Molecular Biology Reports (2024) 51:56
https://doi.org/10.1007/s11033-023-09147-0

ORIGINAL ARTICLE

Comparison of the inhibition effects of naringenin and its glycosides on LPS-induced inflammation in RAW 264.7 macrophages

Shu-Chi Cho[1] · **Shyh-Yu Shaw**[1]

Received: 31 August 2023 / Accepted: 12 December 2023 / Published online: 2 January 2024

Abstract

Background Inflammation is intricately linked to the development of various diseases, such as diabetes, cardiovascular diseases, and cancer. Flavonoids, commonly found in plants, are known for their diverse health benefits, including antioxidant and anti-inflammatory properties. These compounds are categorized into different classes based on their chemical structure. structures. However, limited research has compared the effects of flavonoid aglycones and flavonoid glycosides. This study aims to assess the anti-inflammatory effects of naringenin and its glycosides (naringin and narirutin) in RAW264.7 macrophages.

Methods and Results RAW264.7 cells were treated with naringenin, naringin, and narirutin, followed by stimulation with lipopolysaccharide. The levels of inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2), were assessed. Additionally, the study examined nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation using western blot analysis. Among the compounds tested, narirutin exhibited the most potent anti-inflammatory effect against TNF-α, NO, and iNOS. Naringin and narirutin showed comparable inhibitory effects on IL-1β and COX-2. Both naringin and narirutin suppressed the expression of pro-inflammatory mediators by targeting different levels of the NF-κB and MAPK pathways. Naringenin demonstrated the weakest anti-inflammatory effect, primarily inhibiting NF-κB and reducing the phosphorylation levels of p38.

Conclusions This study suggests that the presence of glycosides on naringenin and the varied binding forms of sugars in naringenin glycosides significantly influence the anti-inflammatory effects compared with naringenin in RAW 264.7 macrophages.

Keywords Anti-inflammation · Naringenin · Naringin · Narirutin · Nuclear factor-κB (NF-κB) · Mitogen-activated protein kinases (MAPK)

Abbreviations

TNF-α	Tumor necrosis factorα
LPS	Lipopolysaccharide
IL-1β	Interleukin-1β
NO	Nitric oxide
NF-κB	Nuclear factor-κB
MAPK	Mitogen-activated protein kinase
iNOS	inducible NOS
COX-2	Cyclooxygenase-2

Introduction

Inflammation, a complex physiological response triggered by injury, infection, or irritation [1], serves as a crucial mechanism of the immune system to eliminate damaged tissue, pathogens, or antigens. Apart from its immediate response to stimuli, inflammation also initiates the healing process, aiding tissue repair and restoring normal function [2]. However, prolonged or uncontrolled inflammation can lead to the development of various diseases [1]. Extensive research underscores inflammation as a pivotal contributor

✉ Shyh-Yu Shaw
syshaw@mail.ncku.edu.tw

Shu-Chi Cho
l38081030@gs.ncku.edu.tw

[1] Department of Chemistry, National Cheng Kung University, No.1, University Road, Tainan City 701, Taiwan (ROC)

to conditions, such as diabetes, cardiovascular diseases, autoimmune disorders, and cancer [3, 4]. Macrophages, integral immune cells distributed throughout the body that mature from circulating monocytes, play pivotal roles in the host's immune defense system during inflammation [5]. Activation of macrophages occurs when stimulated by cytokines or agents, such as tumor necrosis factor α (TNF-α), interferon gamma (IFNγ), or lipopolysaccharide (LPS) [5]. LPS, an endotoxin derived from the cell wall of Gram-negative bacteria, triggers macrophage activation by binding to toll-like receptor 4 (TLR4), prompting the release of proinflammatory cytokines and mediators, including TNF-α, interleukin-1β (IL-1β), and nitric oxide (NO) [6]. LPS-induced secretion of these inflammatory molecules operates via the activation of intracellular signaling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) [7]. Consequently, targeting these cell signaling pathways, specifically NF-κB and MAPK, stands as a potential therapeutic strategy for various inflammatory conditions [8, 9].

Flavonoids, a diverse group of polyphenolic compounds abundantly present in plants commonly consumed in the human diet (such as fruits, vegetables, tea, and dark chocolate) [10], exhibit numerous health benefits, including anticancer, antioxidant, and antihypertensive properties [11, 12]. Scientists are increasingly exploring their pharmacological activities [12], including their noted anti-inflammatory effects [13]. Mechanisms underlying the anti-inflammatory properties of flavonoids involve the inhibition of regulatory enzymes and suppression of transcription factors associated with inflammation [14]. For instance, procyanidin B2 inhibits the kinase activity of MEK1, an upstream activator that tyrosine-phosphorylates extracellular signal-regulated kinase-1 (ERK-1) in the MAPK pathway [15]. Similarly, the anti-inflammatory effect of fisetin involves inhibiting the transcription factor NF-κB [16].

These beneficial properties of flavonoids are related to their molecular structures [17]. Flavonoids are divided into diverse subclasses based on their molecular structure. Normally, the structure of flavonoid aglycones is characterized by two benzene rings (A and B) linked by a heterocyclic ring containing oxygen (C) [17]. However, in plants, flavonoids predominantly exist in glycoside rather than in aglycone form [18]. Flavonoid glycosides, comprising one or more sugar molecules attached to their aglycones, exhibit distinct bioactivities influenced by the presence or absence of sugar molecules [19]. Notably, flavonoid glycosides typically display higher water solubility than their aglycones [19]. For instance, in an in vitro digestion assay, quercetin exhibits significantly higher antioxidant activity than its glycoside isoquercitrin during the oral phase but ultimately demonstrates lower antioxidant activity than isoquercitrin in the intestinal phase [20]. Hence, the varied bioactivities between flavonoids and their glycosides necessitate further research.

Naringenin, a flavonoid belonging to the flavanone subclass widely found in various fruits and herbs, has demonstrated anti-inflammatory effects [21]. Beyond its anti-inflammatory properties, naringenin has exhibited diverse biological effects on the human body, including anti-hepatitis C virus effects and cardioprotective qualities, suggesting its potential clinical utility [21]. Naringenin and its glycosides are abundant in numerous plants, such as grapefruit and tea [22]. Naringin and narirutin, naringenin glycosides, are connected with a disaccharide attached at the C7 of naringenin. Naringin's disaccharide consists of rhamnose and glucose (Ram (1→2) Glc), while narirutin's disaccharide comprises rhamnose and glucose (Ram (1→6) Glc). Apart from their chemical structures, differences among these three compounds have been reported. For example, unlike the tastelessness of naringenin and narirutin, naringin contributes to the bitter taste in fruits rich in this glycoside [23, 24]. Additionally, naringenin and naringin exhibit differing pharmacological activities concerning neurological diseases [25]. Therefore, these minor structural disparities might significantly influence their varied bioactivities. Nonetheless, no comparative research has explored the in vitro anti-inflammatory effects and mechanisms of naringenin, naringin, and narirutin. Consequently, this study aims to investigate the differences in their anti-inflammatory potentials and mechanisms in LPS-induced RAW264.7 cell lines.

Materials and methods

Materials

Naringenin (purity > 98%) was procured from MedChemExpress (Monmouth Junction, NJ, USA), while naringin (purity > 98%) was purchased from TargetMol (Wellesley Hills, MA, US), and narirutin (purity > 98%) was obtained from ChemFaces (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were acquired from Cytiva (Marlborough, MA, US). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) reagent was obtained from Promega (Madison, WI, US). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β were supplied by R&D Systems (Minneapolis, MN, US), and the Griess reagent kit was acquired from Biotium (Fremont, CA, US). Bovine serum albumin (BSA) was purchased from Bio Basic (Markham, Canada). The bicinchoninic acid (BCA) Protein

Assay Kit and blocking buffer were acquired from Visual Protein (Taipei, Taiwan). RIPA Buffer was obtained from Abcam (Waltham, MA, US), while the protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from TargetMol. Polyvinylidene difluoride (PVDF) membranes were acquired from PerkinElmer (Waltham, MA, US). Antibodies against p65, p-p65, IkBα, p38, extracellular signal-regulated kinase (ERK), p-ERK, c-Jun N-terminal kinase (JNK), p-JNK were supplied by Cell Signaling (Beverly, MA, US), whereas antibodies against inducible NOS (iNOS), and cyclooxygenase-2 (COX-2) were purchased from Proteintech (Rosemont, IL, US). The anti-β-actin antibody was obtained from iReal (Hsinchu, Taiwan), and the horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was supplied by Jackson ImmunoResearch (West Grove, PA, US). Additionally, the WesternBright ECL HRP substrate was purchased from Advansta (San Jose, CA, US).

Cell culture

RAW 264.7 cells procured from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan) were cultivated in DMEM supplemented with 10% FBS, penicillin (100 U/mL), sodium bicarbonate (1.5 mg/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM). The cells were incubated in a humidified atmosphere containing 5.0% CO_2.

Cell viability assessment

Cell viability was determined using the MTS assay. RAW264.7 cells were seeded in 96-well plates at a density of 1×10^5 cells/mL in a volume of 100 µL per well. Following a 24-h incubation at 37 °C, cells were treated with varying concentrations of naringenin, naringin, and narirutin for 24 h. Subsequently, 20 µL of MTS reagent was added to each well, and the plates were incubated for an additional 3 h. The absorbance was measured at 490 nm using an ELISA reader (SpectraMax Plus 384, Molecular Devices, San Jose, CA, US).

TNF-α and IL-1β production analysis

RAW264.7 cells, seeded at 1×10^5 cells/mL in 96-well plates, were incubated for 24 h at 37 °C. The cells were then treated with specified concentrations of naringenin, naringin, and narirutin for 1 h before adding LPS (100 ng/mL) for another 24 h. Culture medium from each well was collected for subsequent analysis. TNF-α and IL-1β concentrations were quantified using ELISA kits according to the manufacturer's instructions, with absorbance measured at 520 nm using an ELISA reader.

NO production measurement

RAW264.7 cells, plated at a density of 1×10^5 cells/mL in a volume of 200 µL/well in 96-well plates and incubated for 24 h at 37 °C, were exposed to indicated concentrations of naringenin, naringin, and narirutin for 1 h, followed by the addition of LPS (100 ng/mL) for 24 h. Culture medium from each well was collected for further analysis. Griess reagent was added to the collected medium and incubated for 30 min at room temperature. The absorbance was measured at 548 nm using an ELISA reader, and NO levels were determined by a nitrite standard curve.

Western blot analysis

RAW264.7 cells were seeded in 6-well plates at a density of 3×10^5 cells/mL in a volume of 1000 µL/well and incubated at 37 °C for 24 h. Subsequently, cells were treated with indicated concentrations of naringenin, naringin, and narirutin for 1 h followed by the addition of LPS (100 ng/mL) for specific durations (24 h or 30 min). After the designated incubation periods, cells were washed twice with ice-cold phosphate-buffered saline, harvested, and lysed in ice-cold RIPA buffer supplemented with cocktails of protease and phosphatase inhibitors on ice. Scraped cells were collected and centrifuged at 14,000 × g for 20 min to obtain supernatants. Protein concentrations were determined using the BCA Protein Assay Kit with BSA standards. Equal amounts of protein were loaded onto 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred to PVDF membranes for 2.5 h. Following transfer, membranes were blocked with a blocking buffer for 30 min at room temperature. Incubation with specific primary antibodies was conducted overnight at 4 °C on a shaker. Thereafter, HRP-conjugated anti-rabbit secondary antibodies were applied at room temperature for 1 h following three washes with tris-buffered saline containing 0.1% Tween. Protein visualization was performed using an ECL reagent in an imaging system (Multigel-21 system, TOPBIO, New Taipei City, Taiwan), and ImageJ software was utilized for quantification.

Statistical analysis

All experiments were conducted in triplicate, and results are presented as mean ± standard deviation. GraphPad Prism 9.0 software (GraphPad Software, Carlsbad, CA, US) was used to determine statistical differences among the data.

Statistical significance was assessed by Dunnett' s multiple comparison test ($P < 0.05$).

Results

Effects of naringenin, naringin, and narirutin on RAW264.7 cell cytotoxicity

The impact of naringenin, naringin, and narirutin on RAW264.7 cell cytotoxicity was evaluated through a 24-h treatment using various concentrations assessed via an MTS assay (Fig. 1). Treatment with naringenin at concentrations of 50 μM exhibited a significantly higher cytotoxic effect on RAW264.7 cells than the control group. Conversely, no cytotoxicity was observed for naringin and narirutin, even at concentrations up to 10 times (250 μM) higher than 25 μM. Consequently, concentrations of naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) were selected for subsequent experiments.

Effects of naringenin, naringin, and narirutin on LPS-induced TNF-α and IL-1β production

The impact of naringenin, naringin, and narirutin on the production of LPS-induced cytokines TNF-α and IL-1β in RAW264.7 cells is depicted in Fig. 2. In the LPS-induced group, notably increased levels of TNF-α and IL-1β were observed. Treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) significantly inhibited TNF-α production. At TNF-α expression levels, naringin (25 μM) and narirutin (25 μM) were notably lower than naringenin (25 μM), whereas narirutin (25 μM) showed significantly lower TNF-α expression than naringin (25 μM). Moreover, narirutin (250 μM) exhibited stronger inhibitory effects on TNF-α than naringin (250 μM). Concerning IL-1β levels, significant inhibition was observed with naringin (25 and 250 μM) and narirutin (25 and 250 μM). However, no significant differences were noted in IL-1β levels between naringin and narirutin at concentrations of 25 and 250 μM, respectively.

Effects of naringenin, naringin, and narirutin on LPS-triggered production of NO

The impact of naringenin, naringin, and narirutin on LPS-induced NO production in RAW264.7 cells is illustrated in Fig. 3. The LPS-induced group exhibited a significant increase in NO production compared to the untreated group. Treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) notably suppressed NO production in LPS-stimulated RAW264.7 cells. Specifically, naringenin (25 μM) displayed a higher inhibitory effect on NO compared to naringin (25 μM). However, no significant difference was observed between naringenin and narirutin at the 25 μM concentration. Notably, the most substantial inhibition of NO was observed in the narirutin (250 μM) group.

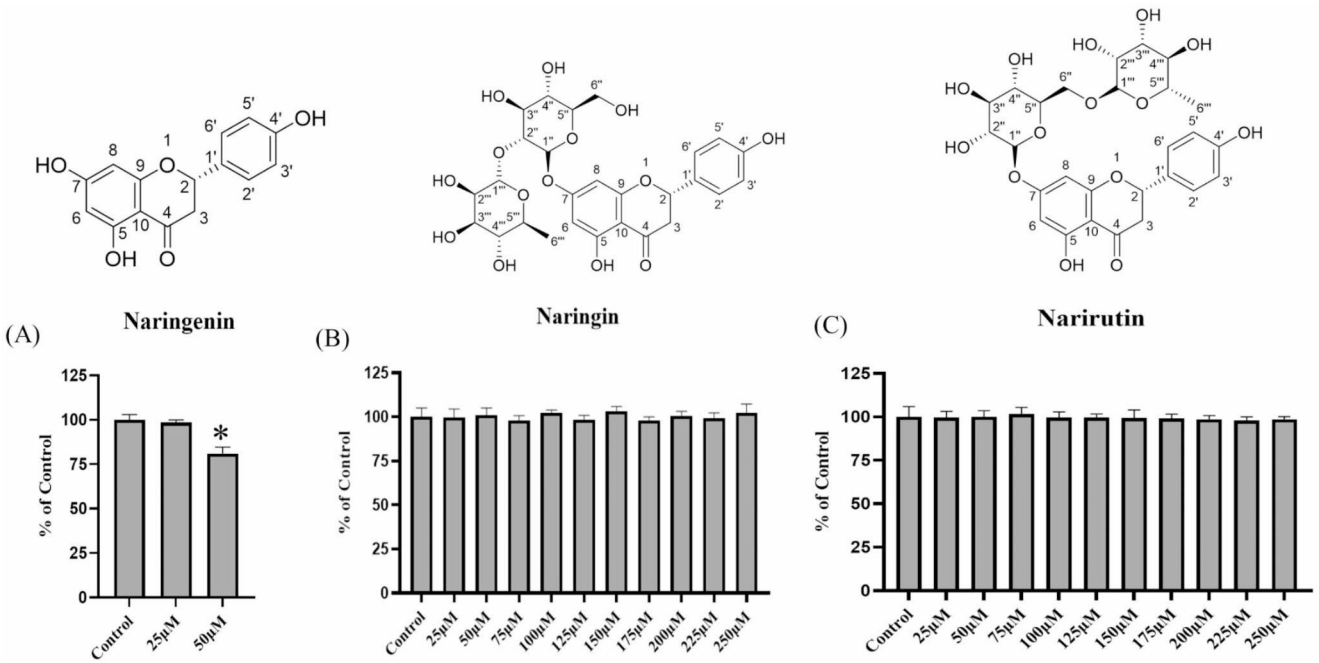

Fig. 1 Cell viability of naringenin, naringin, and narirutin in RAW264.7 cells. (**A**) RAW264.7 cells were treated with varying concentrations of naringenin for 24 h. (**B**) RAW264.7 cells were treated with different concentrations of naringin for 24 h. (**C**) RAW264.7 cells were treated with various concentrations of narirutin for 24 h. * indicates significant differences compared with the control group ($P < 0.05$)

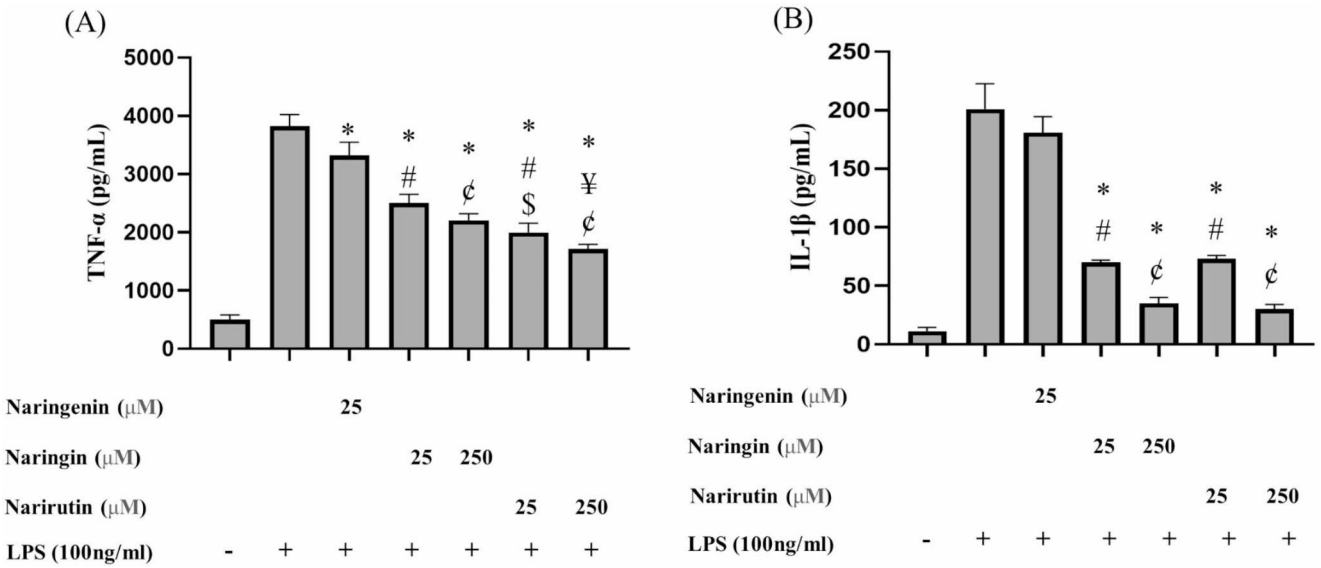

Fig. 2 Inhibition effects of naringenin, naringin, and narirutin on LPS-stimulated production of TNF-α (**A**) and IL-1β (**B**) in RAW264.7 cells. RAW264.7 cells were treated with naringenin (25 μM), naringin (25 and 250 μM), or narirutin (25 and 250 μM) for 1 h followed by LPS induction (100 μg/mL) for 24 h. * indicates groups significantly lower than the LPS-treated group ($P < 0.05$). # indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $ indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥ indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$)

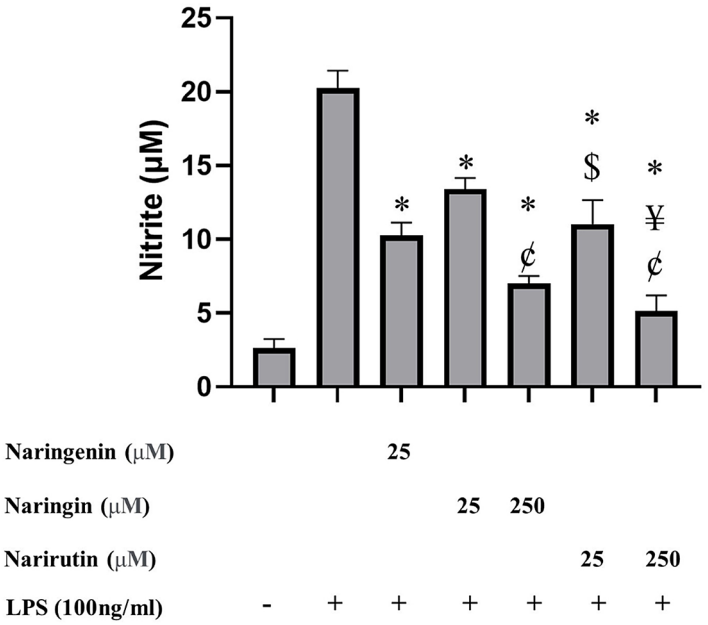

Fig. 3 Inhibition effects of naringenin, naringin, and narirutin on LPSstimulated production of NO in RAW264.7 cells. RAW264.7 cells were treated with naringenin (25 μM), naringin (25 and 250 μM), or narirutin (25 and 250 μM) for 1 h followed by LPS induction (100 μg/mL) for 24 h. * indicates groups significantly lower than the LPS-treated group ($P < 0.05$). # indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $ indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥ indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$)

Effects of naringenin, naringin, and narirutin on LPS-triggered expressions of iNOS and COX-2

Figure 4 illustrates the effects of naringenin, naringin, and narirutin on LPS-induced expressions of iNOS and COX-2 in RAW264.7 cells. Treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) significantly inhibited iNOS expression in LPS-triggered RAW264.7 cells. Specifically, naringenin (25 μM) exhibited a higher inhibitory effect on iNOS than naringin (25 μM), while no significant difference was observed between naringenin and narirutin at the 25 μM concentration. Notably, the most substantial inhibition of iNOS occurred with narirutin (250 μM) in LPS-induced RAW264.7 cells. Furthermore, treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) significantly suppressed COX-2 expression in LPS-stimulated RAW264.7 cells. Particularly, naringin (25 μM) and narirutin (25 μM) displayed

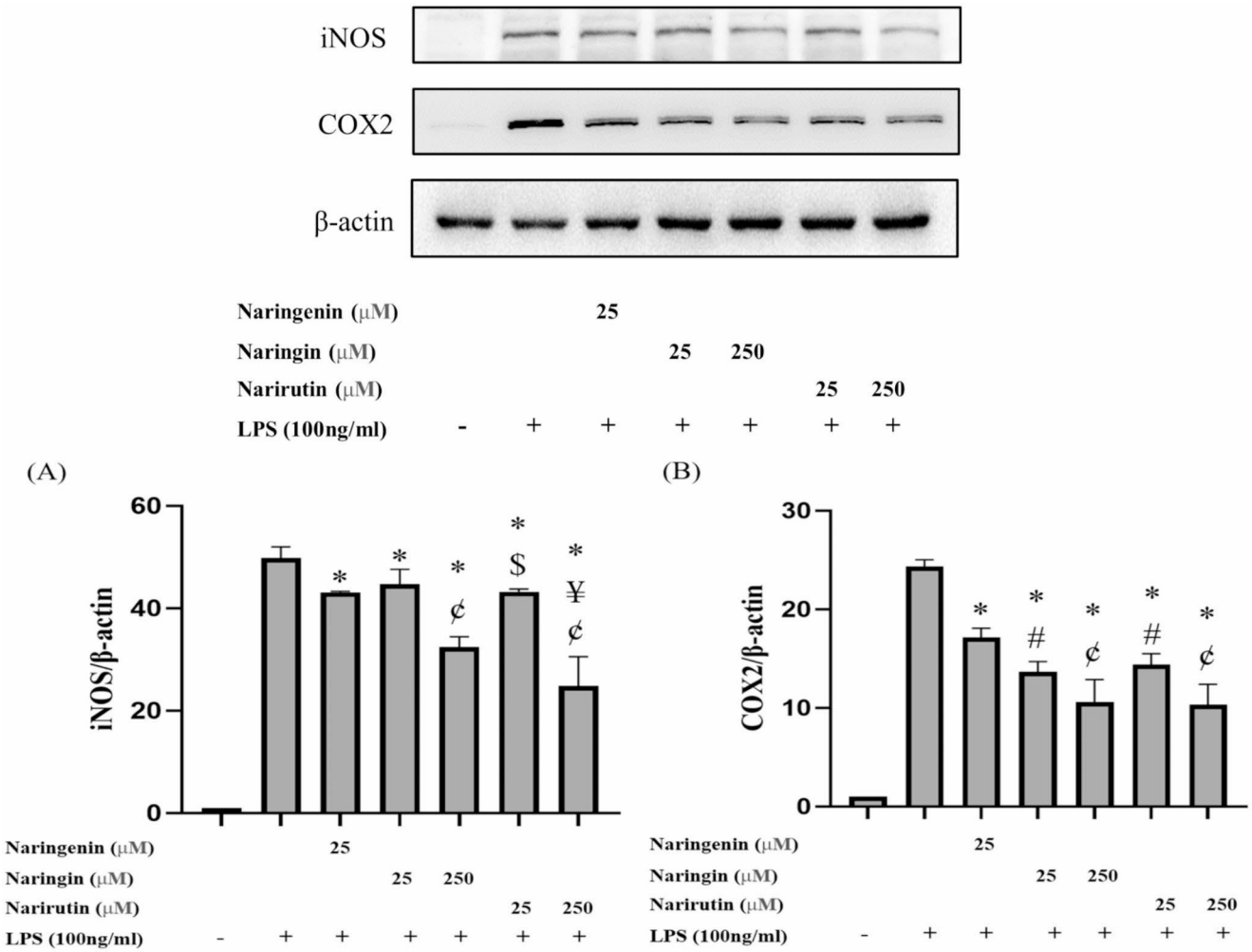

Fig. 4 Inhibition effects of naringenin, naringin, and narirutin on LPS-stimulated expression of iNOS (**A**) and COX2 (**B**) in RAW264.7 cells. RAW264.7 cells were treated with naringenin (25 μM), naringin (25 and 250 μM), or narirutin (25 and 250 μM) for 1 h followed by LPS induction (100 μg/mL) for 24 h. *indicates groups significantly lower than the LPS-treated group ($P < 0.05$). # indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $ indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$)

stronger inhibitory effects on COX-2 than did naringenin (25 μM). No significant difference was noted between the effects of naringin and narirutin at concentrations of 25 and 250 μM, respectively.

Effects of naringenin, naringin, and narirutin on LPS-induced activation of NF-κB

Figure 5 illustrates the effects of naringenin, naringin, and narirutin on LPS-triggered phosphorylation of p65 and degradation of IkBα in RAW264.7 cells. Treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) significantly attenuated LPS-induced phosphorylation level of p65. Notably, naringin (25 μM) and narirutin (25 μM) exhibited significantly lower phosphorylation levels of p65 than naringenin (25 μM), with narirutin (25 μM) showing the lowest phosphorylated p65 levels among the groups. Moreover, at a concentration of 250 μM, narirutin displayed a stronger inhibitory effect on phosphorylated p65 than naringin (250 μM). Furthermore, the degradation of IkBα was notably inhibited by naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM). Specifically, the expression levels of IkBα were significantly higher with naringin (25 μM) and narirutin (25 μM) than with naringenin (25 μM), with narirutin (25 μM) exhibiting higher IkBα expression than naringin (25 μM). At a concentration of 250 μM, narirutin demonstrated a stronger inhibitory effect on IkBα degradation than naringin (250 μM).

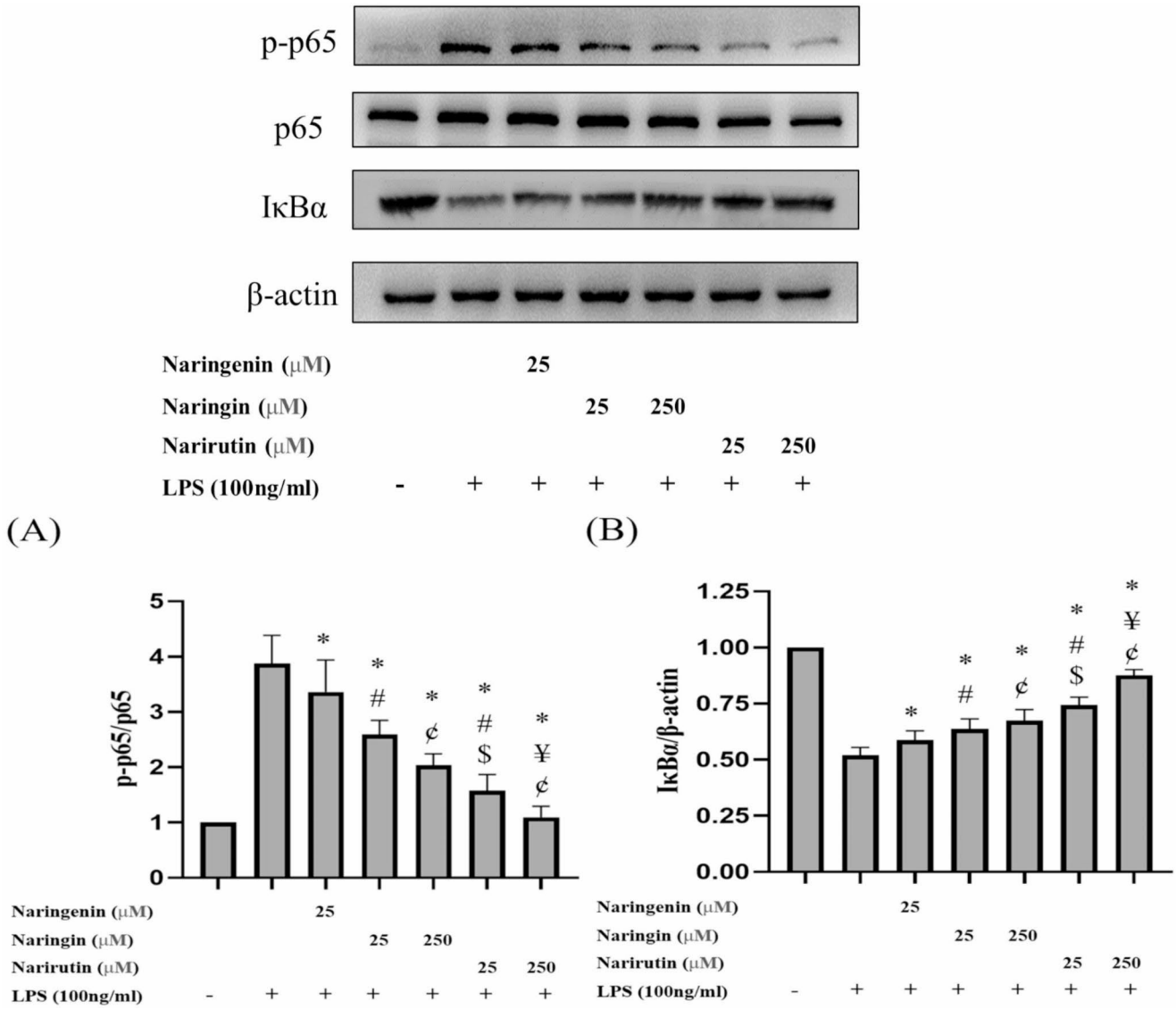

Fig. 5 Inhibition effects of naringenin, naringin, and narirutin on LPS-stimulated phosphorylation of p65 (**A**) and degradation of IκBα (**B**) in RAW264.7 cells. RAW264.7 cells were treated with naringenin (25 μM), naringin (25 and 250 μM), or narirutin (25 and 250 μM) for 1 h followed by LPS induction (100 μg/mL) for 24 h. * indicates groups significantly lower than the LPS-treated group ($P < 0.05$). # indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥ indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). In Fig. 5B, * indicates groups significantly higher than the LPS-treated group ($P < 0.05$). # indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥ indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$)

Effects of naringenin, naringin, and narirutin on LPS-triggered phosphorylation of MAPK

Figure 6 depicts the effects of naringenin, naringin, and narirutin on LPS-induced phosphorylation of JNK, p38, and ERK in RAW264.7 cells. Treatment with naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM) significantly attenuated the LPS-induced phosphorylation of p38. There was no significant difference between the effects of naringenin (25 μM), naringin (25 μM), and naringin (250 μM). Notably, narirutin demonstrated the strongest inhibition of p38 phosphorylation among the treatments. Additionally, the LPS-stimulated phosphorylation of ERK was significantly attenuated by naringin (250 μM), and narirutin (25 and 250 μM). Narirutin (25 and 250 μM) showed higher inhibitory effect on the phosphorylation of ERK than naringin (250 μM). LPS-stimulated phosphorylation

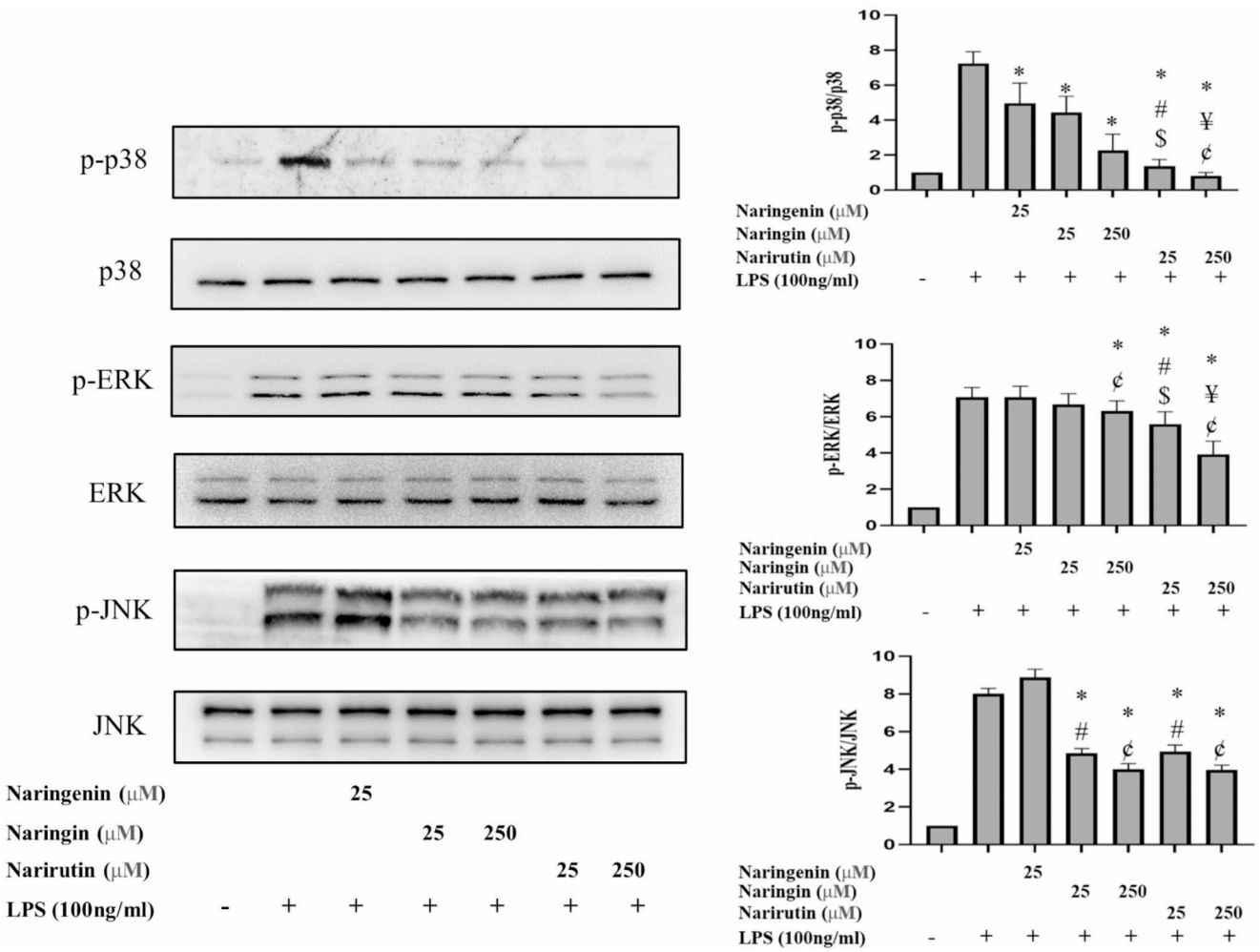

Fig. 6 Inhibition effects of naringenin, naringin, and narirutin on LPSstimulated phosphorylation of p38 (**A**), ERK (**B**), and p38 (**C**) in RAW264.7 cells. RAW264.7 cells were treated with naringenin (25 μM), naringin (25 and 250 μM), or narirutin (25 and 250 μM) for 1 h followed by LPS induction (100 μg/mL) for 24 h. *indicates groups significantly lower than the LPS-treated group ($P < 0.05$). #indicates that naringin (25 μM) or narirutin (25 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$). $indicates that narirutin (25 μM) is significantly lower than naringin (25 μM) ($P < 0.05$). ¥ indicates that narirutin (250 μM) is significantly lower than naringin (250 μM) ($P < 0.05$). ¢ indicates that naringin (250 μM) or narirutin (250 μM) is significantly lower than naringenin (25 μM) ($P < 0.05$)

form of JNK was significantly attenuated by naringenin (25 μM), naringin (25 and 250 μM), and narirutin (25 and 250 μM). Although naringenin (25 μM) showed a higher phosphorylation level of JNK than the LPS-induced group, both naringin (25 and 250 μM) and narirutin (25 and 250 μM) displayed similar inhibitory effects on JNK phosphorylation at the same concentrations.

Discussion

Flavonoids have garnered significant interest due to their diverse health benefits, encompassing antioxidant and anti-inflammatory effect [12, 13]. These compounds, along with their derivatives, are classified based on their chemical structures, such as the arrangement of hydroxyl (OH) moiety, methoxy substitutions, and the presence of aglycone or glycoside forms [17]. Glycosylation, a prevalent biological process, involves enzymatically attaching glycosides to molecules [26], serving various functions, such as stabilizing protein structure, increasing hydrophilicity, and protecting molecules against oxidation [27, 28]. Most natural flavonoids in plants exist in glycoside form, adding one or more sugars to their aglycones [18]. Naringin and narirutin, glycoside forms of naringenin, possess identical molecular weights, chemical formulas, and similar chemical structures [29]. However, the impact of glycosylation on naringenin and its different glycoside forms regarding anti-inflammatory effects remains understudied. This study sought to comparatively analyze naringenin, naringin, and narirutin to assess their anti-inflammatory effects.

Cell viability assay is a common method used to determine cytotoxicity of substances based on the measurement of differences between the treatment and non-treatment groups. In this study, naringenin exhibited stronger cytotoxicity than naringin and narirutin, evident from an approximately 80% survival rate of RAW 264.7 cells after 24 h of treatment with naringenin (50 µM). However, even at five times higher concentrations (250 µM), naringin and narirutin showed no detectable cytotoxicity in RAW 264.7 cells. No apparent difference in cytotoxicity was observed between naringin and narirutin in this study. These findings indicate that naringin and narirutin demonstrated significantly lower cytotoxicity than their aglycone form, naringenin. Similar trends have been observed with other flavonoids such as quercetin, kaempferol, and myricetin, showcasing stronger cytotoxic activity against myeloid lineage blood cancer cells than their glycoside forms [30]. Furthermore, glycoside forms of certain chemotherapy medications, such as glufosfamide derived from ifosfamide, have shown reduced toxicity compared with their original cytotoxic drugs [31]. Thus, glycosylation at the hydroxyl moiety of C7 in naringenin significantly mitigated cytotoxicity in RAW 264.7 cells, whereas the specific binding sites between glucose and rhamnose in naringenin glycosides (naringin and narirutin) did not notably affect cytotoxicity in RAW 264.7 cells.

Cytokines, a group of signaling proteins, regulate immune responses, including infection and inflammation [32]. Proinflammatory cytokines, released by immune cells like helper T cells and macrophages, play pivotal roles in inflammatory responses [33]. Overproduction of proinflammatory cytokines such as TNF-α and IL-1β is linked to inflammatory diseases such as cancer and rheumatoid arthritis [34, 35]. Hence, inhibiting their production presents a promising therapeutic approach. In this study, naringenin, naringin, and narirutin exhibited significant inhibition of LPS-induced TNF-α production in RAW264.7 cells. The inhibition sequence observed was narirutin > naringin > naringenin, suggesting that flavonoid glycosylation and the disaccharide binding sites in naringin and narirutin influenced TNF-α production inhibition. Moreover, unlike naringenin, both naringin and narirutin significantly inhibited IL-1β production at 25 and 250 µM, indicating that glycosylation at the hydroxyl moiety of C7 in naringenin significantly increased its potency in inhibiting IL-1β. Similar trends of higher inhibition effects on TNF-α and IL-1β are observed in myricetin glycosides compared with myricetin [36].

NO, a vital endogenously produced free radical, mediates various body functions [37]. However, excessive NO production has been reported to be associated with diseases, cytotoxicity, and inflammation [38, 39]. Three isoforms of NO synthase (NOS) primarily generate NO: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [37]. Among these three, iNOS is chiefly responsible for inflammatory NO production [40]. Macrophage activation by IFN-γ, IL-1β, TNF-α, and LPS induces iNOS expression, resulting in excessive NO production [41]. This study found that naringenin, naringin, and narirutin could inhibit iNOS expression and suppress NO production in LPS-stimulated RAW264.7 cells. These results demonstrated that naringenin, naringin, and narirutin limited NO overproduction through the inhibition of iNOS expression. At 25 µM, naringenin and narirutin exhibited stronger inhibitory effects than naringin, suggesting naringenin's higher potency in iNOS and NO inhibition compared with its flavonoid glycoside, naringin, in specific cases. Similarly, kaempferol appears to exhibit a stronger inhibitory effect on NO than its glycosides [42], while myricetin displays lower inhibitory effect than its glycosides against NO production in LPS-stimulated RAW264.7 cells [36]. Notably, the stronger inhibition observed with narirutin at 250 µM implies that the glucose and rhamnose binding sites in naringenin glycosides (naringin and narirutin) influenced iNOS and NO inhibition.

COX-2 plays a crucial role in inflammatory responses, converting arachidonic acid into the precursor of prostaglandin E2 (PGE2) [43]. PGE2, abundantly present in inflamed tissues, contributes to inflammatory pain [44]. Overexpression of COX-2 has been reported in inflammatory cells, such as mast cell and macrophages, and certain cancers [45]. This has prompted the use of COX-2 inhibitors as anti-inflammatory drugs to relieve pain and fever and reduce inflammation [43]. In this study, treatments with naringenin, naringin, and narirutin significantly inhibited COX-2 expression in LPS-stimulated RAW264.7 cells. Nevertheless, naringin and narirutin exhibited stronger inhibitory effects than naringenin at same concentration. Similarly, 3-O-glycosylation of myricetin enhances COX-2 inhibition compared to myricetin in LPS-stimulated RAW264.7 cells [36]. Conversely, kaempferol shows higher COX-2 inhibition compared to its glycosides [42]. Moreover, there was no significant difference in COX-2 expression inhibition between naringin and narirutin at 25 and 250 µM. Thus, these results suggest that glycosylation at the hydroxyl moiety of C7 in naringenin significantly inhibited LPS-induced COX-2 expression in RAW264.7 cells compared to naringenin.

The NF-κB and MAPK cell signaling pathways play crucial roles in inflammation when the TLR4 receptor binds to LPS [7]. In the NF-κB pathway, NF-κB is a transcription factor complex regulating genes involved in immune and inflammatory responses. The expression of proinflammatory mediators, including iNOS, COX-2, TNF-α, and IL-1β, is regulated by NF-κB [9]. IκBα, a protein in this pathway, inhibits the NF-κB transcription factor [46]. However, when

stimulated by various factors such as TNF-α and LPS, IκBα dissociates from the NF-κB complex (p65 and p50) and is then degraded via the proteasome-dependent pathway [46]. The active NF-κB complex enters the nucleus, binding to the target gene and initiating the transcription of proinflammatory mediators. This study revealed that naringenin, naringin, and narirutin inhibited p65 phosphorylation by blocking IκBα degradation, suggesting that three compounds reduce the production of proinflammatory mediators, such as iNOS, COX-2, TNF-α, and IL-1β through NF-κB pathway inhibition. Notably, naringenin glycosides displayed significantly higher NF-κB inhibition effects. Moreover, narirutin exhibited stronger NF-κB inhibition than naringin. These results highlight how flavonoid glycosylation, along with the disaccharide binding sites in their glycoside forms, affects NF-κB inhibition in RAW264.7 cells, influencing the magnitude of anti-inflammatory effects.

The MAPK signaling pathway orchestrates various biological processes, including stress response, apoptosis, and inflammation [47]. Within mammalian cells, three MAPK subfamilies—p38, ERKs, and JNKs—have been delineated [47]. Activation of the MAPK pathway occurs in response to diverse extracellular and intracellular stimuli, such as TNF-α, IL-1β, and LPS [7]. This pathway serves as an upstream signal in the inflammatory cascade, impacting the expressions of proinflammatory mediators, including iNOS, COX-2, TNF-α, and IL-1β [48, 49]. In this study, treatments with naringenin, naringin, and narirutin significantly inhibited LPS-induced p38 phosphorylation in RAW264.7 cells. The inhibitory trend for phosphorylated p38 was observed as narirutin > naringin > naringenin at 25 μM and narirutin > naringin at 250 μM, suggesting that flavonoid glycosides (naringin and narirutin) exert stronger inhibition on phosphorylated p38 than flavonoid aglycone (naringenin), indicating that the disaccharide structure of flavonoid glycosides influences this inhibition. However, only naringin (250 μM) and narirutin (25 and 250 μM) inhibited ERK phosphorylation in LPS-stimulated RAW264.7 cells, indicating that the presence and binding form of the disaccharide moiety in flavonoid glycosides (naringin and narirutin) potentially affect ERK phosphorylation inhibition. Furthermore, naringin and narirutin suppressed JNK phosphorylation in LPS-stimulated RAW264.7 cells at 25 μM and 250 μM, while naringenin did not inhibit phosphorylated JNK, instead inducing JNK phosphorylation. These results suggest that the substituent group of the disaccharide moiety and its binding site in flavonoid glycosides might impact phosphorylated JNK inhibition. Despite their similar chemical structures, naringenin and its glycosides (naringin and narirutin) exhibited markedly different effects in inhibiting the MAPK pathway. This implies that variations in the chemical structures of flavonoid glycosides and their aglycones may yield diverse mechanisms and intensities of the anti-inflammatory effect.

This study's limitation lies in the absence of in vivo experiments comparing the anti-inflammatory effects of naringenin and its glycosides. Future studies should focus on conducting these experiments to assess the effects in LPS-induced inflammatory and other pathological animal models [50].

Conclusion

Throughout this study, narirutin emerged as the most potent anti-inflammatory agent among the compounds tested in several assays (TNF-α, NO, and iNOS), when compared with its aglycone (naringenin) and naringin. The observed mechanism behind this effectiveness involved the inhibition of NF-κB and MAPK pathways within RAW 264.7 cells (Fig. 7). Naringin suppressed the expressions of proinflammatory mediators through the inhibition of NF-κB and MAPK pathways in RAW264.7 cells. In contrast, naringenin displayed a comparatively weaker anti-inflammatory effect than its glycosides (naringin and narirutin), primarily by inhibiting NF-κB and reducing phosphorylated forms of p38. This study provides insight into the crucial role of naringenin glycosylation and the differential binding sites within the disaccharide of naringenin glycosides, suggesting their significant influence on modulating the intensity of the anti-inflammatory effects in RAW264.7 cells.

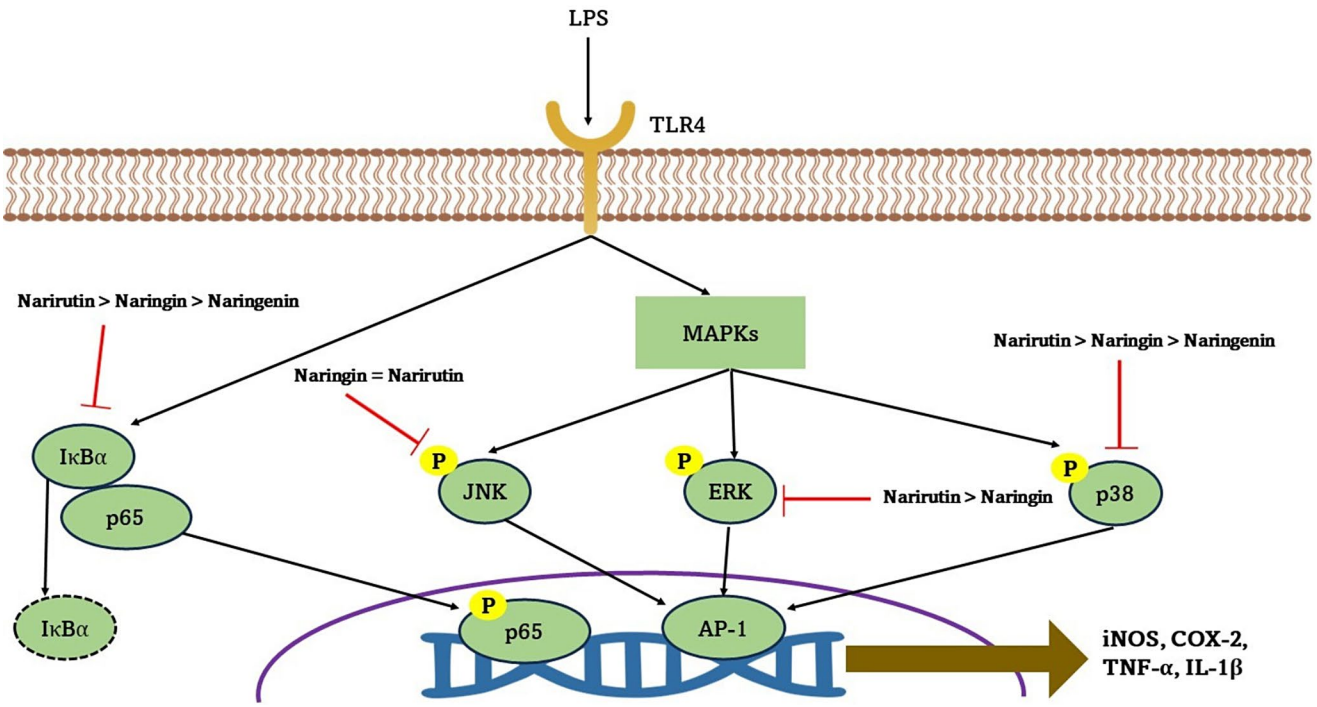

Fig. 7 Anti-inflammatory mechanisms of Naringenin, Naringin, and Narirutin on LPS-stimulated inflammation in RAW264.7 cells. Narirutin exhibited an anti-inflammatory effect via inhibition of the pathways of NF-κB and MAPK in RAW 264.7 cells. Naringin exhibited an anti-inflammatory effect via inhibition of the pathways of NF-κB and MAPK in RAW 264.7 cells. Naringenin exhibited an anti-inflammatory effect via inhibition of the pathways of NF-κB and the phosphorylation level of p38

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-023-09147-0.

Author contributions S-C.C.: designed, performed, and wrote project. S-Y.S.: supervised the project. S-C.C. and S-Y.S.: confirmed the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Funding This study was supported by Interdisciplinary Research Center on Material and Medicinal Chemistry, National Cheng Kung University, Tainan, Taiwan.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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