs) and activate downstream signaling pathways, thereby inducing the release of inflammatory cytokines and immune responses [31]. Our *in vivo* experiments further confirmed that DOP significantly modulates the expression levels of TLR4 and its downstream key proteins in the colonic mucosa of mice, which may help alleviate excessive inflammatory responses and maintain intestinal homeostasis. Additionally, based on literature reports, there is a close interaction between the intestinal microbial community and the TLR4 signaling pathway [32]. We speculate that DOP may indirectly influence TLR4 activity by regulating the microbiota-host immune axis, thereby playing a dual role in intestinal immune defense and balance.

While this study demonstrates the immunomodulatory effects of DOP through NF-κB/MAPK pathways, several methodological limitations warrant discussion. Firstly, although DEAE Sephadex A50 chromatography achieved

95.33% total sugar content (Section 2.2), the HPGPC analysis revealed three distinct molecular weight peaks (1.89×10^5 Da, 549 Da, and 211 Da) in the DOP preparation *(Fig. 2)*. This heterogeneity suggests potential interactions between different polysaccharide fractions that might confound the observed bioactivities. Secondly, batch-to-batch variations inherent to plant-derived polysaccharides were not systematically evaluated. Minor fluctuations in ethanol precipitation conditions (80% ethanol at 4°C for 24 h) and NaCl gradient elution parameters (0.1-2.0 M) during purification could potentially alter polysaccharide composition across production batches. To address these limitations, future studies should: Employ orthogonal purification strategies (e.g., sequential ultrafiltration and HILIC chromatography) to isolate homogeneous polysaccharide fractions; Establish quality control metrics beyond total sugar content, including monosaccharide composition analysis and molecular weight distribution profiling; Conduct inter-batch comparisons using at least three independently prepared DOP batches to verify reproducibility.

In summary, *Dendrobium officinale* polysaccharide (DOP) enhances the immune function of RAW264.7 cells by promoting the secretion of inflammatory cytokines and the expression of inflammatory genes, mediated through modulation of the MAPK and NF-κB signaling pathways.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author (C. Sun).

Acknowledgements: This study was supported by the Key Science Foundation of High Education of Anhui Province (NO. 2022AH051668) and the Geographical Origin and Molecular Evolution of Medicinal Plants of the Dendrobium genus in China: Supported by the High Level Talent Research Initiation Fund of West Anhui University, (NO. WGKQ2023010). We would like to express our sincere gratitude to both funding agencies for their financial support.

Funding Support: The study was supported by Key Science Foundation of High Education of Anhui Province (NO. 2022AH051668); Geographical Origin and Molecular Evolution of Medicinal Plants of the Dendrobium genus in China: Supported by the High Level Talent Research Initiation Fund of West Anhui University (NO. WGKQ2023010).

Ethical Statement: This study does not require ethical permission.

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: GX and YH contributed to the design of this study. GX, LY and JC participated in the sample collection, data analysis. CS provided funding and analytical tools. PJ wrote the original draft. All authors contributed to data collection and discussion.

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