

Integrative discovery of *Licorice*-derived phytochemicals as potential anti-inflammatory adjuvants targeting immune signatures of *Klebsiella pneumoniae* infection identified by single-cell profiling

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Article Info



Article Type:
Original Article

Article History:
Received: 6 Feb. 2026
Revised: 2 Aug. 2026
Accepted: 9 Aug. 2026
ePublished: 3 Oct. 2026

Keywords:
Drug discovery
Molecular dynamics
Glycyrrhiza glabra
Klebsiella pneumoniae
Single-cell RNA-seq analysis

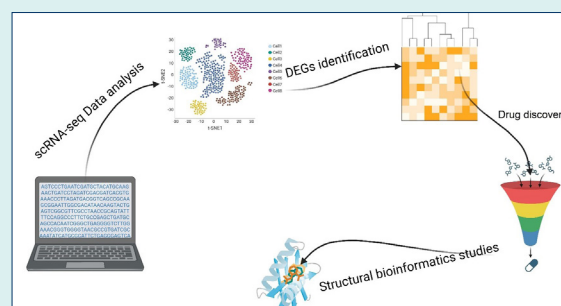
Abstract

Introduction: *Klebsiella pneumoniae* is a major cause of severe pneumonia where dysregulated inflammation contributes to tissue injury. *Glycyrrhiza glabra* (as known licorice) has long been used in traditional medicine to relieve respiratory conditions such as cough, bronchitis, and pneumonia-like symptoms, suggesting the presence of bioactive constituents with immunomodulatory and anti-inflammatory potential.

Methods: We analyzed single-cell RNA sequencing (scRNA-seq) data (GSE220594) from *K. pneumoniae*-infected and control samples to define differentially expressed genes (DEGs) across immune cell populations. Gene ontology (GO) and pathway enrichment analyses were then used to characterize immune response pathways. Subsequently, quantitative structure–activity relationship (QSAR) modeling was combined with molecular docking to clarify which *licorice*-derived phytochemicals might interact with potential key inflammatory triggers identified by DEG signatures. Top ligands were further assessed by all-atom molecular dynamics (MD) simulations to evaluate binding stability and structural dynamics of the ligand–receptor complexes.

Results: We identified 2,633 DEGs across major immune cell subsets. Enrichment analysis suggested strong involvement in cytokine-mediated signaling and host defense responses to bacterial and viral infection. Notably, Guanylate-Binding Protein 5 (GBP5) and its downstream inflammasome-related partners were significantly upregulated, potentially contributing to excessive inflammatory activation. Using QSAR and molecular docking, APIGENIN, NEOISOLQUIRITIN, and NEOLIQIRITIN from *licorice* root emerged as candidate anti-inflammatory compounds, with predicted binding free energies of -7.0 , -6.7 , and -6.0 kcal/mol against GBP5, respectively. Short MD simulations suggested that the APIGENIN–GBP5 complex is stable, hinting at a possible role in modulating inflammasome activation; however, experimental validation is needed.

Conclusion: This computational investigation suggests that *licorice*-derived phytochemicals can potentially modulate excessive inflammatory signaling during *K. pneumoniae*-induced pneumonia. The study provides a foundation for future experimental validation and the development of plant-based adjuvant therapies targeting inflammation in bacterial pneumonia.



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Introduction

Klebsiella pneumoniae holds significant clinical importance as a major pathogen responsible for various infections, especially in healthcare settings. It has gained notoriety due to its increasing antibiotic resistance, including carbapenem-resistant strains, posing serious treatment challenges.¹ This bacterium is characterized by the formation of lobar consolidation, extensive lung tissue damage, and often results in necrotizing pneumonia with hemorrhagic inflammation.² Pneumonia caused by *K. pneumoniae* is a severe lung infection known for its rapid progression and high morbidity and mortality rates.³

Inflammation triggered by pneumonia plays a crucial dual role in host defense and tissue injury. Upon infection, lung immune cells recognize bacterial components via pattern recognition receptors such as Toll-like receptors, leading to the activation of proinflammatory signaling pathways.⁴ This results in the release of cytokines and chemokines that recruit neutrophils and other immune cells to the infection site.⁵ While this inflammatory response is essential for bacterial clearance, uncontrolled inflammation contributes to respiratory failure.^{6,7} So, alleviation of the cytokine storm during the uncontrolled inflammation underscores the urgent need for innovative therapeutic strategies.

There are numerous medicinal plants with significant anti-inflammatory properties, which offers a valuable source of bioactive compounds. *Licorice* root is well known worldwide as a significant medicinal plant, having been used for centuries in traditional anti-inflammatory remedies across Asia and Europe.⁸⁻¹¹ *Licorice* is commonly used to relieve inflammatory conditions and serves as a flavoring and sweetening agent in foods and remedies. Its rich array of phytochemicals, particularly glycyrrhizin and various flavonoids like glabridin, supports multiple pharmacological effects, including antioxidant and anti-inflammatory properties.¹² Recent studies also indicate that phytochemicals derived from *Licorice* have immunomodulatory effects and may help reduce lung and systemic inflammation.¹³ However, the specific molecular mechanisms involved in pulmonary contexts are still being investigated and require further research for clinical application.

Recent advances in computational biology, including high-throughput sequencing and structural bioinformatics, have significantly enhanced our ability to understand disease mechanisms and explore the therapeutic potential of phytochemicals with molecular precision. The emergence of scRNA-seq has enabled transcriptomic profiling at the single-cell level, providing unprecedented insights into cellular diversity and dynamic responses in diseased tissues.¹⁴⁻¹⁶ In infection models, scRNA-seq helps

distinguish subpopulations of immune cells and analyze their specific gene expression profiles during pneumonia, thereby revealing mechanisms that drive individual variation in disease outcomes.

While recent studies have begun to elucidate the diversity and functional responses of immune cells during pneumonia, the molecular mechanisms driving uncontrolled inflammation in *K. pneumoniae* infection remain incompletely understood. Similarly, the specific molecular mechanisms underlying the anti-inflammatory potential of *licorice*-derived phytochemicals in lung inflammation remain poorly characterized. In this study, we analyzed publicly available scRNA-seq datasets from *K. pneumoniae*- and mock-infected lung samples to identify DEGs associated with infection-related immune responses. We then applied a QSAR and structure-based screening pipeline to evaluate *licorice* root-derived phytochemicals with predicted anti-inflammatory activity. Our integrative computational approach identifies apigenin, neoisoliquiritin, and neoliquiritin as promising candidates with predicted binding affinities for inflammasome-related targets, suggesting their potential to modulate excessive inflammatory signaling during *K. pneumoniae* infection.

Materials and Methods

Single cell expression profile analysis

Data acquisition

To clarify the DEGs associated with *K. pneumoniae* infection, we downloaded the transcriptomic dataset generated by Xu et al (GSE220594) from the Gene Expression Omnibus (GEO) repository.¹⁷ This study includes two experimental groups: mock-infected control and *K. pneumoniae*-infected lung tissue, which consists of 11,345 and 11,480 cells, respectively. Both datasets provide comprehensive mRNA expression profiles at single-cell resolution, enabling robust comparative analysis of transcriptional changes induced by the infection. A detailed summary of the datasets is presented in Table 1.

scRNA-seq data processing

The single-cell transcriptomic data were processed using the Seurat package (version 5.1.0) within RStudio (version 4.3.2).¹⁸ After merging the datasets, we excluded cells that exhibited more than 20% mitochondrial gene content, fewer than 800 total RNA counts, and fewer than 500 detected features. Additionally, genes expressed in fewer than 3 cells were removed to enhance the robustness of the analysis.

Normalization was conducted using the SCTransform() function to account for variability in sequencing depth. This was followed by data scaling using the ScaleData() function and the identification of highly variable genes through the FindVariableFeatures() function. Dimensional reduction

Table 1. A detailed description of study samples for identifying cell populations and DEGs

GSE series	Sample	Infection type	Animal model	Number of cells	Reference
GSE220594	GSM6807161	Mock-infected	Mice	11345	17
GSE220594	GSM6807162	<i>K. pneumoniae</i> -infected	Mice	11480	17

was performed using principal component analysis (RunPCA()) and Uniform Manifold Approximation and Projection (RunUMAP()). Potential batch effects were addressed using the Harmony package.¹⁹ Harmony was selected for batch effect correction because it effectively integrates single-cell datasets by repeatedly eliminating batch-related variation in a common low-dimensional representation. This method does not require predefined anchors or complete overlap between conditions. By maintaining genuine biological differences while reducing technical noise, Harmony minimizes the risk of over-correction and enhances the reliability of downstream analyses, such as clustering and differential expression. Lastly, doublet cells were identified and removed with the DoubletFinder package.²⁰

Cell clustering was conducted using the first 15 principal components with a resolution parameter set to 0.2. This was implemented using the FindNeighbors() and FindClusters() functions. For cell-type annotation, we referenced the Mouse Cell Atlas (MCA) with the SingleR package.²¹ To identify conserved marker genes, the FindConservedMarkers() function was utilized for robust comparisons across different conditions. Also, DEGs were identified by comparing *K. pneumoniae*-infected samples with mock-infected samples through the FindMarkers() function, applying the following thresholds: $|\log_2FC| > 1$, minimum expression percentage (min.pct) > 0.1 , and an adjusted *P* value < 0.05 . These thresholds were chosen to create a balance between biological relevance and statistical rigor. Specifically, a $|\log_2FC| > 1$ ensures that only genes with at least a 2-fold change are considered, thereby minimizing the inclusion of biologically insignificant transcriptional noise. An adjusted *p*-value < 0.05 , calculated to control the false discovery rate (FDR), reduces the risk of type I errors arising from multiple hypothesis testing and, additionally, sets a minimum.pct > 0.1 excludes genes expressed in fewer than 10% of cells in either condition, helping eliminate spurious signals from very low or patchy expression.

Functional and pathway enrichment analysis of DEGs

Functional enrichment analyses of DEGs were performed using the ClusterProfiler package.²² This analysis included GO term annotation, focusing on biological processes (BP), cellular components (CC), and molecular functions (MF). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was carried out using the KEGGgraph package.²³ Significantly enriched terms and pathways were identified using a *P* < 0.05 threshold and a minimum of 3 genes per term, ensuring both statistical reliability and biological relevance.

Pathway analysis

To identify key regulatory proteins that modulate downstream pathway components, pathway analysis was performed focusing on the GBP5 within the NOD-like receptor signaling cascade. Using the KEGG Mapper online tool "Search & Color Pathway" (<https://www.genome.jp/kegg/mapper/>), GBP5 was precisely mapped onto the corresponding pathway.²⁴ This approach enabled visualization of upstream and downstream interactors

whose leads to overexpression or suppression of GBP5, thereby providing insights into mechanisms controlling inflammation and highlight potential molecular targets for therapeutic intervention.

Drug discovery studies

The comprehensive list of phytochemicals that identified from *licorice* roots and shown to be active was retrieved from Dr. Duke's Phytochemical and Ethnobotanical Databases (<https://phytochem.nal.usda.gov/>), an authoritative resource for plant-based chemical and bioactivity information.²⁵ This database supports exhaustive queries using both scientific and common names, supplying detailed profiles of phytochemicals, their biological activities, and ethnobotanical uses across a wide range of plant species. Search results can be exported in spreadsheet or PDF formats, making it easier for researchers in herbal medicine, natural product drug discovery, and nutritional science to conduct further bioinformatics and cheminformatics analyses.

Pharmacokinetics analysis

Pharmacokinetic parameters were predicted using the SwissADME web server (<http://www.swissadme.ch/>), a free platform designed for the *in silico* evaluation of small molecules.²⁶ This tool enables thorough assessments of physicochemical properties and pharmacokinetics, including absorption, distribution, metabolism, and excretion (ADME). It also evaluates drug-likeness and medicinal chemistry friendliness using several high-accuracy predictive models.

Acquisition and preparation of three-dimensional (3D) structures of phytochemicals and receptor protein

The 3D structures of drug-like phytochemicals were obtained in SDF format from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>).²⁷ Additionally, the 3D structure of the target receptor, GBP5, was retrieved from the Protein Data Bank (<https://www.rcsb.org/>) under PDB ID 7E59, and its binding pocket was predicted using BSpred neural network based algorithm on Zhang Lab server (<https://aideepmed.com/BSpred/>).^{28,29}

The ligands and receptor structures were preprocessed using Chimera version 1.17.1.³⁰ During this process, nonstandard molecules were removed from the receptor, nonpolar hydrogen atoms were added, and Gasteiger partial charges were assigned to both the ligands and the receptor to enhance molecular docking compatibility. The aromatic carbon rings in both the ligands and the receptor were identified and designated as flexible torsion trees, allowing for conformational flexibility during docking.

Following this, molecular energy minimization was conducted using ChemOffice software utilizing the MM2 and MD modules to ensure that the ligands are energetically favorable.³¹ Additionally, to achieve a stable receptor conformation, energy minimization was performed using the Amber force field for 1,000 steps, thereby ensuring structural refinement before initiating the docking simulations.

Molecular docking

To identify bioactive phytochemicals from *licorice* that may have anti-inflammatory effects through GBP5 inhibition, the molecular docking was conducted to evaluate the binding affinities between the ligands and the receptor. Also, prednisolone, as a food and drug administration (FDA)-approved anti-inflammatory drug, served as a positive control in ligand-receptor interaction. The structures of both the ligands and the receptor were prepared, and the docking was performed using AutoDock Vina version 1.12.1³² via the PyRx version 0.9.2 graphical interface.^{32,33} The receptor was defined as the macromolecule target, while the phytochemicals and prednisolone were considered the ligands. The docking process was confined within a grid box with dimensions $X = 42.56 \text{ \AA}$, $Y = 31.05 \text{ \AA}$, and $Z = 36.10 \text{ \AA}$, using a grid spacing of $0.375 \text{ \AA}$. This configuration enabled efficient exploration of the ligand-binding pocket and accurate predictions of ligand-receptor interactions.

MD simulation

The best ligand, which showed a suitable interaction with high binding affinity and created hydrogen bonds with the receptor (apigenin), and prednisolone were selected for further investigation through MD simulation with the GBP5 in a solvated environment. MD simulations were conducted using the open-source GROMACS software, version 2023.³⁴ Ligands topology files were created using the CHARMM General Force Field (CGenFF) web server (<https://app.cgenff.com/login>), which applies the CHARMM36 force field.³⁵ This same force field was used to generate receptor topology files. The ligand-receptor complexes were positioned centrally within a cubic simulation box with periodic boundary conditions (pbc), maintaining a 1 nm buffer zone from the box edges.

The systems were solvated with explicit TIP3P water molecules and neutralized by adding 0.15 M Na^+ and Cl^- ions. Energy minimization was performed using the steepest descent algorithm over 5,000 steps to eliminate steric clashes. Following this, equilibration was conducted in two phases: a 3.0 ns NVT ensemble using the V-rescale thermostat and a 5.0 ns NPT ensemble with the C-rescale barostat, both maintaining the systems at 310.15 K and 1 bar, respectively. Electrostatic interactions were calculated using the Particle-Mesh Ewald (PME) method, with a 1.2 nm cutoff for both electrostatic and van der Waals (vdW) forces. The LINCS algorithm was employed to constrain hydrogen bonds during the simulations.³⁶ MD simulation was conducted for 100.0 ns, and trajectory coordinates and energy values were recorded every 10.0 ps for further analysis.

Graphical results visualization

The expression profile analyses were visualized using the ggplot2 and pheatmap packages with additional support from the ggVennDiagram package in R.³⁷ Additionally, ligand-receptor interactions were visualized with Discovery Studio Visualizer.³⁸ The MD simulation trajectories were analyzed using built-in GROMACS tools, and the results

were visualized with XMGrace.³⁹ Further graphical representation of the simulation results was carried out using OriginProLab version 2022.

Statistical analysis

DEGs were identified using the Wilcoxon rank-sum test, a nonparametric method particularly suitable for scRNA-seq data. This test compares the rank distributions of gene expression between different groups. The Wilcoxon rank-sum test was selected because scRNA-seq data are often skewed, zero-inflated, and heavy-tailed due to dropout events, which violate the normality assumptions of parametric tests. As a nonparametric test, the Wilcoxon rank-sum test replaces raw expression values with ranks that are uniformly distributed, regardless of the original distribution, eliminating the need for any distributional assumptions. The analysis used default thresholds, including a min.pct of 0.01 and a logFC cutoff of 1. To account for multiple hypothesis testing, P values were adjusted using the Bonferroni correction method. Genes with an adjusted $P < 0.05$ were considered statistically significant.

Pathway and functional analyses were conducted using Ingenuity Pathway Analysis (IPA, Qiagen). For inclusion in IPA, DEGs were filtered based on two criteria, including an adjusted $P < 0.05$ and an absolute average log fold change ($|\text{avg.logFC}| > 1$). In interpreting the IPA results, only pathways that met these statistical thresholds were regarded as significant.

Results

Cell population structure

After preprocessing the datasets and removing low-quality cells, the batch effect was mitigated using the Harmony package to eliminate experimental and other differences that could affect subsequent analysis (Fig. 1). Following rigorous quality filtering, 9,340 out of 10,338 cells in the *K. pneumoniae*-infected sample and 9,872 out of 10,693 cells in the mock-infected control sample were classified as immune cells. Detailed immunocyte abundances are provided in Supplementary file 1, Table S1.

Identification of DEGs in the *K. pneumoniae*-infected immune cells

The host immune response to infection involves dynamic transcriptional changes, which were characterized by identifying DEGs between infected and control samples. Cell-type-specific DEGs profiles were as follows: B cells exhibited 347 DEGs (189 upregulated, 158 downregulated); dendritic cells (DCs) showed 234 DEGs (99 upregulated, 135 downregulated); T cells revealed 568 DEGs (311 upregulated, 257 downregulated); granulocytes demonstrated 1,381 DEGs (494 upregulated, 887 downregulated); monocytes presented 2,381 DEGs (906 upregulated, 1,475 downregulated); natural killer cells (NK Cells) included 974 DEGs (492 upregulated, 482 downregulated); and macrophages showed 282 DEGs (190 upregulated, 92 downregulated). Feature plots indicated

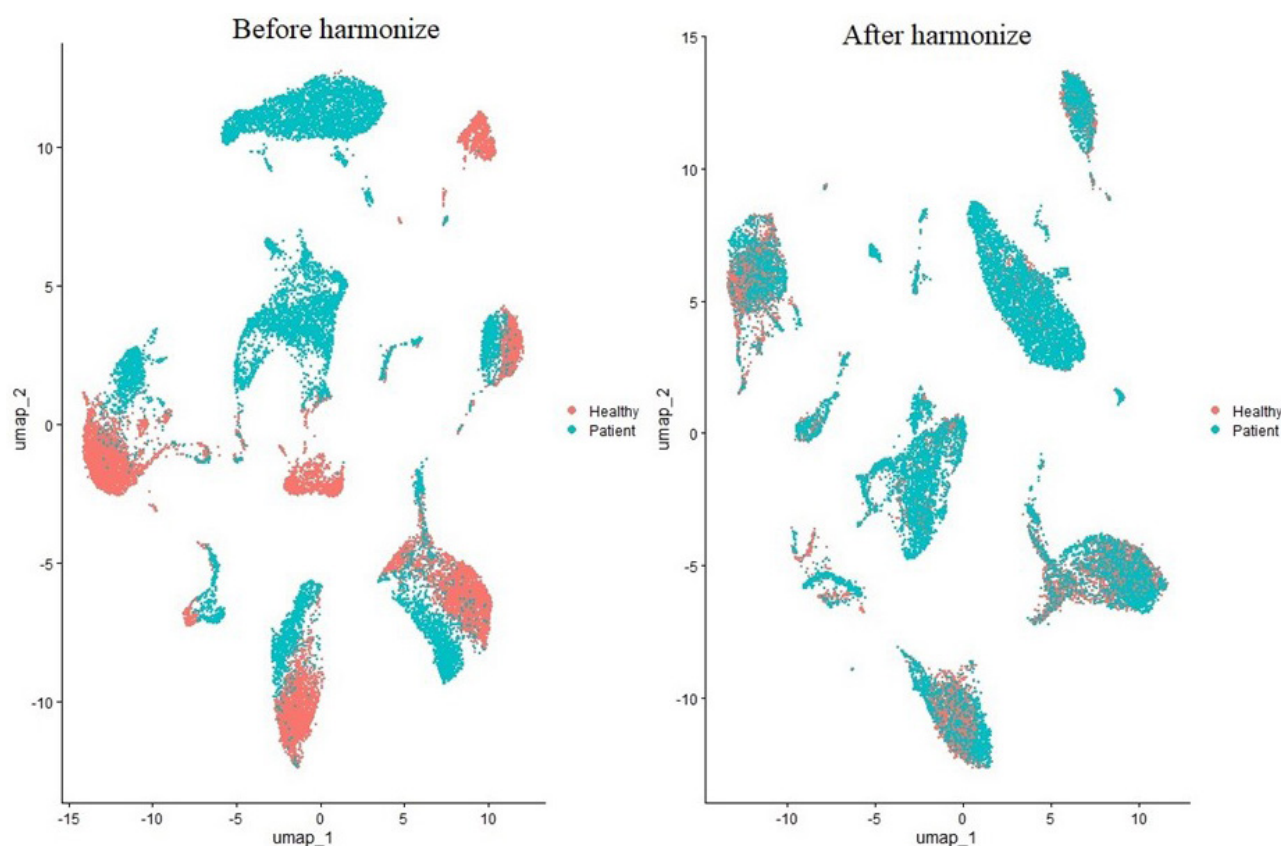


Fig. 1. Cell clustering before and after harmonizing. (Left plot) The distribution of *K. pneumoniae*- and Mock-infected cells before removing batch effects; (Right Plot) The distribution of *K. pneumoniae*- and Mock-infected cells after removing batch effects. In the clusters, *K. pneumoniae* and Mock-infected cells are represented in blue and red colors, respectively, with each dot corresponding to an individual cell.

that the macrophage population was enriched with inflammatory or M1-associated markers, including Nitric Oxide Synthase 2 (Nos2), Interleukin-1 Beta (Il-1 β), Tumor Necrosis Factor (Tnf), C-X-C Motif Chemokine Ligand 9 (Cxcl9), and CD38 Molecule (Cd38). In contrast, M2-associated markers, such as Arginase 1 (Arg1), C-C Motif Chemokine Ligand 17 (Ccl17), and C-C Motif Chemokine Ligand 22 (Ccl22), were either weakly expressed or detected only in a small subset of cells. These findings suggest a predominantly inflammatory, M1-like macrophage state, with limited representation of M2-like characteristics. Furthermore, they indicate a degree of phenotypic heterogeneity rather than a strictly polarized binary state (Supplementary file 2, Fig. S1). Complete up- and down-regulated gene lists for all immune subtypes are accessible in Supplementary file 1, Table S2. These results highlight extensive and cell-specific transcriptional remodeling under infection conditions.

We utilized the msigdb R package to retrieve gene sets from the Molecular Signatures Database (MSigDB) (<https://www.gsea-msigdb.org/gsea/index.jsp>). We then intersected these gene sets with the DEGs identified in our study to provide functional context for the *K. pneumoniae*-infected samples. Our results indicated that most of the DEGs (480 genes) were related to immune functions. This was followed by 256 genes related to metabolic pathways. In contrast, only 14 genes were associated with structural functions.⁴⁰

Functional and pathway enrichment analyses

To decipher the biological significance of DEGs identified in *K. pneumoniae*-infected immune cells, we performed GO annotation and KEGG pathway enrichment analyses using the ClusterProfiler package in R.

GO enrichment analysis revealed that the top-ranked terms were mainly related to innate immune activation and inflammatory signaling. Specifically, the most significant BP terms included "response to lipopolysaccharide," "response to bacterium-derived molecules," and "cytokine-mediated signaling pathway." The enriched CC terms were "lipoprotein particle," "plasma lipoprotein particle," and "high-density lipoprotein particle." Additionally, the leading MF terms included "antioxidant activity," "chemoattractant activity," and "molecular sequencing activity." Furthermore, KEGG analysis identified the Interleukin-17 (IL-17) signaling pathway, TNF signaling pathway, and Influenza A as the most significantly enriched pathways in *Mus musculus*, highlighting the role of the DEGs in immune and inflammatory regulation (Fig. 2A–2D).

Identification of immune system-related common DEGs

The integrative analysis of DEGs across various immune cell subtypes identified 19 genes that were consistently differentially expressed in all significant immune cell populations. While these shared DEGs were observed across all cell types, their expression levels varied among subsets. The heatmap visualization further confirmed that

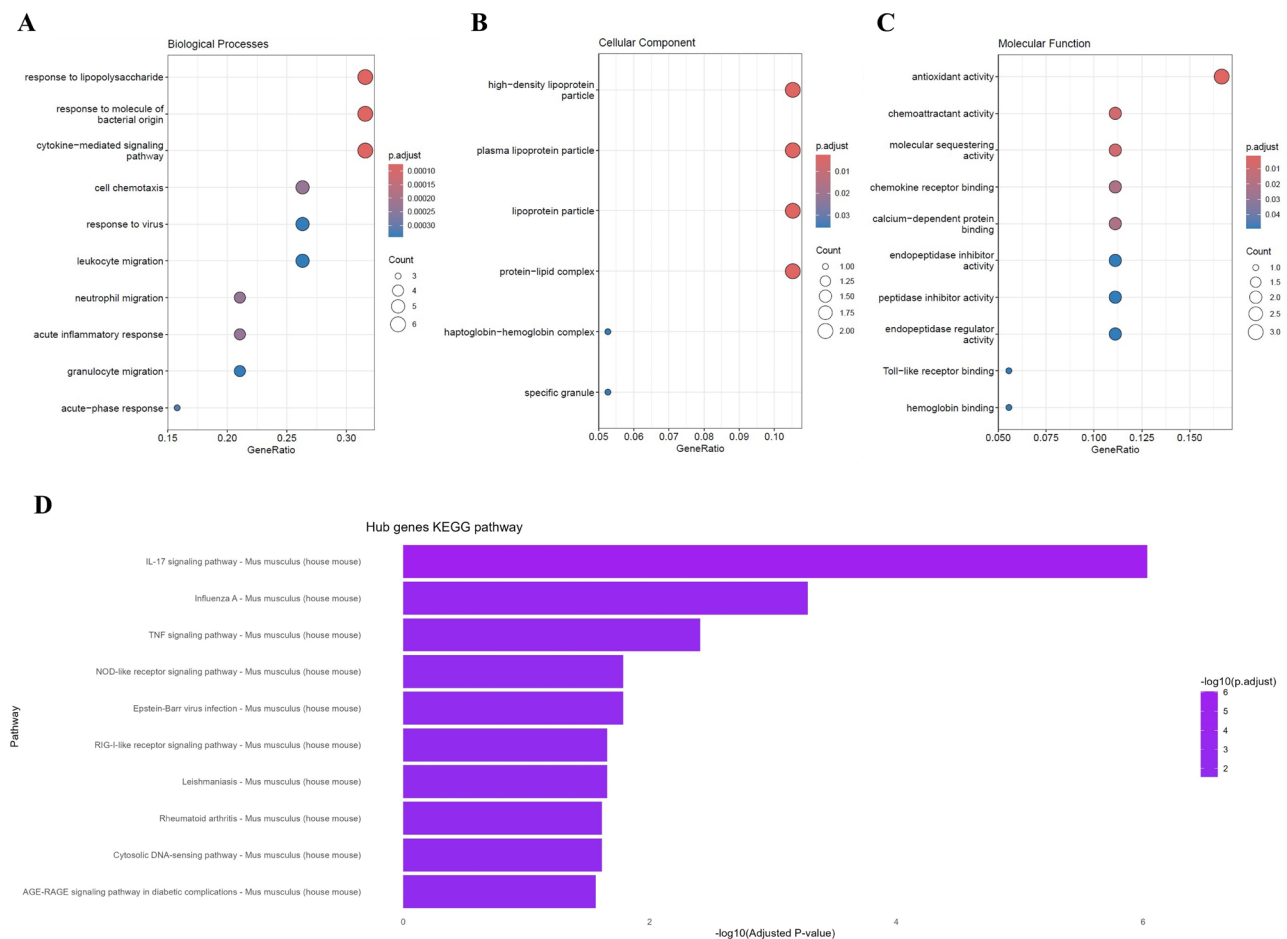


Fig. 2. Functional and pathway enrichment analysis of DEGs in *K. pneumoniae*-infected immune cells. (A) GO enrichment of biological processes highlights immune-related pathways activated upon infection, (B) GO cellular component analysis identifies critical subcellular localization changes in infected cells, (C) GO molecular function enrichment reveals key binding and catalytic activities affected by infection, (D) KEGG pathway enrichment displays significantly impacted signaling pathways associated with infection response; Panels (A–C) utilize bubble plots where bubble size indicates the number of genes involved, and position reflects enrichment significance (*adjusted P-values*) against gene ratio. Panel (D) features a bar plot charting pathway enrichment significance (bar height) alongside a color gradient representing the adjusted $-\log_{10}(p.adjust)$ values. Abbreviations: DEGs: differentially expressed genes

these genes were generally overexpressed in cells infected with *K. pneumoniae* compared to the control group (Fig. 3). To systematically map transcriptional changes related to immune function, we retrieved immune system gene lists from the Pathway Unification Database (<https://pathcards.genecards.org/>). DEGs in each cell type were then individually aligned with this curated resource. This cell-specific classification revealed a significant number of immune-related DEGs: B cells (110 genes), DCs (52 genes), T cells (137 genes), granulocytes (274 genes), macrophages (80 genes), NK cells (179 genes), and monocytes (368 genes). Notably, monocytes displayed the most pronounced immune gene response. Among the identified DEGs, we noted that proteins from the ADGRE family, which modulate leukocyte activation and facilitate phagocytosis during infections, were significantly downregulated in different cell types.⁴¹ In contrast, several genes, including GBP5 and NLR Family Pyrin Domain Containing 3 (NLRP3), together with downstream inflammasome-related effectors such as IL-1 β , were upregulated in multiple immune cell types, including macrophages, dendritic cells, and monocytes. Table 2 provides details on some immune function-related

genes along with their biological roles. A comprehensive list of immune-related DEGs regulated in at least three cell types is also available in Supplementary file 1, Table S3. To further support the immune-activation signature associated with these pathway genes, we examined the expression pattern of inflammasome-related genes across immune-cell subsets in an additional bacterial-infected dataset (GSM8170748) relative to the corresponding mock-infected reference dataset shown in Fig. S2 (Supplementary file 2). The heatmap revealed a clear myeloid-biased inflammatory signature, with increased expression of NLRP3, Caspase 1 (CASP1), and interleucin-18 (IL-18) in patient-derived macrophage and monocyte populations, as well as a prominent NLRP3 signal in granulocytes. These findings support the presence of an activated inflammasome-associated immune milieu and are consistent with the rationale for prioritizing GBP5 as a key upstream regulator. GBP5 was selected as the primary target for *in silico* drug discovery analyses because it is an interferon- γ -inducible GTPase that serves as a critical rheostat for NLRP3 inflammasome activation during bacterial infections. GBP5 promotes NLRP3 inflammasome assembly and CASP1-dependent maturation of IL-1 β in

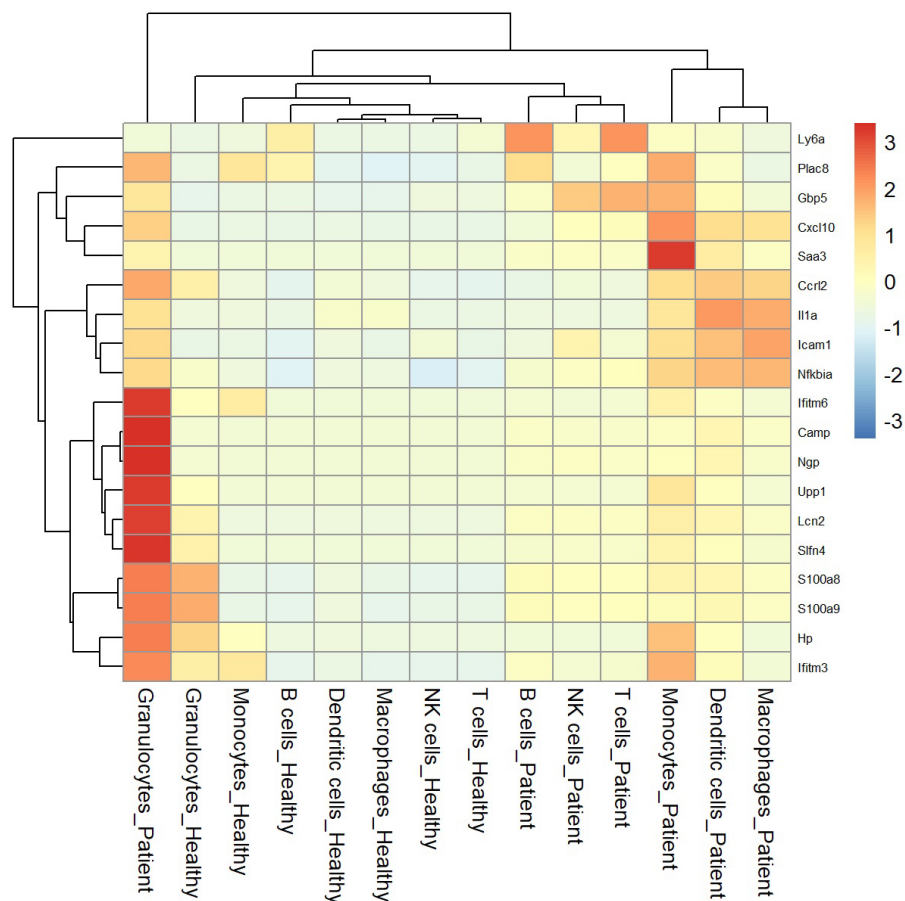


Fig. 3. Immune cell-wide integration of DEGs in *K. pneumoniae* infection. (a) Venn diagram showing the integrated DEGs across B cells, T cell, DCs, NK cells, Macrophages, Monocytes, Granulocytes in *K. pneumoniae*-infected sample. (b) Heatmap depicting the integrated DEGs among the different immune cells. The color gradients in the heatmap indicate relative expression levels (red: high; blue: low), while the branches of the dendrogram demonstrate transcriptional similarities between various cell states. Abbreviations: DEGs: differentially expressed genes.

Table 2. Immune-related genes commonly differentially expressed across multiple immune cell types, with their intersection frequency and immune functions

Genes	Number of cells	Role
Downregulated		
Integrin Subunit Alpha L (ITGAL)	4	Integrin ITGAL mediates leukocyte adhesion and migration during immune surveillance and inflammation.
CD44 Molecule (CD44)	3	CD44 facilitates leukocyte migration and cell adhesion during immune responses to infection.
Protein Kinase C Beta (PRKCB)	3	PRKCB participates in B cell and T cell receptor signaling pathways for host defense.
Ring Finger Protein 125 (RNF125)	3	RNF125 ubiquitinates signaling proteins to negatively regulate antiviral innate immune pathways.
Protein Phosphatase 3 Catalytic Subunit Alpha (PPP3CA)	3	PPP3CA activates NFAT transcription factors crucial for T cell activation.
Upregulated		
Interferon Induced Transmembrane Protein 3 (IFITM3)	6	IFITM3 restricts viral entry into host cells, strengthening antiviral immune defense.
IL-1 β	6	IL-1 β drives fever and leukocyte activation during acute inflammation.
GBP5	6	GBP5 promotes inflammasome assembly and activation for effective pathogen elimination.
TNF	4	TNF is a master pro-inflammatory cytokine, activating many immune cells during infection.
NLRP3	3	NLRP3 forms inflammasomes, triggering inflammation and coordinating pathogen clearance.

response to pathogenic bacteria, thereby driving pro-inflammatory signaling and host defense.⁴² Excessive or prolonged activation of this pathway can lead to tissue-damaging inflammation and negative health outcomes. In the context of bacterial infections, GBP5 has been shown to

regulate inflammation and cell death, making it a potential therapeutic target for inflammatory disorders associated with infections.⁴³⁻⁴⁵ Accordingly, in this study, GBP5 was pursued as a mechanistically relevant target to identify *licorice*-derived compounds with potential adjunctive anti-

inflammatory activity, rather than as a stand-alone strategy to suppress host defense.

Pathway analysis

To contextualize GBP5 within NOD-like receptor signaling, we mapped its reported interactions using the KEGG NOD-like receptor pathway (hsa04621), where GBP5 is annotated alongside NLRP3 inflammasome components (Fig. 4). This is consistent with experimental evidence showing that interferon-induced GBP5 cooperates with the NLRP3 inflammasome and promotes CASP1-dependent maturation of IL-1 β and IL-18 in response to microbial stimuli.⁴⁵

Additionally, scRNA-seq profiling revealed coordinated upregulation of Signal Transducer and Activator of Transcription 1 (STAT1), a key upstream transcriptional activator of interferon-stimulated genes, together with NLRP3-inflammasome-related genes, such as NLRP3, IL-1 β , IL-18, and CASP1, in infection-associated monocytes and macrophages (Fig. 5). This transcriptional pattern is compatible with an inflammasome-prone state. It aligns with GBP5's established role in facilitating inflammasome activation, supporting its candidacy as a therapeutic target within this pathway rather than as the sole upstream driver of NLRP3 activation.⁴⁵

Pharmacokinetic properties

The pharmacokinetic and ADME profiles of 31 phytochemicals derived from *licorice* were assessed using the SwissADME web server (Table 3). An evaluation based on Lipinski's rule of five, which considers molecular weight, the number of hydrogen bond donors and acceptors, and lipophilicity to predict drug-likeness, indicated that 27 out of the 31 compounds, such as APIGENIN and CAMPHOR, *etc.*, met the criteria, suggesting favorable drug-like properties (Table 3). In contrast, APIOGLYCRRHIZIN, ARABOGLYCRRHIZIN, GLYCRRHIZIN, and HEDERASAPONIN-C did not satisfy the Lipinski criteria and were thus classified as non-drug-like phytochemicals. Among the drug-like compounds, 13 were found to have the ability to penetrate the blood-brain barrier (BBB), while 23 exhibited high gastrointestinal absorption. A detailed overview of all compounds' pharmacokinetic properties is provided in Supplementary file 1, Table S4. Compounds that met the drug-likeness criteria were prioritized for further experimental and computational analyses.

Ligand-receptor molecular docking

Molecular docking was performed using AutoDock Vina to evaluate the binding affinity and interaction patterns between the drug-like phytochemicals (Supplementary file 2, Fig. S3) and GBP5. Before docking, we predicted the

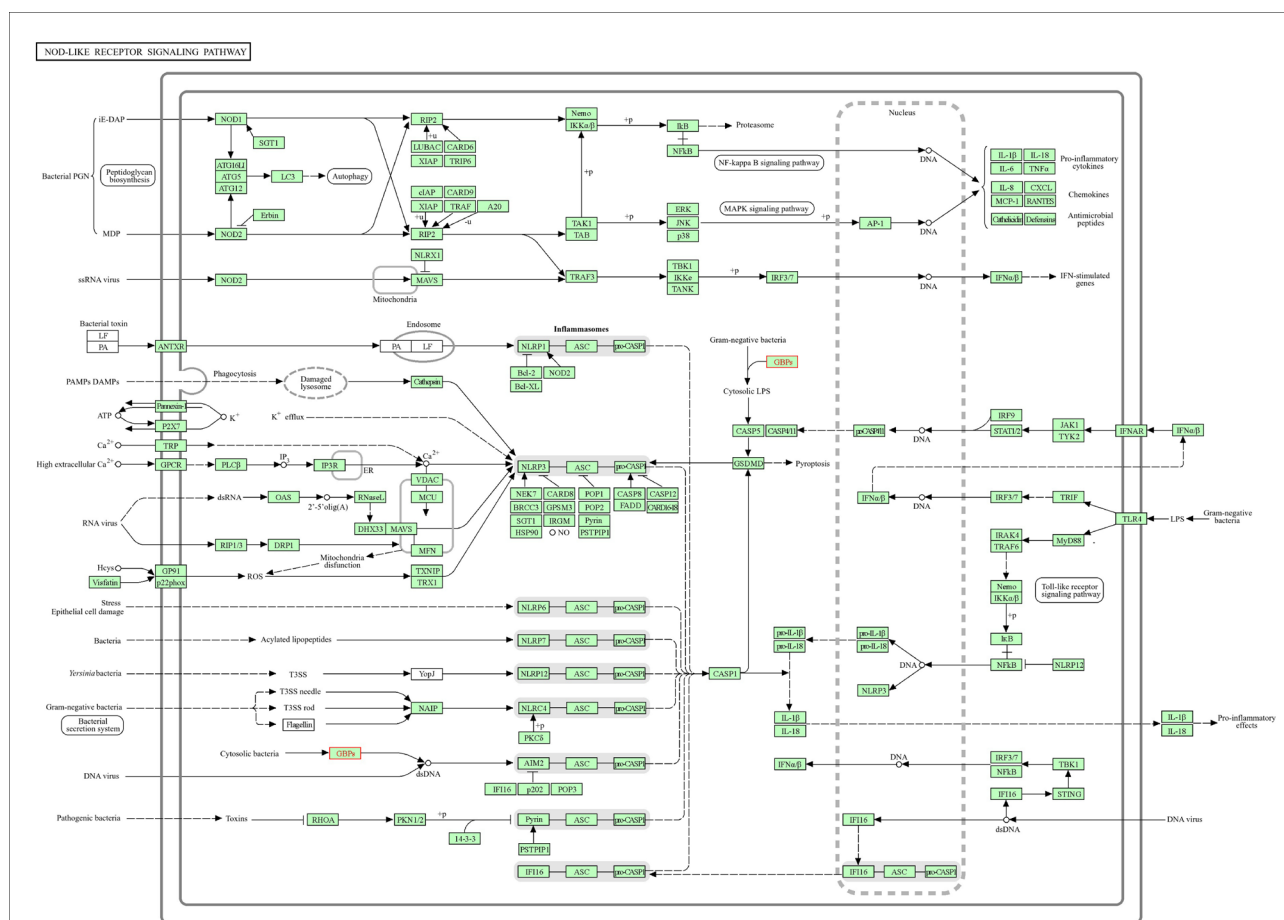


Fig. 4. Visualization of the NOD-like receptor signaling pathway mapped via KEGG. The position of GBP5 within the NOD-like receptor pathway was determined using the KEGG Mapper online tool. Genes bordered in color are those significantly upregulated in this study, suggesting potential roles in regulating pathway activity and inflammatory signaling.

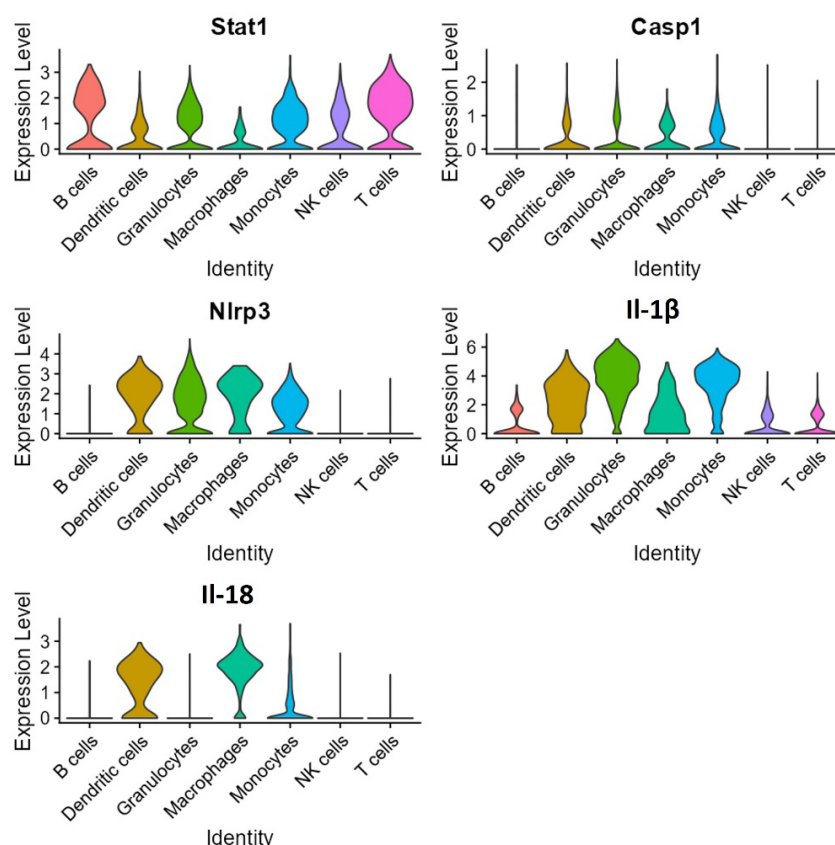


Fig. 5. Single-cell expression profiles of key GBP5 pathway activator and effectors across immune cell types. Violin plots illustrate the distribution of transcript expression levels for STAT1, CASP1, NLRP3, IL-1 β , and IL-18 across various immune cell populations, including B cells, dendritic cells, granulocytes, macrophages, monocytes, NK cells, and T cells. There is increased expression of upstream regulators, such as STAT1. Furthermore, downstream effectors, like IL-1 β , exhibit higher transcript levels in these cell populations.

likely binding pocket of GBP5 using the BSpred server. The docking grid box was centered on this site to include the nucleotide triphosphate (NTP)-binding domain, which is the functionally relevant region associated with GBP5 activity. This design enabled us to evaluate whether the candidate compounds could interact with residues within GBP5's known catalytic pocket, potentially interfering with its functional activity.

The docking simulations predicted ligand binding affinities to GBP5 in the range of -5.0 to -7.4 kcal·mol $^{-1}$, while the FDA-approved anti-inflammatory positive control prednisolone bound at -7.8 kcal·mol $^{-1}$, providing an experimental benchmark for comparison. Additionally, the ligands formed 5-8 hydrogen bonds within the GBP5 binding site (Table 4). Notably, APIGENIN, NEOISOLQUIRITIN, and NEOLIQUIRITIN exhibited the most favorable binding energies of -7.0 , -6.7 , and -6.0 kcal/mol, respectively, and formed 5, 5, and 8 hydrogen bonds with the receptor, as illustrated in Fig. 6. In addition to the favorable drug-likeness and pharmacokinetic properties mentioned in Section 3.6, the leading *licorice*-derived candidates, including APIGENIN, NEOISOLQUIRITIN, and NEOLIQUIRITIN, demonstrated promising *in silico* safety profiles in ADMETlab 3.0 (<https://admetlab3.scbdd.com/>).⁴⁶ These ligands were predicted to have a low risk for major toxicity-related issues, including hERG blockade, drug-induced liver injury, Ames mutagenicity,

and acute oral toxicity. They also showed low predicted risks for nephrotoxicity, neurotoxicity, hematotoxicity, and immunotoxicity. Toxicophore analysis indicates that most major structural alerts are absent, supporting a comparatively favorable toxicity profile for these compounds. Full toxicity details and ADMETlab 3.0 output are provided in Supplementary file 1, Table S5. Overall, the top licorice-derived phytochemicals exhibited interaction strengths comparable to those of prednisolone. Based on ligands predicted interaction profiles and docking scores, APIGENIN was prioritized for subsequent analyses.

MD simulation

To gain a deeper understanding of the binding stability of APIGENIN and prednisolone when complexed with GBP5, we conducted 100.0 ns MD simulations for both systems. The Ca root mean square of deviation (RMSD) profiles revealed that both complexes experienced an initial equilibration phase during the early part of the simulation, followed by relatively stable behavior for the remainder of the trajectory. This pattern indicates that the overall protein framework and ligand-bound state were preserved (Fig. 7A). The solvent-accessible surface area (SASA) and radius of gyration (Rg) profiles remained largely stable, with only minor fluctuations. This suggests that neither ligand caused significant structural expansion, compaction, or unfolding of GBP5 during the simulation (Figs. 7B and

Table 3. Drug likeness prediction of *Licorice*-derived phytochemical using SwissADME

Phytochemicals name	MW	GI absorption	BBB permeant	Lipinski #violations	Drug-like
APIGENIN	244.2	High	No	0	Yes
APIOGLCYRRHIZIN	778.92	Low	No	3	No
ARABOGLCYRRHIZIN	778.92	Low	No	3	No
CAMPHOR	152.23	High	Yes	0	Yes
FORMONONETIN	162.14	High	Yes	0	Yes
GLABRANIN	324.37	High	Yes	0	Yes
GLABRENE	322.35	High	Yes	0	Yes
GLABRIDIN	324.37	High	Yes	0	Yes
GLABROL	392.49	High	No	0	Yes
GLYCYRRHISOFLAVANONE	368.38	High	No	0	Yes
GLYCYRRHISOFLAVONE	354.35	High	No	0	Yes
GLYCYRRHIZIN	822.93	Low	No	3	No
HEDERASAPONIN-C	1230.45	Low	No	3	No
HERNIARIN	176.17	High	Yes	0	Yes
HISPAGLABRIDIN-A	392.49	High	Yes	0	Yes
HISPAGLABRIDIN-B	390.47	High	Yes	0	Yes
ISOLIQUIRITIGENIN	256.25	High	Yes	0	Yes
ISOLIQUIRITIN	418.39	Low	No	1	Yes
ISOMUCRONULATOL	302.32	High	Yes	0	Yes
KAEMPFEROL	286.24	High	No	0	Yes
LICOCHALCONE-A	338.4	High	Yes	0	Yes
LICOCHALCONE-B	286.28	High	No	0	Yes
LICOCUMARONE	340.37	High	No	0	Yes
LICORICIDIN	424.53	High	No	0	Yes
LIQUIRITIGENIN	256.25	High	Yes	0	Yes
LIQUIRITIN	418.39	Low	No	0	Yes
NEOISOLIQUIRITIN	418.39	Low	No	1	Yes
NEOLIQUIRITIN	418.39	Low	No	0	Yes
ONONIN	430.4	High	No	0	Yes
QUERCETIN	302.24	High	No	0	Yes
UMBELLIFERONE	134.13	High	Yes	0	Yes

7C). Additionally, both complexes maintained consistent hydrogen-bond interactions throughout the trajectory, indicating sustained contact between the ligand and protein, and stable binding within the GBP5 binding pocket (Fig. 7D). Notably, APIGENIN displayed a stability profile comparable to that of prednisolone, which further supports its potential as a candidate binder for GBP5.

Discussion

Pneumonia is one of the most significant infections of the lower respiratory tract. It remains a major public health challenge due to its rising global incidence and hospitalization rates.⁴⁷ *K. pneumoniae* is often associated with pneumonia and other inflammatory conditions.⁴⁸ Recent advancements in multi-omics technologies, such as scRNA-sequencing, enable detailed analysis of host immune responses by resolving cellular heterogeneity and defining molecular signatures through DEGs analysis. In this study, we analyzed single-cell expression profiles

from a *K. pneumoniae*-infected dataset to clarify the host's immune response to the pathogen.

Analysis of immune cell populations revealed that infection with *K. pneumoniae* significantly reduces proportions in several immune cells, particularly NK cells. This is consistent with bacterial immune evasion strategies involving secreted factors that impair NK cell viability and function.^{49,50} Single-cell profiling identified 2,633 DEGs across immune cell subtypes, indicating substantial remodeling of host cellular responses.

GO enrichment analysis identified several BP, including "response to lipopolysaccharide" and "cytokine-mediated signaling pathway." These findings indicate activation of innate immune signaling via pattern recognition receptors, particularly in macrophages. This observation aligns with previous studies demonstrating that bacterial endotoxins, such as lipopolysaccharides, initiate inflammatory signaling cascades via classical pattern recognition receptors, particularly in macrophages.^{51,52} Moreover,

the identification of “high-density lipoprotein particle” components in the CC category suggests infection-induced metabolic reprogramming associated with modulation of immune recognition and inflammatory signaling.^{53,54} Also, molecular function terms such as “antioxidant activity” and “chemoattractant activity” further underscore the intricate balance between oxidative stress regulation and leukocyte

Table 4. Molecular docking of drug-like *Licorice*-derived phytochemical with GBP5

Phytochemicals name	Free energy (kcal/mol)	Hbonds with binding pocket
APIGENIN	-7.0	5
CAMPHOR	-5.1	1
FORMONONETIN	-6.4	2
GLABRANIN	-6.4	4
GLABRENE	-7.0	3
GLABRIDIN	-6	3
GLABROL	-6.6	3
GLYCYRRHISOFLAVANONE	-6.8	1
GLYCYRRHISOFLAVONE	-5.0	1
HERNIARIN	-5.0	2
HISPAGLABRIDIN-A	-5.1	0
HISPAGLABRIDIN-B	-5.6	1
ISOLIQUIRITIGENIN	-7.5	4
ISOLIQUIRITIN	-6.8	4
ISOMUCRONULATOL	-5.6	3
KAEMPFEROL	-6.0	4
LICOCHALCONE-A	-5.8	2
LICOCHALCONE-B	-6.8	4
LICOCOUMARONE	-5.4	1
LICORICIDIN	-5.3	1
LIQUIRITIGENIN	-5.2	2
LIQUIRITIN	-6.4	2
NEOISOLIQUIRITIN	-6.7	5
NEOLIQUIRITIN	-6.0	8
ONONIN	-5.1	3
QUERCETIN	-5.0	6
UMBELLIFERONE	-6.6	5

recruitment, both of which are critical for adequate bacterial clearance. Supporting this notion of immunometabolic reprogramming, KEGG pathway enrichment analysis revealed the canonical pro-inflammatory signaling networks, including the IL-17 and TNF pathways. These pathways play central roles in neutrophil mobilization and pathogen elimination, and the enrichment of the “Influenza A” pathway additionally underscores conserved host defense mechanisms shared across different infectious contexts.⁵⁵⁻⁵⁷

This study revealed significant downregulation of immune response-relevant genes, such as CD44, in specific immune cell populations. CD44, a critical transmembrane glycoprotein, regulates leukocyte migration, cellular adhesion, and cytokine synthesis, and its reduction disrupts immune surveillance, attenuates IFN- γ responses, and impairs cell motility, thereby undermining effective immune cell trafficking and tissue infiltration during infection.^{58,59} In contrast, genes essential for inflammasome activation and cytokine production, including GBP5, NLRP3, and IL-1 β were sharply upregulated in infected immune cells. GBP5 upregulation resulted in NLRP3 forms a multiprotein inflammasome complex in response to microbial products or danger signals, and its activation induces CASP1 autocleavage, which processes pro-IL-1 β and pro-IL-18 into their mature, bioactive forms.^{60,61} The inflammasome pathway is essential for enhancing immune responses, helping clear pathogens by increasing neutrophil influx and promoting Th17 cell differentiation. However, uncontrolled inflammasome activity can lead to pro-inflammatory cytokine storms that contribute to several inflammatory diseases. This includes cryopyrin-associated periodic syndromes (CAPS) and neurodegenerative disorders like Alzheimer's and Parkinson's diseases. In addition to monogenic autoinflammatory syndromes, the activation of the inflammasome is linked to chronic inflammatory conditions such as asthma and chronic obstructive pulmonary disease (COPD), underscoring its role in lung health.⁶² These findings suggest that targeting members of this pathway may be an effective therapeutic strategy to reduce excessive inflammation in pneumonia.

Conventional anti-inflammatory treatments, particularly biologic agents targeting IL-1 β , effectively reduce

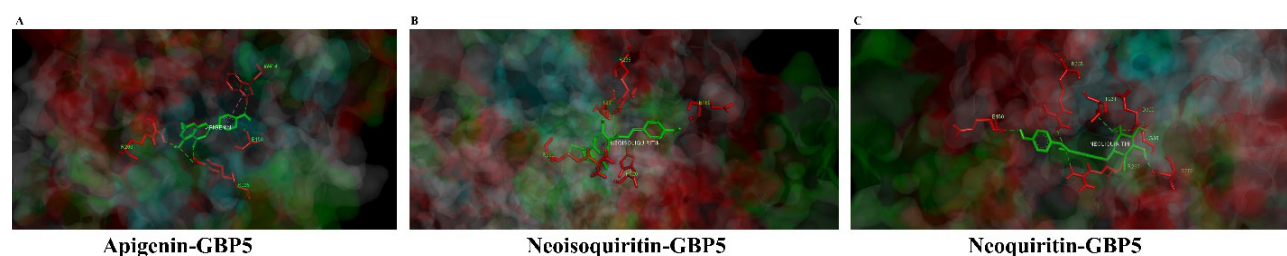


Fig. 6. Visualization of molecular docking results for APIGENIN, NEOISOLIQUIRITIN, and NEOLIQUIRITIN with GBP5. **(A)** Apigenin forms two conventional hydrogen bonds with Arginine 235 (Arg235), and one hydrogen bond each with Glutamic acid 150 (Glu150), Arg260, and Tryptophan 414 (Trp414). **(B)** Neoisoquiritin establishes one hydrogen bond with each of the following amino acids: Glu150, Threonine 231 (Thr231), Arg235, Arg260, and Histidine 520 (His520). **(C)** Neoquiritin forms one hydrogen bond with each of Glu150, Thr231, Arg235, Arg260, twice, Aspartic acid 300 (Asp300), Glycine 301 (Gly301), Asp303, and Thr247. All ligands additionally engage in van der Waals and carbon–hydrogen bonding interactions with GBP5. In each panel, the ligand is displayed as a green stick model, amino acid residues involved in binding are highlighted in red stick representation, and the remaining regions of the receptor are rendered in rainbow-colored surface views.

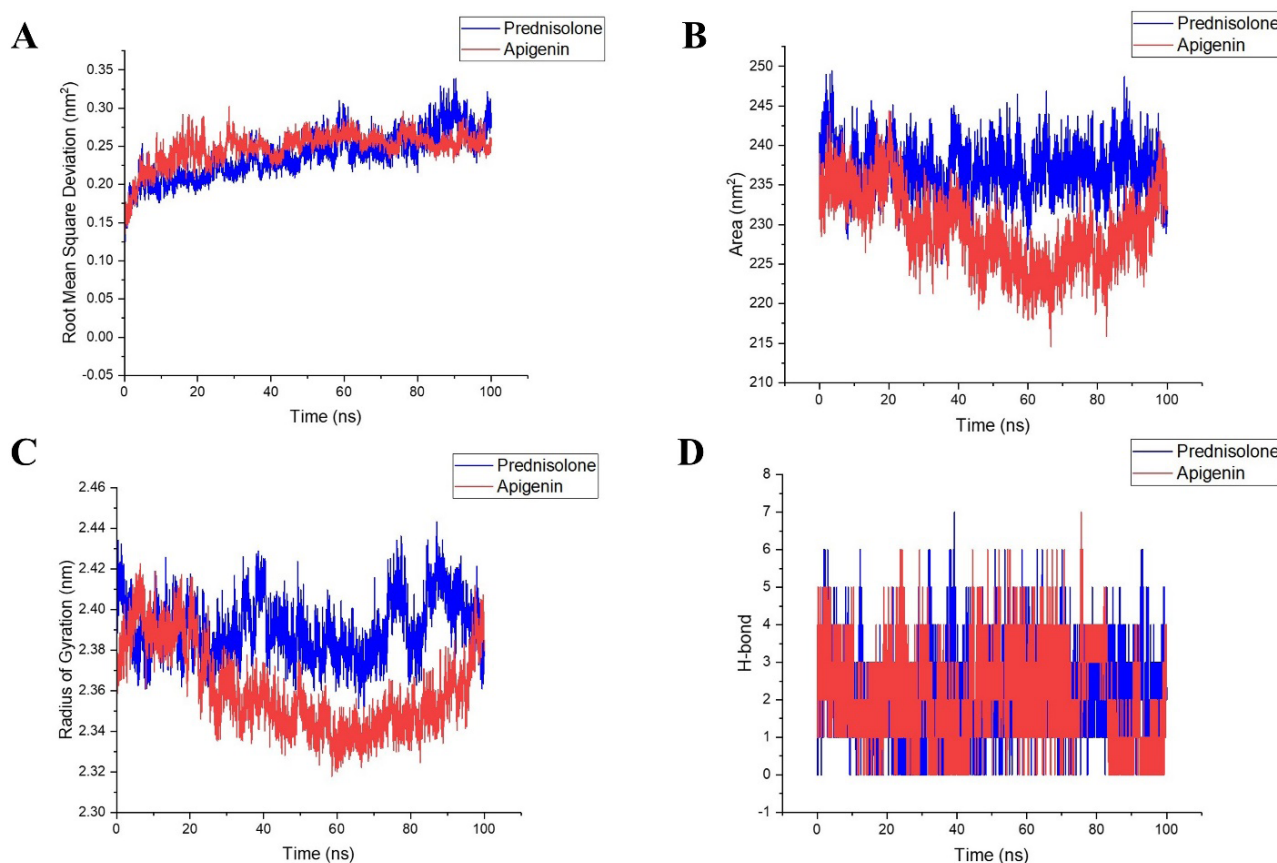


Fig. 7. MD simulation of GBP5–ligand complexes over 100 ns. (A) Ca RMSD of the GBP5–prednisolone and GBP5–apigenin complexes. (B) SASA profiles over time. (C) Rg profiles indicating protein compactness. (D) Hydrogen-bond occupancy between GBP5 and the ligands during the simulation. The results for apigenin and prednisolone are shown in red and blue, respectively.

inflammation but fail to block upstream NLRP3 activation, allowing persistent cytokine production and inflammatory signaling.⁶³ For conditions requiring regulation of the inflammasome, small-molecule inhibitors represent a promising strategy. MCC950 (formerly CRID3), for instance, selectively inhibits NLRP3 by stabilizing its inactive conformation and disrupting ASC speck formation, thereby preventing inflammasome assembly and IL-1 β maturation. Although MCC950 potently suppressed IL-1 β secretion in murine and human immune cells, its clinical development was discontinued due to off-target hepatotoxicity from carbonic anhydrase inhibition.^{64,65} Similarly, oridonin (Ori), a covalent NACHT domain inhibitor, blocks NLRP3-NEK7 interaction and ASC oligomerization.⁶⁶ However, despite preclinical efficacy, concerns persist regarding its cytotoxicity and drug interactions.⁶⁷

Given the lack of GBP5-specific inhibitors and the limitations and side effects of current corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), medicinal herbs such as *licorice* offer a promising alternative for immune modulation in pneumonia.⁶⁸ *Licorice* root extracts exhibit broad-spectrum anti-inflammatory activity and are generally well-tolerated, and their sweet flavor makes them particularly suitable for pediatric populations.⁶⁹

In light of the growing interest in herbal adjuvant therapies that modulate immune responses to improve clinical outcomes, and the rapid advances in structural

bioinformatics, we conducted a QSAR screening to identify potential anti-inflammatory adjuvants derived from *licorice* root. We assessed their ability to interact with the GBP5 and their potential to inhibit its activity. Our drug discovery studies indicate that *licorice*-derived phytochemicals, such as APIGENIN, NEOISOLQUIRITIN, and NEOLQUIRITIN, exhibit strong binding affinity and stabilizing interactions with the GBP5, supporting their potential as inflammasome inhibitors. Consistent with our *in silico* findings, recent studies show that APIGENIN exerts marked anti-inflammatory effects by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and Mitogen-Activated Protein Kinase (MAPK) signaling, thereby reducing TNF- α , IL-6, and IL-1 β production and attenuating oxidative stress by downregulating cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).⁷⁰⁻⁷² NEOLQUIRITIN has also been reported to reduce nitric oxide and cytokine production, particularly in microglial and hepatic cells, further supporting its immunomodulatory properties.^{73,74} Although direct evidence for NEOISOLQUIRITIN remains limited, studies on its parent compound, ISOQUIRITIN, demonstrate inhibition of NF- κ B-mediated release of inflammatory mediators, suggesting a similar anti-inflammatory potential.^{75,76} Current computational drug discovery research indicates that phytochemicals derived from *licorice* root are promising candidates for adjunctive therapies to control inflammation in pneumonia caused

by *K. pneumoniae*. Additionally, recent studies have emphasized that bioinformatics algorithms, machine learning, and multi-omics integration are revolutionizing modern biopharmaceutical design. These innovations are accelerating target identification, virtual screening, and mechanism-informed prioritization of drug candidates, while also reducing both time and experimental costs.^{77,78} However, these approaches are still limited by factors such as dataset availability, model interpretability, batch effects, and the necessity for rigorous experimental validation before clinical application. Therefore, our findings should be considered as hypothesis-generating and provide a foundation for future experimental studies to confirm their efficacy, safety, and therapeutic relevance.

Conclusion

This computational study combined scRNA-seq data analysis with QSAR screening to explore host immune responses and define potential anti-inflammatory adjuvants derived from *licorice* root for treating *K. pneumoniae*-induced pneumonia. Expression profiling revealed significant changes in immune gene expression, notably an increase in GBP5 and NLRP3-related inflammasome genes. The computational screening of *licorice*-derived phytochemicals identified APIGENIN, NEOISOLQUIRITIN, and NEOLISOLQUIRITIN as promising candidates with favorable binding affinities to GBP5, suggesting their potential as effective anti-inflammatory adjuvants.

However, several important considerations must be addressed before advancing these compounds toward clinical application. First, GBP5 plays a crucial role in host defense against bacterial pathogens; thus, its inhibition could compromise antimicrobial immunity if not carefully regulated. It is essential to define the therapeutic window for GBP5-targeted adjuvants to balance inflammation control and pathogen clearance. Second, while our *in silico* findings are encouraging, *in vitro* and *in vivo* experimental validation is necessary to confirm anti-inflammatory efficacy, evaluate toxicity and biocompatibility, and establish optimal dosing regimens that achieve the desired anti-inflammatory effects without hindering host defense. Third, pharmacokinetics and pharmacodynamics studies are required to determine the bioavailability, tissue distribution, and safety profiles of these compounds in the context of infection. Only after comprehensive preclinical evaluations can these *licorice*-derived phytochemicals be considered safe and effective adjunctive therapies for infection-associated inflammation.

These findings highlight the importance of integrating systems biology and drug discovery approaches to define molecular targets and treatments for infectious diseases. While this study provides valuable mechanistic insights and candidate compounds, experimental validation is crucial to confirm their efficacy and safety. Future research should investigate the immunomodulatory effects of these phytochemicals in preclinical and clinical pneumonia models, ultimately paving the way for new adjuvant therapies.

Acknowledgements

We would like to thank Professor Azam Bolhassani from the Department of Hepatitis, AIDS, and Blood-borne Diseases, Pasteur Institute of Iran, Tehran, Iran, for her valuable assistance in reviewing the manuscript.

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Competing Interests

All authors declare that they have no conflicts of interest.

Data Availability Statement

The raw expression profile datasets are available in the GEO database under the accession numbers provided in the manuscript. The phytochemical structures and related information are accessible through the PubChem database. All analyzed data generated during this study are presented in the main manuscript and the supplementary materials.

Declaration of AI-assisted Tools in the Writing Procedure

The authors declare that Grammarly (an AI-based writing assistant) has been utilized to improve the language and clarity of the manuscript during drafting. Following the use of this tool, the authors reviewed and revised the content as necessary and take full responsibility for the final version of the published paper.

Ethical Approval

Not applicable.

Funding

The authors affirm that this manuscript was prepared without any financial support, funding, or grants from any organization or institution.

Supplementary files

Supplementary file 1 contains Tables S1-S5 and Supplementary file 2 contains Figs. S1-S3.

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