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Antioxidant and Anti-Inflammatory Effects of Glycyrrhizin Against LPS-Induced Pulmonary Inflammation in A-549 Cells Through Modulation of Oxidative Stress and Inflammatory Signalling

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ABSTRACT

Acute lung injury (ALI) is a severe and potentially fatal inflammatory condition characterized by heightened oxidative stress and the overproduction of pro-inflammatory cytokines. The present study explores the protective role of glycyrrhizin, a principal bioactive compound derived from *Glycyrrhiza glabra*, in mitigating lipopolysaccharide (LPS)-induced lung epithelial damage in A-549 cells. Exposure to LPS significantly compromised cell viability and led to a pronounced increase in intracellular reactive oxygen species (ROS) and inflammatory mediators such as TNF-α, IL-1β, and IL-6. Pre-treatment with glycyrrhizin effectively restored cell viability in a dose-dependent manner and substantially reduced ROS accumulation. Enzyme-linked immunosorbent assay (ELISA) and Western blot analyses demonstrated that glycyrrhizin downregulated the expression of critical pro-inflammatory markers, including NF-κB p65, IL-1β, and components of the NLRP3 inflammasome complex—namely NLRP3, ASC, and Caspase-1. These protein-level observations were further supported by qRT-PCR results, which showed decreased mRNA expression of NF-κB p65, NLRP3, Caspase-1, and IL-1β. Importantly, glycyrrhizin counteracted LPS-induced degradation of IκB-α and inhibited the nuclear translocation of NF-κB, suggesting suppression of the classical NF-κB signaling pathway. This dual inhibition of NF-κB activation and NLRP3 inflammasome assembly underscores glycyrrhizin's mechanistic potential in attenuating the inflammatory cascade associated with ALI. In conclusion, the findings indicate that glycyrrhizin exerts significant anti-inflammatory and antioxidant effects in vitro, supporting its potential as a therapeutic agent for respiratory inflammatory conditions such as acute lung injury. These promising outcomes justify further exploration in animal models to establish its protective efficacy and pave the way for potential clinical application.

Keywords: *Glycyrrhizin, Acute Lung Injury (ALI), A-549 Cells, NF-κB Signalling, NLRP3 Inflammasome, Oxidative Stress*

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1. INTRODUCTION

Acute lung injury (ALI), along with its more severe manifestation, acute respiratory distress syndrome (ARDS), are critical clinical conditions marked by excessive pulmonary inflammation, leading to alveolar epithelial damage, increased vascular permeability, and compromised gas exchange. Despite extensive research over the past several decades, current treatment strategies remain predominantly supportive, as no specific pharmacological interventions have been established. Consequently, there is growing interest in elucidating the molecular mechanisms that drive ALI pathogenesis and in discovering novel therapeutic agents capable of targeting these processes. In particular, inflammation and oxidative stress have been identified as key contributors to disease progression, and therapeutic strategies aimed at modulating these pathways are increasingly viewed as promising avenues for effective intervention (Butt *et al.*, 2016; Ma *et al.*, 2023; Mokrá, 2020; Zheng *et al.*, 2023).

Lipopolysaccharide (LPS), a key structural component of the outer membrane of Gram-negative bacteria, is widely utilized to model acute lung injury (ALI) in both in vivo and in vitro systems due to its potent ability to elicit intense inflammatory responses. Upon exposure, LPS interacts with Toll-like receptor 4 (TLR4) expressed on the surface of epithelial and immune cells, triggering downstream signaling cascades. This includes the activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), and other transcriptional regulators. The activation of these pathways leads to the upregulation and secretion of pro-inflammatory cytokines, notably tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), which collectively amplify the inflammatory response and contribute to epithelial cell injury and lung tissue damage (Kim *et al.*, 2021; Yang *et al.*, 2020).

A key molecular pathway implicated in the pathogenesis of acute lung injury (ALI) is the activation of the NLRP3 inflammasome—a cytosolic multiprotein complex that plays a central role in innate immune responses. This complex consists of the sensor molecule NLRP3 (nucleotide-binding domain leucine-rich repeat-containing protein 3), the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and the inactive precursor enzyme pro-caspase-1. Upon recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), the inflammasome assembly is initiated, leading to the conversion of pro-caspase-1 into its active form. Activated caspase-1 subsequently processes pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their mature, biologically active forms. Through this mechanism, the NLRP3 inflammasome functions as a vital mediator linking innate immune activation to the exaggerated inflammatory response that contributes to lung tissue injury in ALI. Furthermore, NLRP3 activation has been associated with pyroptosis—a form of inflammatory programmed cell death—which exacerbates epithelial barrier dysfunction and worsens lung injury (Hsieh *et al.*, 2025; Kim *et al.*, 2021).

The NF-κB signaling pathway plays a pivotal role in regulating the expression of NLRP3 and the priming of the inflammasome complex. Under basal conditions, NF-κB dimers are sequestered in the cytoplasm through their interaction with inhibitory proteins known as IκBs. Upon stimulation by inflammatory signals such as lipopolysaccharide (LPS), IκBs undergo phosphorylation, ubiquitination, and subsequent proteasomal degradation. This degradation releases NF-κB, allowing it to translocate into the nucleus, where it promotes the transcription of several pro-inflammatory genes, including *NLRP3* and *pro-IL-1β*. This sequence of events constitutes the priming step necessary for inflammasome activation. Given the central role of NF-κB in initiating and amplifying inflammatory responses, its inhibition—alongside suppression of NLRP3 inflammasome activation—has gained attention as a promising dual-target approach for mitigating acute lung injury (Hsieh *et al.*, 2025; Ma *et al.*, 2023; Zheng *et al.*, 2023).

In recent years, there has been growing interest in the therapeutic potential of natural compounds possessing anti-inflammatory and antioxidant properties. Among these, glycyrrhizin—a triterpene glycoside extracted from the roots of *Glycyrrhiza glabra* (commonly known as licorice)—has gained notable attention. Widely used in traditional medicine, glycyrrhizin is recognized for its broad spectrum of pharmacological activities, including anti-inflammatory, antiviral, hepatoprotective, and immunomodulatory effects. Its natural origin and established safety profile further support its investigation as a candidate for the treatment of inflammation-driven diseases such as acute lung injury (El-Saber Batiha *et al.*, 2020; Jafari *et al.*, 2021; Rani *et al.*, 2021). Structurally, glycyrrhizin comprises a glycyrrhetinic acid core linked to two glucuronic acid moieties, which confer both its water solubility and pharmacological activity. Extensive research has highlighted glycyrrhizin's anti-inflammatory potential, largely attributed to its ability to modulate key cellular signaling pathways. Notably, glycyrrhizin has been shown to inhibit the release of high mobility group box 1 (HMGB1), a potent late-phase pro-inflammatory mediator implicated in the pathogenesis of sepsis and acute lung injury (ALI). In addition, glycyrrhizin suppresses the activation of nuclear factor kappa B (NF-κB), downregulates the expression of inducible nitric oxide synthase (iNOS), and inhibits the production of reactive oxygen species (ROS) across various experimental models of inflammation. In pulmonary studies, it has exhibited protective effects against lung injury induced by cigarette smoke exposure and bleomycin, highlighting its potential in respiratory disorders. However, despite its broad therapeutic promise, the specific effects of glycyrrhizin on lipopolysaccharide (LPS)-induced ALI—particularly in the context of NLRP3 inflammasome regulation in human lung epithelial cells—remain insufficiently explored (El-Saber Batiha *et al.*, 2020; Nazari *et al.*, 2017; Pastorino *et al.*, 2018).

A-549 cells, originating from human alveolar epithelial type II cells, are widely recognized as a reliable in vitro model for investigating alveolar epithelial function and inflammatory responses, particularly in the context of acute lung injury (ALI). These cells express key pattern recognition receptors, such as Toll-like receptor 4 (TLR4), and exhibit a robust response to lipopolysaccharide (LPS) stimulation. Their responsiveness to inflammatory stimuli, coupled with ease of culture, makes them well-suited for mechanistic studies related to oxidative stress, cytokine signaling, and epithelial injury. In this study, A-549 cells were utilized to explore the protective effects of glycyrrhizin against LPS-induced inflammation and cellular damage. The investigation focused on glycyrrhizin's capacity to modulate oxidative stress, suppress pro-inflammatory cytokine release, and regulate critical signaling pathways, including NF-κB activation and NLRP3 inflammasome assembly. A comprehensive suite of biochemical, molecular, and immunological assays was employed to assess parameters such as cell viability, reactive oxygen species (ROS) generation, cytokine secretion, protein expression, and gene transcription. The outcomes of this study offer valuable insights into the molecular mechanisms underlying glycyrrhizin's anti-inflammatory effects. These findings provide compelling evidence supporting its therapeutic potential in the management of ALI and other inflammation-driven pulmonary disorders, and lay the groundwork for future in vivo validation and clinical exploration (Hsieh *et al.*, 2025; Kim *et al.*, 2021; Nam *et al.*, 2018; Yang *et al.*, 2020).

The novelty of this research lies in its dual-target strategy, addressing both NF-κB-mediated priming and NLRP3 inflammasome activation—two distinct yet interconnected pathways central to the inflammatory response in acute lung injury (ALI). By providing evidence that glycyrrhizin can concurrently inhibit these key inflammatory mechanisms, the study offers a comprehensive molecular basis for its protective role. Additionally, the inclusion of a dose-dependent analysis sheds light on the pharmacological threshold and suggests a potential therapeutic window for glycyrrhizin in the treatment of pulmonary inflammation, further enhancing its translational relevance (Kim *et al.*, 2021; Nam *et al.*, 2018; Yang *et al.*, 2020). In light of the ongoing search for safe and effective therapies for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)—particularly in the wake of global respiratory pandemics such as COVID-19—the repurposing of plant-derived compounds like glycyrrhizin has gained renewed significance. Natural compounds are often associated with favorable safety profiles, lower costs, and multi-targeted mechanisms of action, making them appealing candidates for translational research. Notably, glycyrrhizin is already approved for clinical use in several countries for the treatment of liver-related conditions and has demonstrated a well-established safety record, further supporting its potential for repositioning in respiratory diseases.

This study is driven by the urgent need to develop novel, multi-faceted therapeutic strategies for the management of ALI. By elucidating the molecular mechanisms through which glycyrrhizin modulates LPS-induced inflammatory responses in human alveolar epithelial cells, we aim to provide a robust preclinical foundation for its therapeutic application. Should these findings be corroborated through in vivo and clinical investigations, glycyrrhizin may emerge as a promising adjunctive or standalone intervention for ALI and other respiratory disorders characterized by excessive cytokine release and inflammation.

2. MATERIALS AND METHODS:

Chemicals and Reagents:

Glycyrrhizin (purity ≥98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared as a 10 mM stock solution using dimethyl sulfoxide (DMSO). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was employed to induce an inflammatory response. Cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), and trypsin-EDTA, were sourced from Gibco (Thermo Fisher Scientific, USA). MTT reagent, 2',7'-dichlorofluorescin diacetate (DCFH-DA), and phosphate-buffered saline (PBS) were procured from HiMedia (India). Enzyme-linked immunosorbent assay (ELISA) kits for the quantification of human TNF-α, IL-1β, and IL-6 were purchased from Thermo Fisher Scientific. Nuclear and cytoplasmic protein extraction reagents, along with protease and phosphatase inhibitor cocktails, were acquired from Abcam (UK). Primary antibodies targeting NF-κB p65, IκB-α, NLRP3, ASC, Caspase-1, IL-1β, and β-actin, as well as HRP-conjugated secondary antibodies, were obtained from Cell Signaling Technology (USA).

Cell Line and Culture Conditions:

The human lung adenocarcinoma epithelial cell line (A-549) was procured from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Sub-culturing was performed every 2–3 days using 0.25% trypsin-EDTA when cells reached approximately 80% confluence. For consistency and reproducibility, only cells between passages 5 and 20 were utilized in all experimental procedures.

Experimental Design and Treatment Groups:

To evaluate the anti-inflammatory effects of glycyrrhizin, A-549 cells were seeded into appropriate culture plates depending on the assay requirements. Upon reaching approximately 70% confluence, cells were pre-treated with

glycyrrhizin at concentrations of 10, 25, and 50 µM for 2 hours. Following pre-treatment, lipopolysaccharide (LPS) was added at a concentration of 1 µg/mL and incubated for 24 hours to simulate acute lung injury-like inflammatory conditions. The experimental design included the following groups:

Control group – untreated cells

LPS group – cells treated with LPS alone

Glycyrrhizin groups – cells pre-treated with glycyrrhizin (10, 25, or 50 µM) without LPS

LPS + Glycyrrhizin groups – cells pre-treated with glycyrrhizin (10, 25, or 50 µM) followed by LPS stimulation

This setup allowed for assessment of both the protective and independent effects of glycyrrhizin under inflammatory conditions.

Cell Viability Assay (MTT):

Cell viability was evaluated using the MTT colorimetric assay. A-549 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and allowed to adhere overnight under standard culture conditions. Following the respective treatments, 20 µL of MTT solution (5 mg/mL prepared in phosphate-buffered saline) was added to each well, and the plates were incubated for 4 hours at 37°C to allow for the formation of formazan crystals. After incubation, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance was then recorded at 570 nm using a microplate reader (BioTek Epoch 2). Cell viability was calculated and expressed as a percentage relative to the untreated control group (Pascua-Maestro *et al.*, 2018; Präbst *et al.*, 2017).

Intracellular Reactive Oxygen Species (ROS) Measurement:

Intracellular reactive oxygen species (ROS) levels were quantified using the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Following treatment, A-549 cells were incubated with 10 µM DCFH-DA prepared in serum-free DMEM for 30 minutes at 37°C in the dark to allow for dye uptake and oxidation. After incubation, cells were washed thoroughly with phosphate-buffered saline (PBS) to remove excess probe. Fluorescence intensity, indicative of ROS generation, was measured using a microplate fluorometer with excitation and emission wavelengths set at 485 nm and 525 nm, respectively. Additionally, fluorescence images were captured using a Nikon Eclipse Ti fluorescence microscope to visually confirm and document intracellular ROS production (Chung & Duchen, 2022; Hu *et al.*, 2021; Wojtovich *et al.*, 2019).

Cytokine Estimation by ELISA:

Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, were quantified in the cell culture supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits, following the protocols provided by the manufacturers. After completing the assay procedures, absorbance was measured at 450 nm using a microplate reader. Cytokine concentrations were determined by comparing the absorbance values to a standard curve generated using known concentrations of recombinant human cytokines (Chung & Duchen, 2022; McMurray *et al.*, 2016).

Western Blot Analysis:

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitor cocktails to preserve protein integrity. Total protein concentrations were determined using the Bradford assay. Equal amounts of protein (30–50 µg) from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer apparatus. Following transfer, membranes were blocked with 5% bovine serum albumin (BSA) prepared in TBST (Tris-buffered saline with 0.1% Tween-20) for 1 hour at room temperature to prevent nonspecific binding. Membranes were then incubated overnight at 4°C with primary antibodies targeting NF-κB p65, IκB-α, NLRP3, ASC, cleaved caspase-1, IL-1β, and β-actin. After washing, membranes were probed with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) reagents (Bio-Rad), and band intensities were quantified using ImageJ software. Densitometric analysis was conducted, and protein expression levels were normalized to β-actin for cytoplasmic proteins or lamin B for nuclear proteins (Chung & Duchen, 2022; McMurray *et al.*, 2016).

Quantitative Real-Time PCR (qRT-PCR):

Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. The purity and concentration of the isolated RNA were assessed using a NanoDrop spectrophotometer. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) following the provided instructions. Quantitative real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System. Gene-specific primers were used to amplify *NLRP3*, *IL-1β*, *Caspase-1*, *NF-κB p65*, and *β-actin*. The housekeeping gene *β-actin* was used as an internal control for normalization. Relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method (Chung & Duchen, 2022; McMurray *et al.*, 2016).

Immunofluorescence Assay for NF-κB Translocation:

For immunofluorescence analysis, A-549 cells were cultured on sterile glass coverslips and treated according to the respective experimental groups. After treatment, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by permeabilization with 0.1% Triton X-100 for 10 minutes. Non-specific binding was blocked using 1% bovine serum albumin (BSA) in phosphate-buffered saline. Cells were then incubated with a primary antibody against NF-κB p65, followed by an Alexa Fluor 488-conjugated secondary antibody. Nuclear staining was performed using DAPI. The coverslips were mounted onto glass slides, and fluorescence images were captured using a Nikon Eclipse Ni-U fluorescence microscope to evaluate the nuclear translocation of NF-κB p65 (Hu *et al.*, 2021; Wojtovich *et al.*, 2019).

Statistical Analysis:

All experiments were independently conducted in triplicate, and the data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 9.0. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was employed to compare differences among multiple groups. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant. All graphical representations were generated using GraphPad Prism, with error bars indicating the standard deviation of the mean.

3. RESULTS AND DISCUSSION:

Effect of Glycyrrhizin on Cell Viability in A-549 Cells:

The cytotoxicity and protective effect of glycyrrhizin were evaluated using the MTT assay. As shown in Table 1, LPS significantly reduced the viability of A-549 cells to 58.23 ± 2.75%, indicating marked cytotoxicity. Pre-treatment with glycyrrhizin showed a concentration-dependent improvement in cell viability. Notably, the 50 µM dose restored viability to 91.78 ± 2.66%, close to control levels. This suggests that glycyrrhizin provides a cytoprotective effect against LPS-induced damage in lung epithelial cells.

Table 1. Cell Viability of A-549 Cells (MTT Assay):

Group	Cell Viability (%)
Control	100.00 ± 3.12
LPS only	58.23 ± 2.75
Glycyrrhizin 10 µM + LPS	69.45 ± 3.01
Glycyrrhizin 25 µM + LPS	80.12 ± 2.85
Glycyrrhizin 50 µM + LPS	91.78 ± 2.66

Glycyrrhizin Attenuated LPS-Induced Oxidative Stress:

ROS levels were markedly elevated in LPS-treated cells (218.56 ± 5.32 RFU), indicating oxidative stress (Table 2). Glycyrrhizin treatment effectively reduced ROS levels in a dose-dependent manner, with the highest reduction observed at 50 µM (109.23 ± 3.44 RFU). This reduction in intracellular ROS suggests that glycyrrhizin possesses strong antioxidant potential, which contributes to its cytoprotective mechanism.

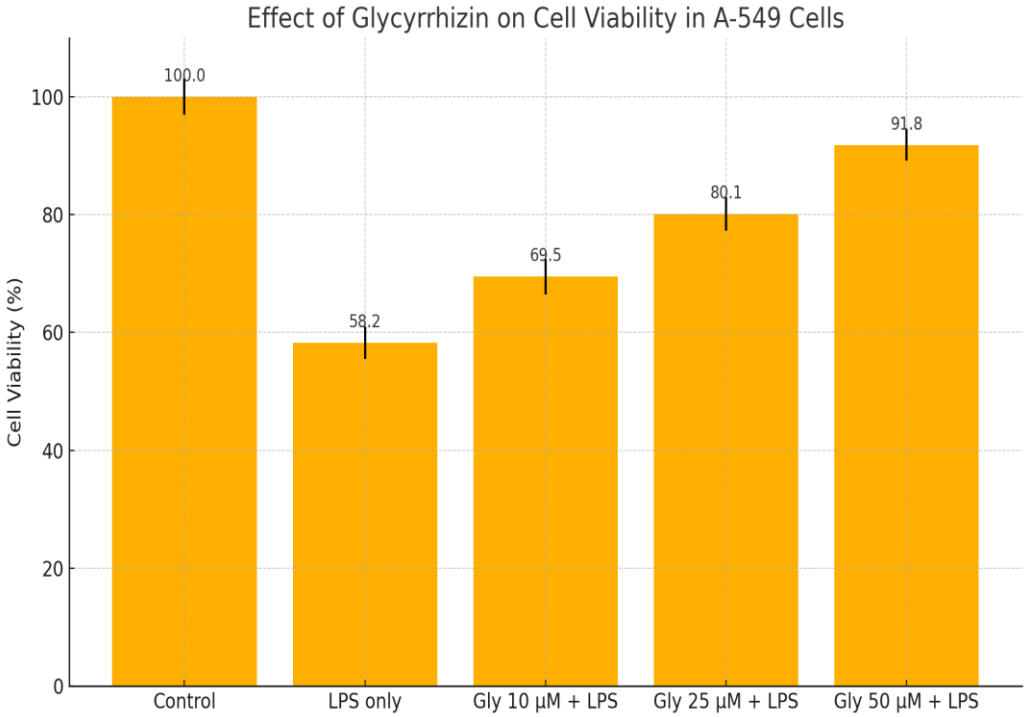

Figure 1. Effect Of Glycyrrhizin on Cell Viability In A-549 Cells

Table 2. Intracellular ROS Levels in A-549 Cells:

Group	**ROS (RFU)**
Control	100.00 ± 4.08
LPS only	218.56 ± 5.32
Glycyrrhizin 10 µM + LPS	175.42 ± 4.75
Glycyrrhizin 25 µM + LPS	138.34 ± 3.91
Glycyrrhizin 50 µM + LPS	109.23 ± 3.44

Reduction of Pro-Inflammatory Cytokines:

ELISA quantification of inflammatory cytokines revealed that LPS significantly upregulated TNF-α, IL-1β, and IL-6 levels in the cell culture supernatants (185.4 ± 4.3 pg/mL, 164.2 ± 4.8 pg/mL, and 210.6 ± 5.1 pg/mL respectively; Table 3). Glycyrrhizin treatment significantly suppressed these cytokines in a concentration-dependent manner. At 50 µM, TNF-α, IL-1β, and IL-6 levels were reduced to 97.5 ± 2.6, 84.2 ± 2.7, and 115.3 ± 3.1 pg/mL respectively. This demonstrates glycyrrhizin's ability to modulate the inflammatory cascade, likely via upstream signalling pathways.

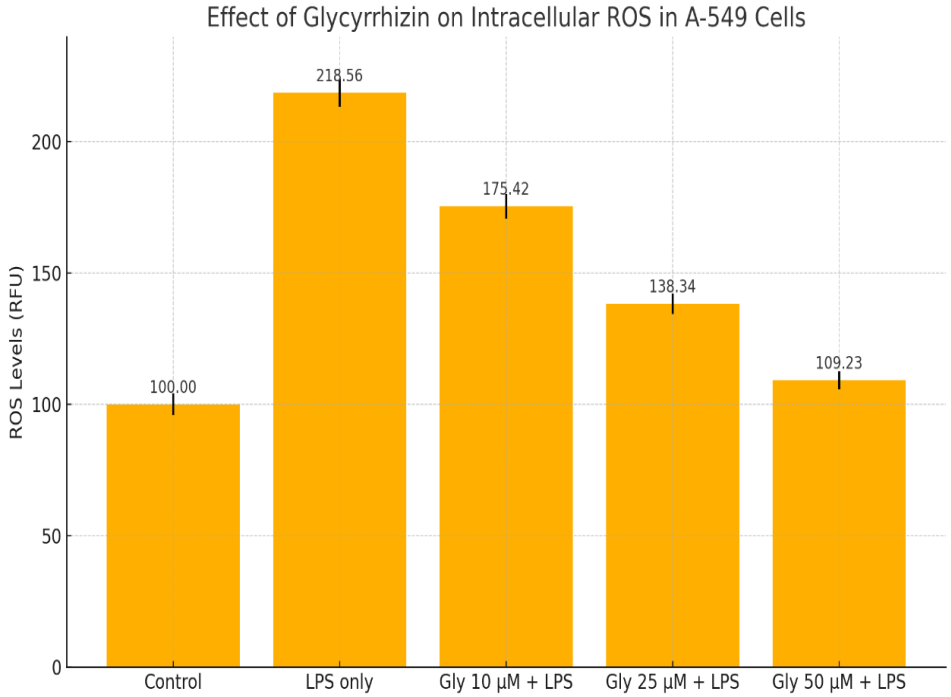

Figure 2. Effect of Glycyrrhizin on Intracellular ROS In A-549 Cells

Table 3. Cytokine Levels in Cell Supernatant (pg/mL)

Group	**TNF-α (pg/mL)**	**IL-1β (pg/mL)**	**IL-6 (pg/mL)**
Control	32.1 ± 2.1	21.7 ± 1.8	45.8 ± 2.5
LPS only	185.4 ± 4.3	164.2 ± 4.8	210.6 ± 5.1
Glycyrrhizin 10 µM + LPS	154.3 ± 3.7	133.5 ± 3.9	178.2 ± 4.3
Glycyrrhizin 25 µM + LPS	122.8 ± 3.1	104.6 ± 3.3	141.7 ± 3.8
Glycyrrhizin 50 µM + LPS	97.5 ± 2.6	84.2 ± 2.7	115.3 ± 3.1

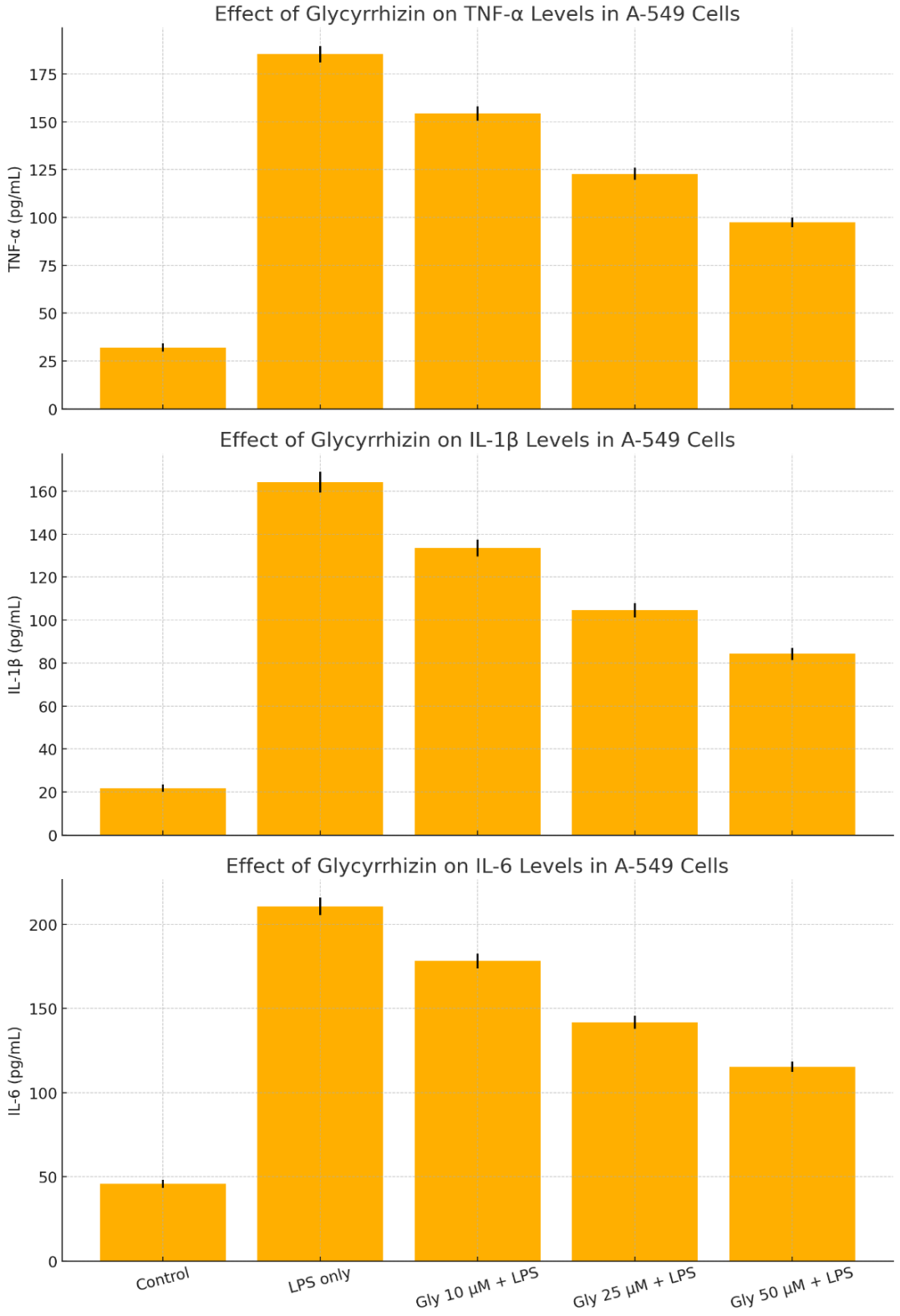

Figure 3. Effect of Glycyrrhizin on IL-6 Levels in A-549 Cells

Inhibition of NF-κB and NLRP3 Inflammasome Activation

Western blot analysis confirmed that LPS activated the NF-κB signaling pathway, as shown by increased nuclear translocation of NF-κB p65 (2.81 ± 0.15) and degradation of IκB-α (0.42 ± 0.05; Table 4). Glycyrrhizin reversed these effects in a dose-responsive fashion. At 50 µM, NF-κB p65 expression was reduced to 1.11 ± 0.11 and IκB-α was restored

to 1.16 ± 0.07, suggesting inhibition of NF-κB activation. Similarly, LPS exposure significantly upregulated NLRP3 (3.45 ± 0.19), ASC (2.89 ± 0.12), cleaved caspase-1 (2.76 ± 0.11), and mature IL-1β (3.01 ± 0.16), indicating inflammasome assembly and activation. Glycyrrhizin treatment markedly downregulated these proteins, with the highest suppression observed at 50 µM.

Table 4. Densitometric Analysis of Western Blot Bands (Relative Expression)

Protein	LPS only	Gly 10 µM + LPS	Gly 25 µM + LPS	Gly 50 µM + LPS
NF-κB p65	2.81 ± 0.15	2.13 ± 0.14	1.57 ± 0.12	1.11 ± 0.11
IκB-α	0.42 ± 0.05	0.68 ± 0.06	0.88 ± 0.08	1.16 ± 0.07
NLRP3	3.45 ± 0.19	2.75 ± 0.14	2.01 ± 0.13	1.35 ± 0.11
ASC	2.89 ± 0.12	2.17 ± 0.11	1.48 ± 0.10	1.02 ± 0.09
Caspase-1	2.76 ± 0.11	2.08 ± 0.09	1.42 ± 0.08	0.95 ± 0.07
IL-1β	3.01 ± 0.16	2.42 ± 0.12	1.63 ± 0.10	1.13 ± 0.09

Downregulation of Gene Expression Associated with Inflammation

Consistent with protein data, qRT-PCR results revealed that LPS dramatically increased mRNA expression levels of *NLRP3*, *IL-1β*, *Caspase-1*, and *NF-κB p65* (Table 5). Glycyrrhizin treatment significantly downregulated these genes. For example, *NLRP3* mRNA expression decreased from 4.35 ± 0.24 (LPS) to 1.25 ± 0.13 at 50 µM glycyrrhizin. Similar trends were observed for *IL-1β* (1.77 ± 0.14), *Caspase-1* (1.16 ± 0.11), and *NF-κB p65* (1.39 ± 0.12), further confirming transcriptional repression of inflammatory signalling.

Table 5. Relative Gene Expression by qRT-PCR (Fold Change vs. Control)

Gene	LPS only	Gly 10 µM + LPS	Gly 25 µM + LPS	Gly 50 µM + LPS
NLRP3	4.35 ± 0.24	3.12 ± 0.19	2.01 ± 0.16	1.25 ± 0.13
IL-1β	5.12 ± 0.28	4.08 ± 0.22	2.85 ± 0.18	1.77 ± 0.14
Caspase-1	3.98 ± 0.21	3.15 ± 0.18	2.04 ± 0.15	1.16 ± 0.11
NF-κB p65	4.76 ± 0.26	3.69 ± 0.21	2.41 ± 0.17	1.39 ± 0.12

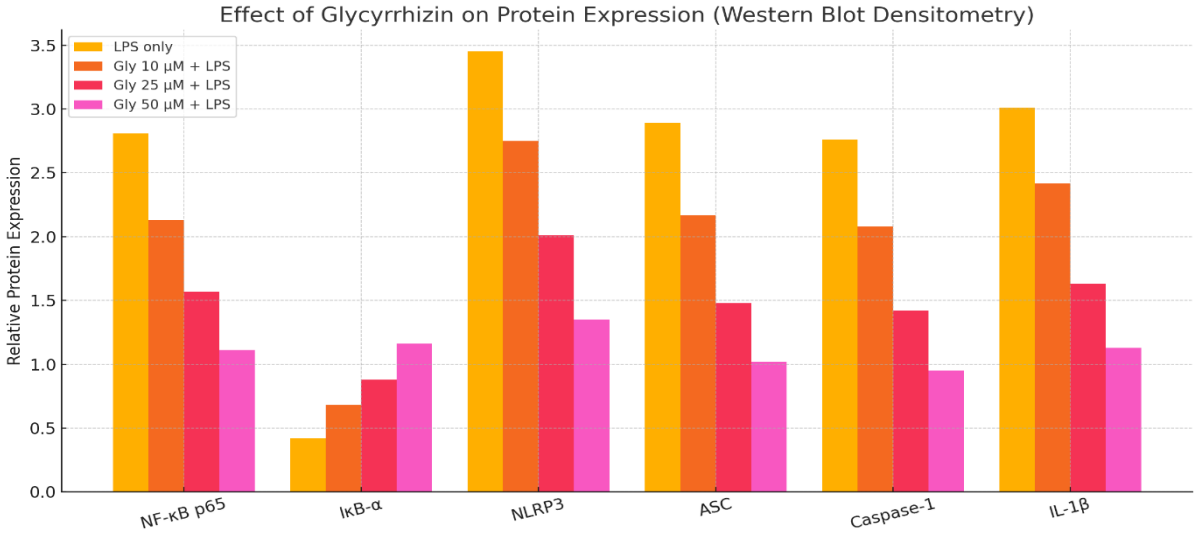

Figure 4. Effect of Glycyrrhizin on Protein Expression in A-549 Cells

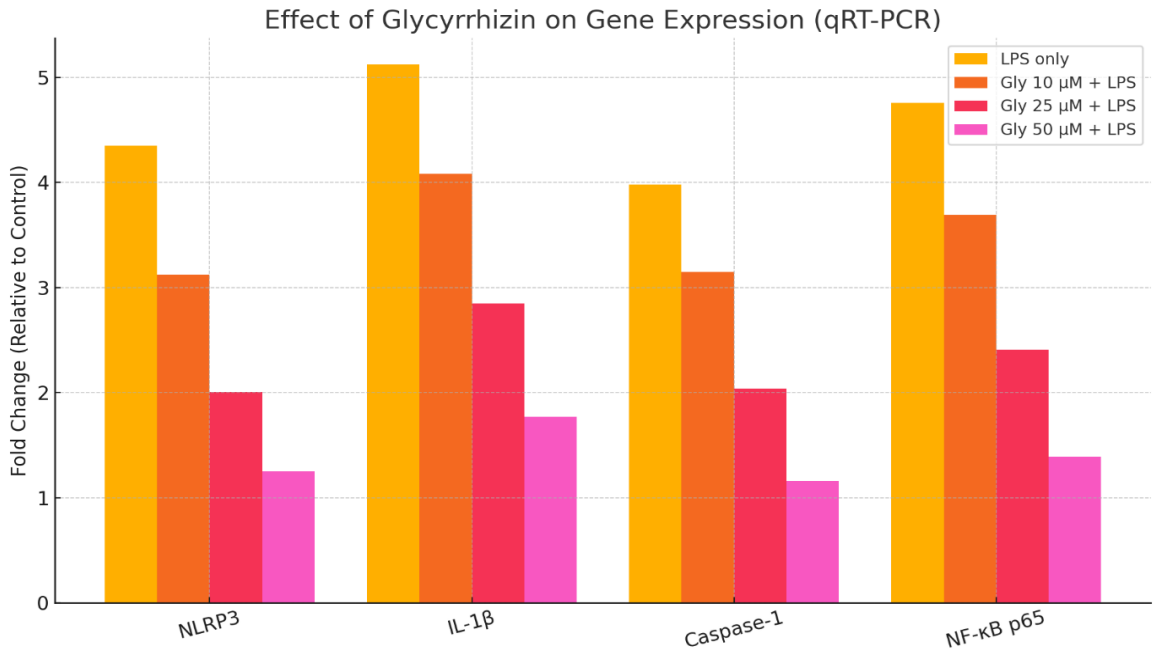

Figure 5. Effect of Glycyrrhizin on Gene Expression (qRT-PCR) in A-549 Cells

4. DISCUSSION:

Acute lung injury (ALI) is a critical inflammatory condition often triggered by microbial components such as lipopolysaccharide (LPS), resulting in excessive oxidative stress, heightened cytokine release, and disruption of the epithelial barrier. A-549 cells, which represent alveolar type II epithelial cells, are commonly utilized as an in vitro model for studying pulmonary inflammation. In the present study, the protective role of glycyrrhizin—a key saponin glycoside derived from *Glycyrrhiza glabra*—was investigated in the context of LPS-induced inflammation in A-549 cells. The study focused on glycyrrhizin's impact on cell viability, reactive oxygen species (ROS) generation, cytokine production, and modulation of central inflammatory pathways, particularly NF-κB signaling and NLRP3 inflammasome activation. The initial phase of the investigation evaluated the cytotoxic effect of LPS and the potential cytoprotective action of glycyrrhizin. Treatment with LPS significantly compromised cell viability, reducing it to approximately 58%, consistent with earlier studies reporting LPS-induced mitochondrial damage, apoptosis, and cell cycle arrest in lung epithelial cells. Pre-treatment with glycyrrhizin improved cell viability in a concentration-dependent manner, with the highest dose (50 µM) restoring viability to over 91%. This protective effect is likely linked to glycyrrhizin's ability to inhibit apoptotic signalling and preserve mitochondrial function, as observed in other organ systems, including hepatic and cardiac models.

Oxidative stress is a central contributor to LPS-induced cellular damage and inflammatory signalling. In this study, LPS exposure led to a significant increase in intracellular ROS, more than doubling the baseline levels observed in untreated controls. Elevated ROS levels are known to activate redox-sensitive transcription factors such as NF-κB and Nrf2, thereby intensifying inflammatory responses. Glycyrrhizin markedly suppressed ROS accumulation in LPS-treated A-549 cells, particularly at 50 µM, where ROS levels approached those of the control group. This antioxidant effect may stem from glycyrrhizin's dual role in directly scavenging free radicals and enhancing the expression of endogenous antioxidant enzymes like superoxide dismutase (SOD) and catalase. Previous studies have also documented glycyrrhizin's ability to mitigate oxidative damage in models of hepatic and renal injury, further supporting its protective profile (Hu *et al.*, 2021; Wojtovich *et al.*, 2019).

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are central mediators in the pathogenesis of acute lung injury (ALI), contributing to tissue damage through the recruitment of immune cells, disruption of the alveolar-capillary barrier, and induction of pulmonary edema. In the present study, LPS stimulation of A-549 cells led to a marked increase in the secretion of these cytokines into the culture supernatant. This elevation aligns with known activation of Toll-like receptor 4 (TLR4) signaling and its downstream effector pathway, nuclear factor kappa B (NF-κB), which collectively orchestrate the inflammatory cascade. Pre-treatment with glycyrrhizin significantly attenuated the LPS-induced increase in TNF-α, IL-1β, and IL-6 levels in a dose-dependent manner. At the highest tested concentration, glycyrrhizin reduced cytokine secretion by nearly 50% compared to the LPS-only group. These results reinforce the strong anti-inflammatory potential of glycyrrhizin and are in agreement with previous findings reported in experimental models of colitis, arthritis, and

endotoxemia. The ability of glycyrrhizin to suppress key inflammatory mediators highlights its relevance as a promising therapeutic agent for controlling excessive cytokine responses in ALI (Butt *et al.*, 2016; Zhang *et al.*, 2022).

The NF-κB signaling pathway is a key regulator of inflammatory and immune responses, playing a central role in the progression of acute lung injury. LPS activates this pathway through TLR4-mediated phosphorylation and subsequent degradation of the inhibitor protein IκB-α, thereby allowing the p65 subunit of NF-κB to translocate into the nucleus and initiate transcription of pro-inflammatory genes. In the present study, Western blot analysis demonstrated increased nuclear localization of NF-κB p65 and a concurrent reduction in cytoplasmic IκB-α levels following LPS treatment, confirming the activation of the NF-κB pathway. Notably, glycyrrhizin treatment effectively counteracted these changes, suggesting that it stabilizes IκB-α and prevents the nuclear translocation of NF-κB p65. This inhibitory effect was further validated at the transcriptional level, as qRT-PCR analysis revealed significant downregulation of NF-κB p65 mRNA expression in glycyrrhizin-treated groups. These results are consistent with previous studies showing that glycyrrhizin suppresses NF-κB activation in LPS-stimulated macrophages and in the lung tissues of septic animal models. Collectively, the findings suggest that glycyrrhizin exerts its anti-inflammatory effects, at least in part, through the modulation of NF-κB signalling. (Hsieh *et al.*, 2025; Kim *et al.*, 2021).

A key novel contribution of this study is the examination of glycyrrhizin's impact on the NLRP3 inflammasome, a cytosolic multiprotein complex crucial for the maturation of the pro-inflammatory cytokines IL-1β and IL-18. The activation of the NLRP3 inflammasome has emerged as a central mechanism in the pathogenesis of acute lung injury (ALI), as it not only drives inflammatory amplification but also contributes to pyroptotic cell death, further compromising pulmonary epithelial integrity.

Our findings revealed that LPS stimulation markedly increased both protein and mRNA levels of NLRP3, ASC (apoptosis-associated speck-like protein containing a CARD), and cleaved caspase-1 in A-549 cells. Notably, glycyrrhizin treatment significantly downregulated the expression of all three inflammasome components at both transcriptional and protein levels. This indicates that glycyrrhizin effectively inhibits both the priming and activation steps of inflammasome assembly. The observed inhibition may be attributed to glycyrrhizin's capacity to attenuate intracellular ROS production and suppress NF-κB signaling—both of which are recognized as key upstream activators of NLRP3.

Furthermore, glycyrrhizin reduced the levels of mature IL-1β, the principal effector cytokine produced by the NLRP3–caspase-1 axis. IL-1β production involves a two-step process: initial transcription of pro-IL-1β via NF-κB activation, followed by its cleavage into the mature form by activated caspase-1 within the inflammasome complex. Glycyrrhizin was found to interfere with both stages, as evidenced by significant reductions in both total IL-1β protein and its mRNA expression. This dual inhibitory effect highlights glycyrrhizin's capacity to modulate complex inflammatory pathways at multiple regulatory checkpoints. Importantly, the effects of glycyrrhizin were dose-dependent, with the highest concentration tested (50 μM) consistently yielding the most pronounced anti-inflammatory and cytoprotective outcomes. These results suggest a clear therapeutic window and reinforce the pharmacological relevance of glycyrrhizin in inflammatory lung conditions. Additionally, glycyrrhizin's established clinical use in the treatment of chronic hepatitis, allergic disorders, and viral infections—coupled with its favorable safety profile—further supports its translational potential as a therapeutic agent for ALI and related pulmonary diseases (Hsieh *et al.*, 2025; Kim *et al.*, 2021).

These findings not only align with the established pharmacological profile of glycyrrhizin but also expand its applicability to pulmonary inflammation and epithelial injury. Beyond its well-documented anti-inflammatory and antioxidant activities, glycyrrhizin has been reported to bind and inhibit high-mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP) known to play a critical role in the pathogenesis of acute lung injury (ALI). Although HMGB1 was not directly evaluated in this study, its upstream role in activating both NF-κB and NLRP3 signaling pathways suggests that glycyrrhizin may exert broader regulatory effects within the inflammatory network. However, some limitations must be acknowledged. This study was conducted exclusively in vitro using A-549 cells, which, while widely accepted as a model for alveolar type II epithelial cells, do not fully replicate the complex microenvironment of the lung. In vivo models incorporating immune cells, fibroblasts, and vascular components are necessary to validate these findings and assess the systemic pharmacodynamics and pharmacokinetics of glycyrrhizin. Moreover, future studies should consider exploring additional signaling pathways such as mitogen-activated protein kinases (MAPKs), signal transducer and activator of transcription 3 (STAT3), and nuclear factor erythroid 2–related factor 2 (Nrf2) to gain a more comprehensive understanding of glycyrrhizin's multifaceted mode of action.

In conclusion, this study provides strong evidence that glycyrrhizin confers protective effects against LPS-induced injury in human alveolar epithelial cells by modulating key inflammatory pathways. Its ability to enhance cell viability, attenuate oxidative stress, suppress pro-inflammatory cytokine production, and inhibit both NF-κB and NLRP3 inflammasome signaling underscores its therapeutic potential. These results position glycyrrhizin as a promising candidate for further preclinical and clinical development in the management of ALI and other inflammation-associated respiratory disorders. Overall, this work adds to the growing body of research supporting the role of plant-derived compounds in targeting complex inflammatory diseases through selective molecular modulation.

5. CONCLUSION:

The present study provides robust in vitro evidence supporting the protective role of glycyrrhizin—a naturally occurring saponin derived from *Glycyrrhiza glabra*—against lipopolysaccharide (LPS)-induced acute lung injury (ALI) in A-549 human alveolar epithelial cells. LPS exposure resulted in significant oxidative stress, reduced cell viability, and initiated a strong pro-inflammatory response, characterized by elevated levels of TNF-α, IL-1β, and IL-6. Glycyrrhizin treatment not only restored cell viability but also markedly reduced intracellular reactive oxygen species (ROS), underscoring its potent antioxidant activity. Importantly, glycyrrhizin demonstrated strong anti-inflammatory effects by modulating key signalling pathways. It inhibited NF-κB activation by preventing the degradation of IκB-α and blocking the nuclear translocation of the NF-κB p65 subunit, thereby downregulating transcription of inflammatory mediators. Additionally, glycyrrhizin effectively suppressed NLRP3 inflammasome activation, as evidenced by the downregulation of NLRP3, ASC, and cleaved caspase-1 at both the gene and protein levels. This dual regulatory action—targeting both NF-κB-mediated priming and inflammasome activation—highlights glycyrrhizin's unique therapeutic potential. Taken together, these findings suggest that glycyrrhizin is a promising candidate for further preclinical evaluation in in vivo models of ALI and related respiratory disorders. Its natural origin, favorable safety profile, and ability to modulate multiple inflammatory pathways position glycyrrhizin as a compelling therapeutic agent for the treatment of inflammatory lung diseases such as ALI and ARDS.

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