

Article

Three Polyphenolic Compounds from *Inonotus obliquus*: Antioxidant Activity, Xanthine Oxidase Inhibition, and Regulatory Effects on MyD88/TLR4/NF- κ B Pathway in MSU-Induced RAW 264.7 Macrophages

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Abstract

Background: *Inonotus obliquus* (Chaga), a medicinal and edible macrofungus abundant in bioactive polyphenols, is a potential source of natural antioxidants and anti-inflammatory agents for functional foods. This study aimed to evaluate the antioxidant capacity of three key polyphenols (osmundacetone [OS], protocatechuic aldehyde [PAH], protocatechuic acid [PA]) from *I. obliquus* and decipher their anti-inflammatory mechanisms via the MyD88/TLR4/NF- κ B pathway in a gout-related model. **Methods:** Antioxidant activity was assessed by xanthine oxidase (XO) inhibition (IC_{50}), superoxide anion (O_2^-) scavenging, and structure–activity relationship (SAR) analysis; in a monosodium urate (MSU)-induced acute gout cell model, reactive oxygen species (ROS), nitric oxide (NO), lactate dehydrogenase (LDH), superoxide dismutase (SOD), pro-inflammatory cytokines (TNF- α , IL-1 β) were quantified, and MyD88/TLR4/NF- κ B pathway proteins were analyzed by Western blot. **Results:** OS showed the strongest XO inhibition (IC_{50} = 4.91 mM), followed by PAH (IC_{50} = 5.92 mM) and PA (IC_{50} = 26.53 mM); OS exerted dual redox effects by scavenging O_2^- and suppressing XO-mediated O_2^- generation, with its conjugated C=C-carbonyl system and PAH's aldehyde group enhancing XO binding. All polyphenols and *I. obliquus* crude extract significantly reduced ROS, NO, LDH, and cytokines ($p < 0.05$), increased SOD, and downregulated TLR4, MyD88, and NF- κ B expression. **Conclusions:** *I. obliquus*-derived polyphenols exhibit obvious antioxidant and xanthine oxidase inhibitory effects, and regulate oxidative stress, pro-inflammatory mediators, and the MyD88/TLR4/NF- κ B signaling pathway in monosodium urate-stimulated RAW 264.7 inflammatory macrophages, supporting their development as natural functional food ingredients and potential candidates for gout-related and oxidative stress-associated inflammatory cellular disorders.

Keywords: *Inonotus obliquus*; polyphenols; xanthine oxidase; antioxidant activity; inflammatory mediator regulation; natural food additive; gout



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1. Introduction

XO is an important enzyme in purine metabolism, catalyzing the generation of uric acid (UA) from xanthine in the body [1]. The structure of XO has four active sites in the form of molybdopterin (Mo-Pt), two [2Fe-2S] clusters and flavin adenine dinucleotide (FAD) [2]. XA and O_2 together act as substrates to produce uric acid, superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) in an XO-catalyzed process. It is worth noting that the Mo-Pt center and the FAD center are the sites for the generation of uric acid and the conversion of oxygen molecules, respectively. Prolonged maintenance of excessive levels of uric acid in the body can lead to hyperuricaemia, which may further develop into gout, and, on the other hand, O_2^- radicals generated by the system can also induce inflammatory reactions [3]. Acute gouty arthritis closely related to this pathologic process. Therefore, inhibiting XO is one of the key therapeutic targets for treating gout. XO inhibitors typically act through competitive or non-competitive inhibition of XO, thereby suppressing uric acid production and reducing serum uric acid levels to achieve the goal of treating hyperuricemia. Currently available XO inhibitors include allopurinol, febuxostat [4], topiroxostat, and benzbromarone. These drugs demonstrate significant clinical efficacy in inhibiting XO. Therefore, inhibition of XO activity has become an important target for the treatment of hyperuricaemia and gout in recent years [5]. Potential XO inhibitors in natural plants have received strong attention from researchers relative to the adverse side effects of synthetic drugs.

Polyphenols are not only found in common foods such as onions, tea, blueberries, etc. [6,7], but also in many medicinal and edible fungi such as *Sanghuangporus*, *I. obliquus*, *Ganoderma lucidum* [8,9]. It is one of the more common complex secondary metabolites with a variety of biological activities, for instance, anti-inflammatory, anticancer, antioxidant and anti-aging [10]. Meanwhile, there are some reports in the previous literature on the ability of polyphenols to effectively inhibit XO activity, and possess good antioxidant activity [11,12]. *I. obliquus* is a natural edible and medicinal fungus, which contains many phenolic substances such as catechols, protocatechuic aldehydes, quercetin, gallic acid, protocatechuic acid, and osmundacetone [8,13–16]. Many studies have shown that *I. obliquus* had good XO inhibitory and antioxidant ability [8,17–19] and anti-inflammatory properties [20,21], but the studies that simultaneously analyze a single polyphenol component of it in terms of both XO inhibitory activity and in vitro antioxidant activity are fewer and incomplete, and need to be further explored. At the antioxidant level, polyphenolic compounds scavenge reactive oxygen species (ROS) through their phenolic hydroxyl structures and enhance the activity of antioxidant enzymes, thereby mitigating oxidative stress damage induced by monosodium urate (MSU) crystals. Oxidative stress and inflammatory response are interconnected in gout, and blocking the former can lower the activation threshold of inflammatory signaling pathways. At the anti-inflammatory level, the inflammatory cascade of gout depends on the initiation and amplification effects of the TLR4/MyD88/NF- κ B pathway [22,23], TLR4 recognizes MSU crystals to activate NF- κ B, which drives the transcription of pro-inflammatory cytokines such as IL-1 β and TNF- α , thereby exacerbating the inflammatory response. However, the specific regulatory mechanism of polyphenols from *I. obliquus* on this core pathway remains unclear.

Acute gouty arthritis is a common inflammatory response caused by elevated blood uric acid levels and the deposition of MSU crystals in the joints and soft tissues [24]. In severe cases, it can lead to joint deformities and multi-organ damage. Manifestations include joint redness, swelling, heat, pain, neutrophil infiltration, and the release of numerous pro-inflammatory factors, such as IL-1 β [25] and TNF- α . The phagocytosis of MSU by macrophages plays a crucial role in the pathogenesis of acute gouty arthritis [26]. External stimuli can accelerate the production of ROS and exacerbate lipid oxidation [27].

Conversely, nitric oxide (NO), a key inflammatory mediator involved in the regulation of immune responses and inflammatory cascade reactions, is closely associated with inflammatory responses [28]. Lactate dehydrogenase (LDH) serves as a key biomarker for assessing cell viability and membrane integrity [29]. Furthermore, the crude extract of *I. obliquus*, which showed potential XO inhibitory activity in previous study, has not been investigated regarding the structure–activity relationship of its bioactive constituents. In addition, the mechanism of O_2^- radical scavenging during the inhibition of XO has not been reported yet. In consideration of its anti-gout and antioxidant mechanisms, we present to elucidate the anti-gout and antioxidant mechanisms of polyphenolic compounds in *I. obliquus*, especially focused on the protective effect of them on the acute gouty cell model.

In this study, high-performance liquid chromatography (HPLC) was employed to quantify three target bioactive compounds—osmundacetone (OS), protocatechuic aldehyde (PAH), and protocatechuic acid (PA)—in *Inonotus obliquus* crude extract, a matrix that preserves the macrofungus’s natural bioactive profile; to systematically dissect their antioxidant potential, we evaluated superoxide anion (O_2^-) scavenging activity via in vitro antioxidant assays and assessed xanthine oxidase (XO) inhibitory capacity (a key mechanism for oxidative stress alleviation, as XO overactivation triggers ROS burst), followed by structure–activity relationship (SAR) analysis to correlate their distinct chemical moieties with XO inhibitory and O_2^- scavenging efficacies, which clarifies underpinning antioxidant mechanisms and informs rational optimization of bioactive agents for redox regulation; additionally, to explore their role in mitigating oxidative stress-related inflammation (a core pathological link between redox imbalance and chronic disorders), MSU-induced RAW264.7 macrophage model was established (recapitulating ROS-amplified inflammatory responses typical of oxidative stress-driven pathologies) to investigate how *I. obliquus* crude extract and the three compounds modulate the MyD88/TLR4/NF- κ B pathway (a pivotal axis bridging oxidative stress and inflammatory cascades), with a specific focus on analyzing their multi-target effects including suppression of TLR4-mediated ligand recognition and attenuation of MyD88-dependent NF- κ B signal transduction; overall, this work aims to unravel the “antioxidation-anti-inflammation” synergistic axis of *I. obliquus*-derived OS and polyphenols, validate their potential as safe, multi-target natural agents for regulating redox homeostasis and associated inflammatory responses, and lay a foundation for advancing natural bioactives in the intervention of oxidative stress-related disorders.

2. Materials and Methods

2.1. Chemicals and Reagents

XO (200 U/L, X1875-25UN) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the following XO was diluted using Tris-HCl (50 mM, pH 7.5) buffer solution. *Inonotus obliquus* was purchased from Yanbian Huaxia Co., Ltd. (Dunhua, China). Methanol (LC grade) was purchased from Fisher Chemicals (Pittsburgh, PA, USA). Osmundacetone (purity $\geq 98\%$, PU0788) was purchased from Chengdu Push Bio-technology Co., Ltd. (Chengdu, China). Xanthine (S18024, B20561), protocatechuic aldehyde (B21613), protocatechuic acid (B21614), DPPH (0.3 mM, B25609), and PMS (0.15 mM, all $\geq 98\%$ purity, S30523) were all purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). NADH (1.22 mM, N8120) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). RAW264.7 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). NO content determination kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). The reactive oxygen species (ROS, S0033S) assay kit and lactate dehydrogenase (LDH, C0016) cytotoxicity detection kit were procured from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China). The superoxide dismutase (SOD, A001-3-2) activity assay kit was procured from

Nanjing Jianjian Bioengineering Research Institute Co., Ltd. (Nanjing, China). TNF- α assay kit (JM-02415M1) and IL-1 β assay kit (JM-02323M1) were purchased from Jiangsu Jingmei Biological Technology Co., Ltd. (Yancheng, China). Anti-Beta Actin antibody (ab6276), Anti-NF- κ B antibody(ab32536), and Anti-MyD88 antibody (ab2064) were purchased from Abcam. TLR4 Polyclonal antibody (19811-1-AP) was purchased from Proteintech Group Co., Ltd. (Wuhan, China).

2.2. Preparation of Crude Extracts of *I. obliquus* and Quantitative Analysis by HPLC

Inonotus obliquus was purchased from Yanbian Huaxia Co., Ltd. (Dunhua, China) and authenticated by Researcher Fanlei Meng (Institute of Agricultural Quality Standard and Testing Technology, Jilin Academy of Agricultural Sciences, Changchun 130033, China). *I. obliquus* was sourced from Siberia with a growth cycle of approximately 8 years. A total of 5.001 g of *I. obliquus* powder was weighed, submerged with 100 mL of analytical grade methanol, and extracted via an ultrasonic-assisted method using a Numerical Control Ultrasonic Cleaner (Model: KH-250DB, Kunshan Hechuang Ultrasonic Instrument Co., Ltd.) at a working frequency of 40 kHz and an output power of 400 W for 1 h. Subsequently, filtration was performed, and the filtered residue was re-extracted under the identical conditions twice more, resulting in three total extraction cycles. The filtrates from the three extractions were combined and concentrated under reduced pressure. When the solvent was nearly completely evaporated, 4 mL of chromatographic grade methanol was added to dissolve the concentrate, and the resulting solution was filtered through a 0.45 μ m filter membrane and stored at 4 °C for subsequent use.

HPLC analysis was performed on an RP-C18 column (250 $\times$ 4.6 mm, 5 μ m, Agilent, USA) with chromatography-grade methanol (A) and 0.5% aqueous acetic acid (B) as mobile phases. The column temperature was maintained at about 25 °C, the flow rate was set at 0.50 mL/min and the injection volume was 5 μ L, which was detected at 280 nm. Gradient elution conditions were as follows: 0 min-20% A, 20 min-40% A, 50 min-80% A, 58 min-100% A, and 60 min-20% A. The crude extract was diluted to half of the original concentration with methanol.

Compound standards were configured to different concentrations using methanol, and analyzed by HPLC. Concentration–peak area standard curves were plotted, allowing a quantitative analysis of single compounds in the crude extracts.

2.3. Determination of XO Inhibitory Activity of PAH, PA and OS

PAH, PA and OS were dissolved to obtain different concentrations of inhibitor solutions. Tris-HCl (50 mM, pH 7.5) solution, inhibitor solution (different concentrations) and XO solution (200 U/L) were sequentially added to the 96-well UV plate, and after the reaction was carried out for 15 min in a 37 °C water bath, 100 μ L of XA (250 μ M) was added to initiate the reaction as a substrate, with a final total volume of 200 μ L per well. The blank group used a buffer solution instead of the inhibitor solution. In addition, each concentration of the samples, including the blank group, corresponded to a control group, which used the Tris-HCl solution instead of XO. The XO inhibition rate was calculated by Equation (1), and the IC_{50} value of the inhibitor against XO was obtained using the software GraphPad Prism 8.

$$\text{Inhibition rate \%} = \left(1 - \frac{C_a - C_b}{C_0 - C_i} \right) \times 100\% \quad (1)$$

where C_0 , C_i , C_a , and C_b refer to the uric acid content (μ g/mL) corresponding to the blank group, blank control group, sample group, and sample control group, respectively.

2.4. Measurement of DPPH Inhibitory Activity

The DPPH scavenging capacity of the three compounds was determined with reference to the method of Soetan et al. [30] with slight modifications, again using chromatographic methanol as the solvent. First, 190 μL of DPPH (0.3 mM) and 10 μL of PAH (or PA/OS) solution at different concentrations were sequentially added to the 96-well plate. After incubation for 30 min at room temperature, the absorbance of the samples was read at 517 nm. Each sample was measured three times in parallel. The blank and sample control groups were replaced with chromatography-grade methanol for the sample solution and DPPH solution, respectively.

$$\text{DPPH clearance rate \%} = \left(1 - \frac{A_i - A_c}{A_0}\right) \times 100\% \quad (2)$$

where A_0 = absorbance of blank group; A_i = absorbance of sample group; and A_c = absorbance of sample control group.

2.5. Determination of O_2^- Inhibitory Activity in Non-Enzymatic Systems

O_2^- is produced by the PMS-NADH system, and the resulting free radical reduces NBT to blue methylhydrazine, which has a characteristic absorption at 560 nm [31]. Tris-HCl (80 μL , 50 mM), sample solution (10 μL , different concentrations), NBT (50 μL , 0.31 mM), and NADH (30 μL , 1.22 mM) were added sequentially to the 96-well plate, and the plate was allowed to stand in the dark at an ambient temperature of 25 $^\circ\text{C}$ for 5 min. Subsequently, PMS (30 μL , 0.15 mM) was added to initiate the reaction, and the absorbance values of different concentrations of samples were measured at 560 nm after 15 min of reaction at 25 $^\circ\text{C}$ in dark. The blank control group was used to replace the samples with chromatographic methanol.

2.6. Determination of O_2^- Inhibitory Activity in XO Systems

O_2^- in the enzyme system is produced by XO catalyzing the reaction of the substrate. XO (20 μL , 200 U/L), samples of different concentrations (10 μL) and NBT were mixed well, with the Tris-HCl solution added to make up to 100 μL , and left to stand at 37 $^\circ\text{C}$ for 5 min. Subsequently, 100 μL of XA was added to initiate the reaction, and the absorbance at 560 nm was measured after 15 min of reaction at 37 $^\circ\text{C}$.

2.7. Cell Culture

RAW 264.7 cells were cultured in a humidified incubator at 37 $^\circ\text{C}$ with 5% CO_2 for 24 h, then seeded into 96-well plates (6×10^4 cells/mL, 100 μL per well) and 6-well plates (1.5×10^5 cells/mL, 2 mL per well) at the specified densities for subsequent experiments.

2.8. Determination of Optimal MSU Crystal Concentration for Cell Model Establishment

Following the method in Section 2.7, cells were seeded in 96-well plates at a density of 6×10^4 cells/mL and exposed to MSU crystals at concentrations of 0, 50, 100, 150, and 200 $\mu\text{g/mL}$, followed by 24 h incubation at 37 $^\circ\text{C}$. Cell viability was subsequently assessed via MTT assay [32], with absorbance measured at 490 nm. The relative cell survival rate was calculated using Equation (3):

$$\text{Relative cell survival/\%} = \frac{\text{OD}}{\text{OD}_{\text{kb}}} \times 100\% \quad (3)$$

2.9. Cytotoxicity Assay of Monomer Compounds, Allopurinol

The cytotoxicity of monomer compounds, including allopurinol, was evaluated using a colorimetric assay. Cells were seeded in 96-well plates at a density of 6×10^4 cells/mL

following the protocol described in Section 2.7. Test compounds were administered at various concentrations: allopurinol (6.25, 12.5, 25, and 50 µg/mL), PAH (1.25, 2.5, 5, 10, 20, and 40 µM), PA (12.5, 25, 50, 100, 200, and 400 µM), and OS (1.25, 2.5, 5, 10, 20, and 40 µM). Following a 24 h incubation period at 37 °C, cell viability was assessed by measuring absorbance at 490 nm. The optimal concentrations for subsequent experiments were determined based on cell viability results, ensuring >80% viability for test compounds and extracts.

2.10. Determination of ROS Levels

Referring to the method in Section 2.7, cells were inoculated in black 96-well plates at a density of 6×10^4 cells/mL, and the following experimental groups were set up: (1) blank control group; (2) model group (MSU-treated); (3) positive control group (allopurinol + MSU); and (4) experimental group (active ingredient + MSU, with three dose gradients of low, medium and high). After polyphenols intervention, the cells were incubated at 37 °C for 24 h. The medium was discarded, and the DCFH-DA fluorescent probe was diluted to the working concentration using a serum-free DMEM medium; according to the instructions of the ROS assay kit, 100 µL of probe working solution was added to each well, and the cells were incubated at 37 °C for 20 min in an incubator protected from light with 5% CO₂. The probe solution was discarded, and the cells were gently washed with pre-warmed serum-free medium 3 times and immediately detected using a fluorescence enzyme marker (excitation wavelength of 488 nm, emission wavelength of 525 nm); all the operations were done under the condition of light avoidance.

2.11. Determination of LDH Release

Following 24 h polyphenols incubation in 96-well plates, 70 µL aliquots of supernatant were collected from each well and transferred to corresponding positions in fresh 96-well plates. Cytotoxicity assessment was performed using the LDH cytotoxicity detection kit according to the manufacturer's protocol. Absorbance measurements at 490 nm were recorded, with cytotoxicity percentage calculated as Equation (4):

$$\text{Cytotoxicity}(\%) = \frac{\text{OD}_{\text{treated}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{maxenzyme activity}} - \text{OD}_{\text{sample control}}} \times 100\% \quad (4)$$

2.12. Determination of NO Level

Cells were seeded in 6-well plates at a density of 1.5×10^5 cells/mL following the protocol outlined in Section 2.7. After polyphenols treatment, the cells were maintained under standard culture conditions (37 °C, 5% CO₂) for 24 h. Subsequently, culture supernatants were collected and analyzed for NO levels using a commercial NO content determination kit purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer's protocol.

2.13. Determination of SOD Activity in RAW 264.7 Cells After Polyphenol Treatment

Cells were seeded in 6-well plates at a density of 1.5×10^5 cells/mL following the protocol described in Section 2.7. After polyphenols treatment, the cells were incubated under standard culture conditions (37 °C, 5% CO₂) for 24 h. Subsequently, cellular supernatants were removed, and cells were lysed for SOD activity analysis. SOD activity was quantified using a commercial SOD assay kit according to the manufacturer's instructions, with absorbance measured at 450 nm.

2.14. ELISA Assay for Detecting Levels of Inflammatory Cytokines TNF- α and IL-1 β

RAW264.7 macrophage suspension was prepared as described in Section 2.7. A 2 mL aliquot of the cell suspension (containing approximately 1.5×10^5 cells per mL) was seeded into 6-well plates and incubated at 37 °C for 24 h. Subsequently, *Inonotus obliquus* extract (12.5, 25, 50 μ g/mL), PAH (2.5, 5, 10 μ M), PA (12.5, 25, 50 μ M), OS (2.5, 5, 10 μ M), and allopurinol solution (12.5 μ g/mL) were added separately, followed by incubation at 37 °C for 1 h. After that, 100 μ g/mL MSU solution was supplemented, and the incubation was continued for another 24 h. Cells were scraped off with a cell scraper and transferred to centrifuge tubes, then they were centrifuged at $10,000 \times g$ and 4 °C for 10 min. The contents of TNF- α and IL-1 β in the supernatant were detected in accordance with the instructions of the ELISA kit.

2.15. Western Blot Analysis of Protein Expression Levels in Inflammatory Signaling Pathways

Cell lysate was prepared by adding a protease inhibitor (1 mM PMSF) at a 1:99 ratio (inhibitor:lysate). Cells were lysed on ice for ~20 min, followed by centrifugation at $13,000 \times g$ and 4 °C for 5 min, and the supernatant was collected for subsequent processing. After electrophoresis and membrane transfer, PVDF membranes were incubated overnight at 4 °C with primary antibodies against TLR4, MyD88, NF- κ B, and β -actin (1:1000 dilution). Post-washing, membranes were incubated with peroxidase-conjugated secondary antibodies for 45 min, protein signals were detected using an enhanced chemiluminescence (ECL) system, and band intensities were analyzed, quantified, and normalized to β -actin using Quantity One software (V4.6.8, Bio-Rad Laboratories, Hercules, CA, USA).

2.16. Statistical Analysis

All experimental data were presented as the mean $\pm$ standard deviation (SD) derived from 3 to 5 independent experimental replicates. Between-group comparisons for two datasets were carried out with Student's *t*-test, whereas multi-group comparisons were assessed via one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. All statistical analyses were implemented in GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was defined as statistically significant.

3. Results and Discussion

3.1. Qualitative and Quantitative Analysis of the Components in Crude Extracts

Three active components, PA, PAH, and OS, were identified in the extract of *I. obliquus* using an established HPLC method [16]. As the major polyphenol components with relatively high contents in the extract, these three compounds also share high structural similarity, which provides an ideal model for investigating the structure–activity relationship between their chemical structures and bioactivities. Thus, they were selected as the research objects to lay a solid material foundation for subsequent studies on the interaction between polyphenol monomers and enzymes, cell experiments, and anti-inflammatory activity evaluation. The reference standards of the three polyphenols were used for HPLC analysis, and the resulting chromatograms are shown in Figure 1a–c. Specifically, peaks 1, 2, and 3 correspond to PAH, PA, and OS, respectively. The standard curves obtained for PA, PAH and OS were $y = 83,014x - 115,383$, $y = 190,043x + 470,804$, and $y = 95,935x - 90,765$, respectively, corresponding to correlation coefficients *r* of 0.9996, 0.9983, and 0.9998, respectively. Figure 1d shows the chromatographic peaks of the crude extract. In comparison, the crude extract contained PAH, PA, and OS, and all of them were well separated from each other. The contents of PAH, PA, and OS in the crude extract were determined to be $0.0210 \pm 0.0006\%$, $0.0108 \pm 0.0002\%$, and $0.0172 \pm 0.0009\%$, respectively, representing the mass percentages of the target compounds relative to the dry weight of the crude extract.

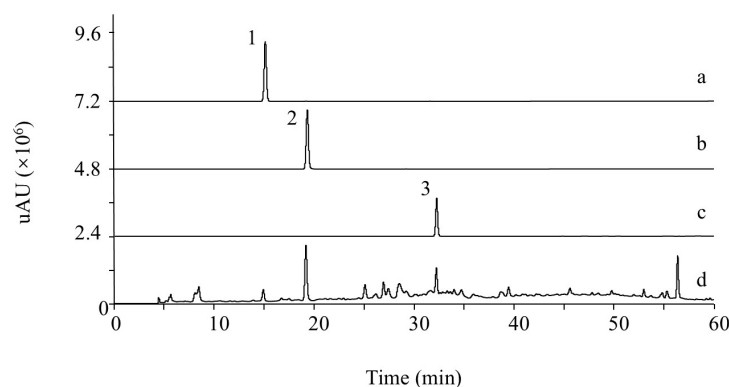


Figure 1. HPLC graphs of crude extracts and standards (a–d are the HPLC graphs of PA, PAH, OS and the crude extracts).

3.2. XO Inhibitory Activity of PAH, PA and OS

Figure 2 demonstrates the inhibition of XO activity by the three compounds, as shown in a concentration-dependent trend. Comparison of XO inhibitory capacity based on IC_{50} values: OS ($IC_{50} = 4.91$ mM) > PAH ($IC_{50} = 5.92$ mM) > PA ($IC_{50} = 26.53$ mM). The three compounds exhibited good XO inhibition, with OS showing the best inhibition and PAH demonstrating similar inhibition.

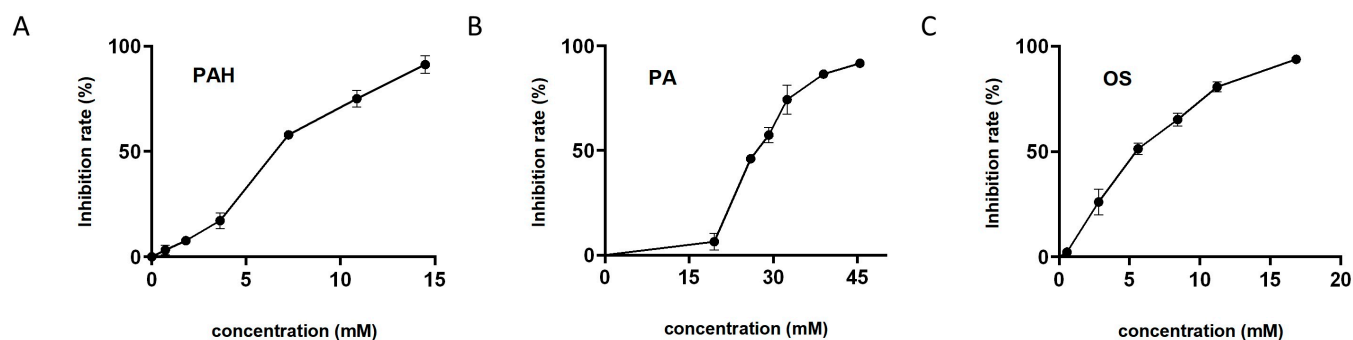


Figure 2. XO inhibition activity of PAH, PA and OS. (A) XO inhibitory activity of PAH; (B) XO inhibitory activity of PA; (C) XO inhibitory activity of OS.

3.3. DPPH Scavenging Activity of PAH, PA and OS

Shown as Figure 3, it can be observed that PAH, PA and OS were all better scavengers of DPPH radicals, and all of them were able to scavenge more than 90% of DPPH radicals when their concentrations reached 1.45 mM, 19.47 mM and 2.81 mM, respectively. This indicates that all of the compounds had strong antioxidant properties. The median effective concentrations (EC_{50}) were calculated to be 0.38 mM, 1.88 mM and 0.83 mM for PAH, PA and OS, respectively. It can be seen that the DPPH radical scavenging ability of PAH was stronger than that of OS, which was different from the ordering of the inhibitory ability of XO. This discrepancy may be attributed to the stronger binding affinity of OS with the amino acid residues at the active site of XO than PAH, as supported by our subsequent SAR analysis, which revealed that OS's carbonyl group and C=C-conjugated system facilitate stronger hydrogen bonding and electron-withdrawing interactions with XO, thereby conferring greater enzyme inhibitory activity.

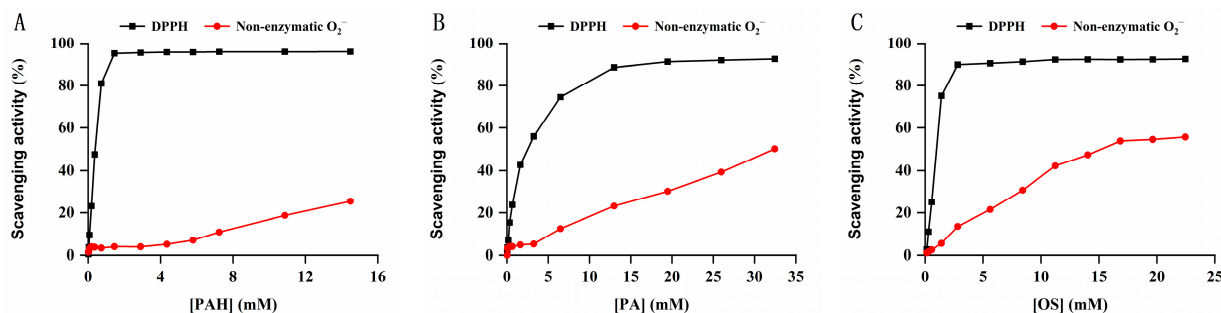


Figure 3. Scavenging effects of PAH, PA and OS on DPPH radicals and O_2^- radicals generated by the PMS-NADH system. (A) Scavenging effects of PAH on DPPH radicals and non-enzymatic O_2^- radicals; (B) Scavenging effects of PA on DPPH radicals and non-enzymatic O_2^- radicals; (C) Scavenging effects of OS on DPPH radicals and non-enzymatic O_2^- radicals.

3.4. O_2^- Scavenging Activity in a Non-Enzymatic System

The FAD center is a key site for the generation of O_2^- and H_2O_2 by oxygen molecules as electron acceptors. There are three main ways to inhibit O_2^- production or scavenge O_2^- [33]: (1) inhibiting uric acid production, which leads to the inhibition of O_2^- and H_2O_2 production; (2) antioxidants reduce XO molecules, catalyzing the production of H_2O_2 for the purpose of reducing O_2^- production; and (3) antioxidants have the ability to directly scavenge O_2^- . Figure 3 demonstrates the scavenging activity of the three compounds against O_2^- radicals generated by the PMS-NADH system. It can be observed that the removal of O_2^- by PAH, PA and OS was significantly weaker than the removal of DPPH with increasing concentration. As the removal of DPPH approached 100%, the three compounds were far less than 50% for O_2^- removal. In summary, it is demonstrated that all of PAH, PA and OS had a certain ability to scavenge O_2^- radicals, but direct scavenging of O_2^- is not the main method and can better function in enzymatic systems.

3.5. Scavenging Activity of O_2^- in Enzymatic Reaction Systems

The scavenging ability of PAH, PA and OS at different concentrations to O_2^- radicals produced by the XO-catalyzed reaction was determined. The results are shown in Figure 4, where all of the three compounds have the ability to rapidly inhibit the production of O_2^- radicals from XO-catalyzed reactions. PAH, PA and OS reached 80% O_2^- scavenging at 0.18, 1.62 and 0.14 mM, respectively, and were eventually maintained at around 90% over the range of concentrations measured, with EC_{50} values for the scavenging of XO-generating O_2^- radicals of 0.06, 0.4 and 0.05 mM, respectively. The results showed that PAH, PA and OS were more inclined to achieve the reduction in O_2^- radical production by inhibiting XO activity than the non-enzymatic system.

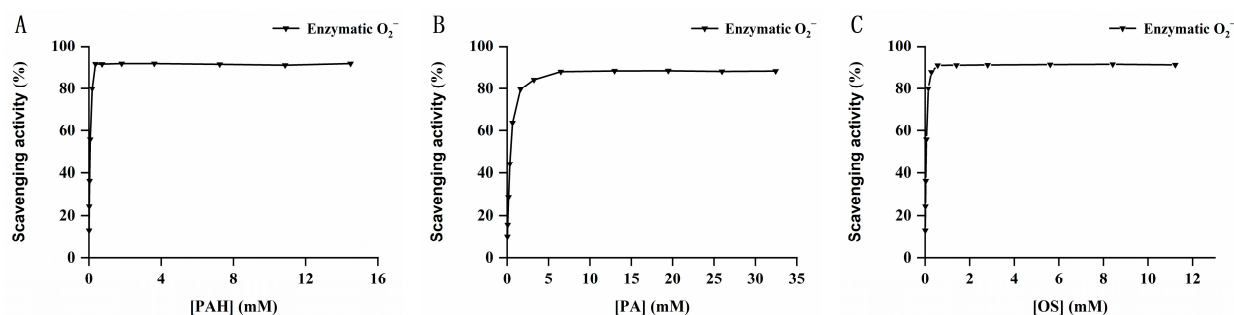


Figure 4. Scavenging effects of PAH, PA and OS on O_2^- radicals generated by the enzymatic reaction of XO. (A) Scavenging effect of PAH on O_2^- radicals generated by the enzymatic reaction of XO; (B) Scavenging effect of PA on O_2^- radicals generated by the enzymatic reaction of XO; (C) Scavenging effect of OS on O_2^- radicals generated by the enzymatic reaction of XO.

3.6. Structure–Activity Relationship Analysis

The structures of PAH, PA and OS are shown in Figure 5, and their structures are very similar, with the difference being the different functional groups R attached to the basic backbone (catechol). For PA, the presence of -OH in the carboxyl group results in a weaker electron-withdrawing property than that of the aldehyde group, so that its ability to form hydrogen bonds with amino acid residues would also be relatively weaker, and it is hypothesized that may be the reason why XO inhibitory activity of PA was significantly weaker than PAH. Zhang et al. [34] compared the antioxidant activity of protocatechualdehyde (PAH) and protocatechuic acid (PA) and found that the strong electron-withdrawing property of the -CHO group positively affected the free radical scavenging activity of the compounds. Joshi et al. [35] studied the inhibition of XO by 3,5-diaryl-4,5-dihydro-1H-pyrazole carbaldehyde derivatives, and analyzed the inhibitors with inhibitory activity. Then, Joshi et al. found that the inhibition was weak or ineffective when the functional group had a carboxyl group, but there was a strong inhibition when the functional group had an aldehyde group.

The carbonyl group has a strong electron-withdrawing effect, and is prone to form hydrogen-bonding interactions with the amino acid residues of the enzyme, a property that greatly increases the ability of OS to inhibit the enzyme. Studies on the conformational relationships of flavonoids have shown that the presence of a carbonyl group was one of the most important reasons for better enzyme inhibition, and many polyphenols had stronger XO inhibition when contain a carbonyl structure than other structures [36]. Meanwhile, the C=C double bond and carbonyl group contained in OS form a conjugated system [37], which could make OS more precise and better bind to the active site on XO protein.

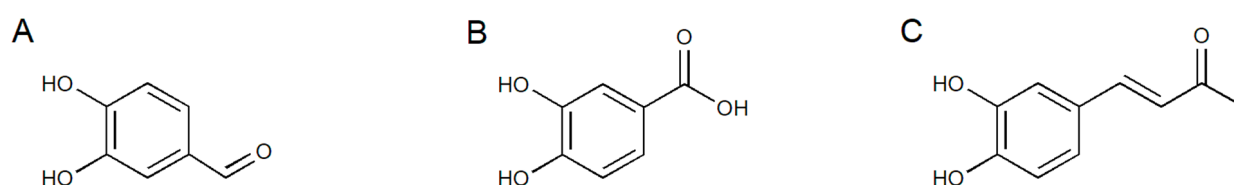


Figure 5. (A–C) are the structural formulas of PAH, PA and OS.

3.7. Screening of Acute Gout Cell Model and Determination of Safe Polyphenols Concentrations

RAW 264.7 cells were injured by MSU stimulation to establish an acute gout cell model. The cell survival rate was significantly reduced when 200 µg/mL MSU was added after 24 h of culture compared with the control group; therefore, a safe concentration of 150 µg/mL was selected for subsequent experiments.

Compared with the control group, the relative cell survival rate showed a significant decrease after adding 25 µg/mL allopurinol, 20 µM PAH, 100 µM PA, and 20 µM OS solution. Therefore, 12.5 µg/mL allopurinol solution, 12.5, 25, and 50 µg/mL *I. obliquus* extract, 2.5, 5, and 10 µM PAH, 12.5, 25, and 50 µM PA, and 2.5, 5, and 10 µM OS solutions were selected to exclude the interference of polyphenols toxicity on cells and to provide a suitable polyphenols concentration basis for subsequent inflammatory index studies in cell models.

3.8. Protective Effects of Polyphenols in the Acute Gout Cell Model

As shown in Figure 6, the cell viability was highly and significantly reduced to $59.93 \pm 4.32\%$ when MSU was added up to a concentration of 150 µg/mL compared to the control group, and the cell viability was significant or highly significant after the addition of 12.5, 25, and 50 µg/mL *I. obliquus* extract, 2.5, 5, and 10 µM PAH, 12.5, 25, and 50 µM PA,

and 2.5, 5, and 10 μM OS compared to the model group, respectively. It indicated that *I. obliquus* extract, PAH, PA and OS had a protective effect on the acute gout cell injury model.

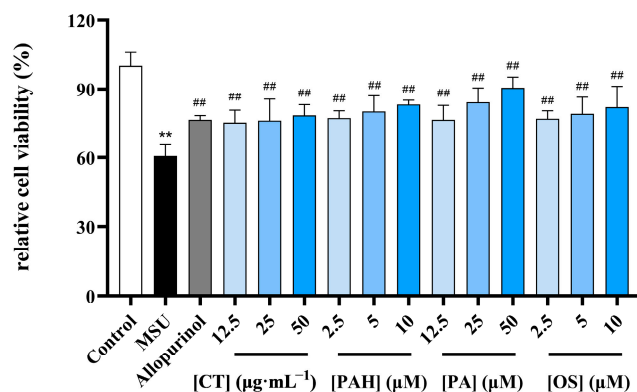


Figure 6. Relative cell survival rate after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by MTT assay. Positive control: allopurinol (12.5 $\mu\text{g}/\text{mL}$); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with the control group: ** $p < 0.01$; Comparison with the model group: ## $p < 0.01$.

3.9. Effect of Polyphenols on RAW 264.7 ROS Production

RAW264.7 cells are subjected to oxidative stress induced by MSU stimulation, which generates excessive ROS—this process can potentially activate the NLRP3 inflammasome, thereby triggering gouty attacks [38]. As shown in Figure 7, compared with the control group, the model group exhibited a highly significant elevation in ROS levels. In contrast, the low-, medium-, and high-concentration dosing groups of PAH, PA, and OS all significantly reduced ROS content relative to the model group. This result indicates that *I. obliquus* extract, PAH, PA, and OS possess the ability to counteract oxidative stress and maintain the balance of cellular functions, which may further contribute to inhibiting NLRP3 inflammasome activation and alleviating gout-related inflammation.

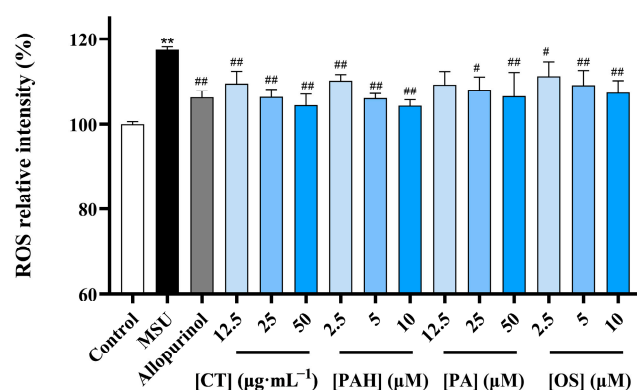


Figure 7. Relative ROS fluorescence intensity after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by ROS fluorescent probe assay. Positive control: allopurinol (12.5 $\mu\text{g}/\text{mL}$); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with the control group: ** $p < 0.01$; comparison with the model group: # $p < 0.05$, ## $p < 0.01$.

3.10. Effect of Polyphenols on NO Secretion in RAW 264.7 Cells

NO is a biological signaling molecule widely present in the cell with important biological regulatory functions and has an important role in inflammatory disease processes. As shown in Figure 8, NO content was highly significantly increased in the model group

compared to the control group. Compared with the model group, the content of NO in the *I. obliquus* extract, PAH, PA and OS dosing group could be significant or highly significant decrease, indicating that *I. obliquus* extract, PAH, PA and OS could inhibit the release of NO in the MSU-induced macrophage RAW 264.7 model.

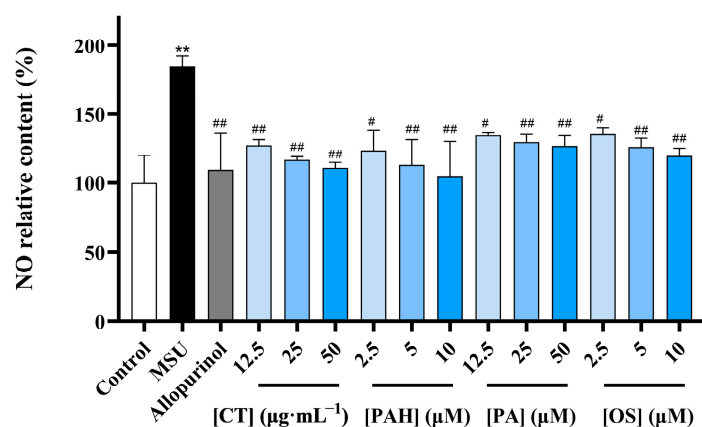


Figure 8. Relative NO content after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by NO assay. Positive control: allopurinol (12.5 μg/mL); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with control group: ** $p < 0.01$; comparison with model group: # $p < 0.05$, ## $p < 0.01$.

3.11. Effect of Polyphenols on RAW 264.7 LDH Release

When cell damage or apoptosis occurs, cell membrane permeability is altered and LDH will be released outside the cell and thus detected in the cytotoxic sample [39]. As can be seen in Figure 9, LDH release from cells in the model group compared to the control group was significantly increased ($p < 0.01$), indicating that MSU can effectively disrupt the RAW 264.7 cell membrane and cause cellular damage. LDH release was significantly reduced in the low-, medium- and high-dose groups of *I. obliquus* extract, PAH, PA and OS in the polyphenols group compared to the model group. It indicated that *I. obliquus* extract and all three polyphenol compounds could reduce cell damage and increase cell survival in gout model.

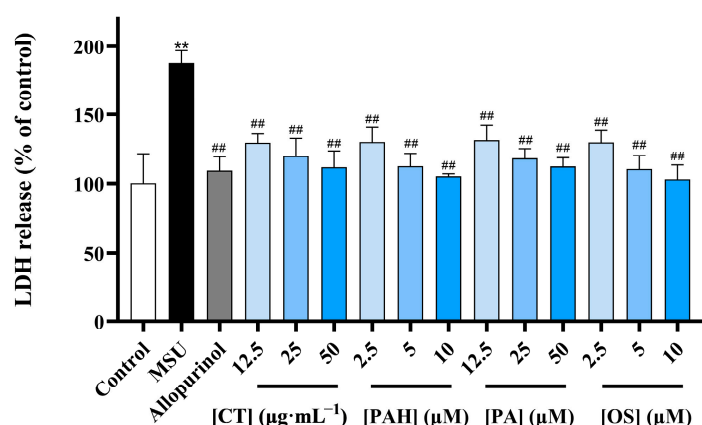


Figure 9. LDH release after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by LDH assay. Positive control: allopurinol (12.5 μg/mL); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with control group: ** $p < 0.01$; comparison with model group: ## $p < 0.01$.

3.12. Effect of Polyphenols on SOD Activity in RAW 264.7 Cells

SOD is a critical protective enzyme against oxidative damage in humans: it efficiently scavenges excess oxygen free radicals, thereby protecting cells from oxidative damage and regulating the metabolic activity of organisms [40]. As shown in Figure 10, compared with the control group (untreated RAW 264.7 cells), the model group (RAW 264.7 cells induced by MSU crystals) exhibited a highly significant decrease in SOD activity. After treatment with different concentrations of *I. obliquus* extract, PAH, PA, and OS, SOD activity increased significantly or highly significantly. This result indicated that *I. obliquus* extract, PAH, PA, and OS could elevate SOD activity in the MSU-induced acute gout model of RAW264.7 cells, further supporting their protective effects against oxidative stress in gout-related cellular damage.

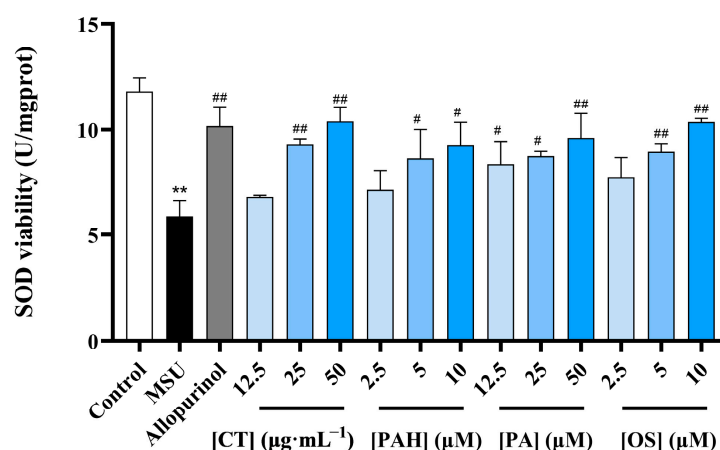


Figure 10. Relative SOD activity after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by SOD assay. Positive control: allopurinol (12.5 $\mu\text{g}/\text{mL}$); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with control group: ** $p < 0.01$; comparison with model group: # $p < 0.05$, ## $p < 0.01$.

3.13. Effect of TNF- α and IL-1 β in MSU-Induced RAW264.7 Cells

MSU triggers the release of inflammatory cytokines, including IL-1 β and TNF- α , from RAW264.7 macrophages, thereby inducing cellular apoptosis and driving the cascade amplification of inflammatory responses [41]. The expression profiles of TNF- α and IL-1 β post MSU stimulation serve as a reliable readout to evaluate the anti-inflammatory activities of *I. obliquus* extract, PAH, PA, and OS. As illustrated in Figure 11, the model group exhibited an extremely significant elevation in IL-1 β and TNF- α levels compared to the control group. In contrast, treatment with low-, medium-, and high-concentrations of *I. obliquus* extract, PAH, PA, or OS resulted in an extremely significant reduction in the contents of these two cytokines relative to the model group, demonstrating the potent anti-inflammatory properties of these substances.

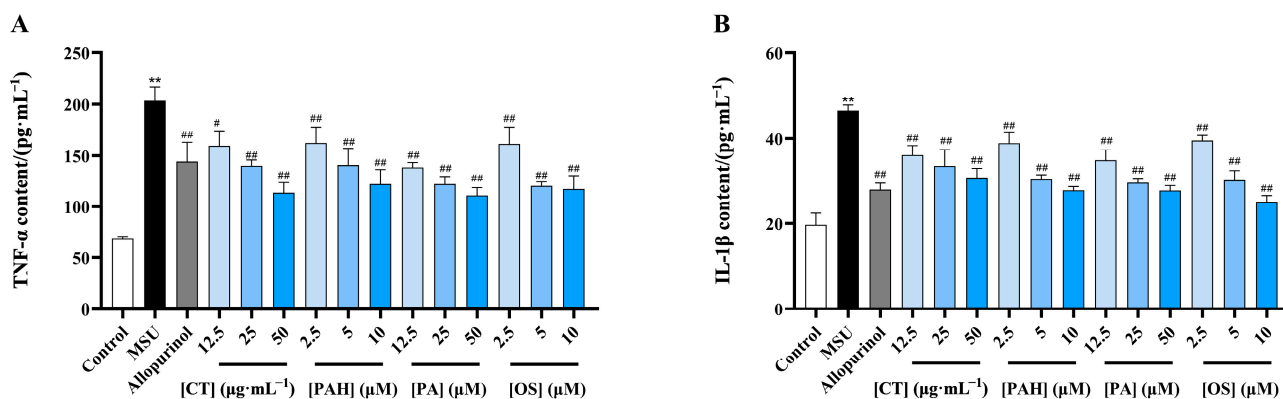


Figure 11. Relative contents of TNF- α and IL-1 β after treatment with CT (*I. obliquus* extract), PAH, PA and OS determined by ELISA assay. (A) Relative content of TNF- α after treatment with CT, PAH, PA and OS; (B) Relative content of IL-1 β after treatment with CT, PAH, PA and OS. Positive control: allopurinol (12.5 $\mu\text{g}/\text{mL}$); control: untreated RAW 264.7 cells; model: MSU-stimulated cells; CT/PAH/PA/OS: MSU-stimulated cells treated with corresponding sample. Comparison with control group: ** $p < 0.01$; comparison with model group: # $p < 0.05$, ## $p < 0.01$.

3.14. Effects of the Extract on TLR4, MyD88 and NF- κB Protein Expression in Acute Gouty Arthritis Cell Models

The TLR4/NF- κB signaling cascade constitutes a fundamental inflammatory mediator and exerts a profound influence on ischemic pathogenesis in cerebral, cardiac, and hepatic tissues [42]. Under basal, unstimulated conditions, NF- κB remains cytoplasmically sequestered through its canonical association with I κB proteins. Upon exposure to stressful stimuli, phosphorylation and subsequent activation of I κB kinase- β (IKK β) triggers phosphorylation-dependent inactivation of I $\kappa\text{B}\alpha$, thereby releasing NF- κB for nuclear translocation and transcriptional upregulation of multiple pro-inflammatory cytokines [43]. TLR4 orchestrates NF- κB activation via both MyD88-dependent and MyD88-independent pathways, with the former specifically facilitating NF- κB -mediated secretion of inflammatory mediators including TNF- α , interleukin-1 (IL-1), IL-6, and IL-8 [44]. To elucidate whether *I. obliquus* extract elicits its anti-inflammatory effects through modulation of the TLR4/MyD88/NF- κB axis, the present study focused on quantifying the protein expression profiles of TLR4, MyD88, and NF- κB .

As shown in Figure 12, Western blotting and quantitative analysis revealed that compared with the normal group, the protein expression of TLR4, MyD88, and NF- κB was significantly upregulated in the MSU-induced model group, indicating activation of the TLR4/NF- κB inflammatory signaling pathway. Conversely, relative to the model group, the 12.5, 25, and 50 $\mu\text{g}/\text{mL}$ *I. obliquus* extract treatment groups showed a marked reduction in the expression of these three proteins. These results confirm that *I. obliquus* extract exerts anti-gout activity by downregulating the protein expression of key molecules (TLR4, MyD88, NF- κB) in the MyD88/TLR4/NF- κB inflammatory signaling pathway.

Consistent with the findings of Storniolo et al. [45] who investigated structurally similar polyphenols (naringenin, hydroxytyrosol) from tomato-based soffrito, our study confirms that polyphenols derived from *Inonotus obliquus* (OS, PAH, PA) share conserved phenolic structural backbones and exert analogous antioxidant and anti-inflammatory effects. Both studies demonstrate that polyphenols can effectively suppress ROS burst, reduce excessive NO production, and mitigate the release of pro-inflammatory mediators in activated macrophages—Storniolo et al. reported the inhibition of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) in oxidized low-density lipoprotein (oxLDL)-stimulated THP-1 macrophages, while our work shows downregulation of TNF- α and IL-1 β in MSU-induced RAW264.7 cells. This consistency validates the universal structure–activity relationship

of polyphenols, wherein phenolic hydroxyl groups and electron-withdrawing functional moieties (e.g., aldehyde group in PAH and naringenin, conjugated C=C-carbonyl system in OS) are critical for scavenging free radicals and modulating inflammatory responses.

Notably, our study extends the understanding of polyphenols' biological functions by addressing a distinct pathological context and novel mechanisms. While Storniolo et al. focused on atherosclerosis-related inflammation triggered by oxLDL, we targeted gout-related inflammation induced by MSU crystals, a key pathological stimulus in acute gouty arthritis. Beyond the shared antioxidant and anti-inflammatory activities, our *Inonotus obliquus* polyphenols exhibit an additional XO inhibitory capacity, which directly reduces uric acid production—the core pathogenic factor of gout. This dual regulatory effect (XO inhibition + inflammation modulation) distinguishes our findings from the prior work, which did not explore XO as a target. Furthermore, we supplemented detailed SAR analysis, revealing that the aldehyde group in PAH (vs. the carboxyl group in PA) and the conjugated system in OS enhance binding to XO's active site, thereby boosting bioactivity. This SAR insight complements Storniolo et al.'s observations on polyphenol structure-function correlations and provides a basis for rational optimization of natural bioactives for gout management.

Together, these studies collectively support the broad potential of plant/fungal polyphenols as natural modulators of oxidative stress-related inflammation, with our work expanding their application scope to gout through a unique multi-target mechanism. Future studies could explore the additive effects of *Inonotus obliquus* polyphenols (as observed in Storniolo et al.'s sofrito compound combinations) and their in vivo efficacy in gout animal models to further validate translational potential.

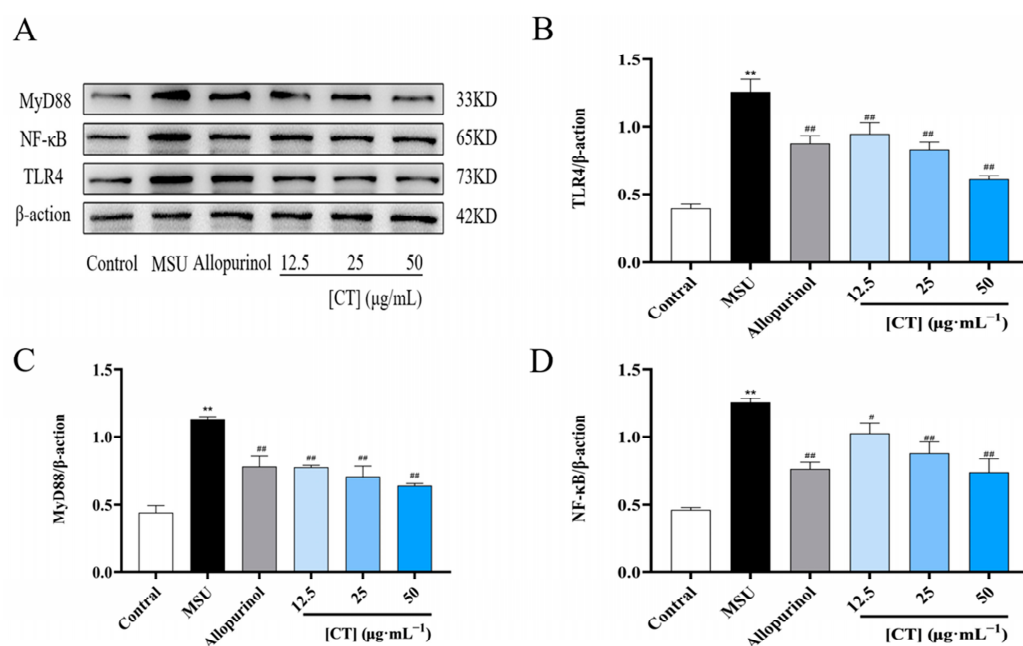


Figure 12. Regulation effect of *Inonotus obliquus* extract on TLR4/NF-κB pathway-related proteins in MSU-induced RAW 264.7 cells. Allopurinol (12.5 μg/mL) was used as the positive control. (A) Western blot bands of TLR4, MyD88, NF-κB, and the internal control β-actin in the control group, MSU model group, positive control group, and 12.5, 25, 50 μg/mL *Inonotus obliquus* extract groups. (B–D) Quantitative analysis of relative protein expression levels normalized to β-actin. Comparison with control group: ** $p < 0.01$; comparison with model group: # $p < 0.05$, ## $p < 0.01$.

4. Conclusions

HPLC was used for the qualitative and quantitative analysis of three polyphenols (PAH, PA, OS) from *Inonotus obliquus*, a medicinal and edible macrofungus with potential as a source of natural functional food ingredients. Subsequent assays confirmed that these polyphenols exhibit obvious antioxidant and XO inhibitory effects—XO inhibitory activity followed the order OS > PAH > PA (OS: $IC_{50} = 4.91$ mM), while DPPH radical scavenging ranked PAH > OS > PA. Notably, their primary pathway for attenuating superoxide anion (O_2^-) is XO inhibition (EC_{50} : 0.14–1.62 mM) rather than direct scavenging, which is related to their structural features: PA's carboxyl hydroxyl weakens XO binding, while PAH's aldehyde group and OS's carbonyl-C=C conjugated system enhance it.

An acute gout cell model was established by stimulating RAW264.7 macrophages with MSU crystals. Cytotoxicity assays verified the safety of *I. obliquus* extract (12.5–50 μ g/mL) and the three polyphenols (2.5–50 μ M). Treatment with these compounds effectively regulated oxidative stress (reduced ROS, increased SOD activity), suppressed pro-inflammatory mediators (IL-1 β , TNF- α , NO, LDH), and downregulated the MyD88/TLR4/NF- κ B signaling pathway via Western blot analysis, exerting protective effects against gout-related cellular damage. Notably, the bioactivity of *I. obliquus* crude extract may involve synergistic interactions between the three polyphenols and other trace components (e.g., polysaccharides, terpenoids), forming a multi-component, multi-target regulatory network. Future studies will focus on isolating these uncharacterized components and verifying their synergistic mechanisms.

Collectively, *I. obliquus*-derived polyphenols exhibit obvious antioxidant and xanthine oxidase inhibitory effects, and regulate oxidative stress, pro-inflammatory mediators, and the MyD88/TLR4/NF- κ B signaling pathway in MSU-stimulated RAW264.7 inflammatory macrophages. These findings deepen the understanding of their structure–activity relationship, support their development as natural functional food ingredients, and lay a theoretical foundation for their potential application in gout-related and oxidative stress-associated inflammatory disorders.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	One-way analysis of variance
BBA	Biochimica et Biophysica Acta
CO ₂	Carbon dioxide
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC ₅₀	Median effective concentration
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide
H ₂ O ₂	Hydrogen peroxide
HPLC	High-performance liquid chromatography
IC ₅₀	Half-maximal inhibitory concentration
IKK β	I κ B kinase- β
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-8	Interleukin-8
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MSU	Monosodium urate
Mo-Pt	Molybdopterin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
NADH	Nicotinamide adenine dinucleotide hydride
NF- κ B	Nuclear factor-kappa B
NBT	Nitroblue tetrazolium
NO	Nitric oxide
NLRP3	NOD-like receptor pyrin domain-containing 3
OD	Optical density
OS	Osmundacetone
O ₂ [−]	Superoxide anion radical
PMS	Phenazine methosulfate
PMSF	Phenazine methosulfate
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RP-C18	Reversed-phase C18
SOD	Superoxide dismutase
TNF- α	Tumor necrosis factor- α
TLR4	Tumor necrosis factor- α
Tris-HCl	Tris (hydroxymethyl) aminomethane-hydrochloric acid
UA	Uric acid
XO	Xanthine oxidase
XA	Xanthine

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