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ORIGINAL RESEARCH

Using the Phillygenin Ameliorates the Severe Acute Pancreatitis in Rats by Inhibiting TLR4/NF-κB Pathway

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Background: There is no effective agent against the inflammation in severe acute pancreatitis (SAP). Phillygenin (PHI) exhibits potent anti-inflammatory activity. However, the molecular mechanisms underlying its effects on SAP remain unclear. The aim of the study was to explore the therapeutic effects and regulatory mechanism of PHI on SAP.

Methods: Thirty male SD rats were randomly divided into control, SAP and PHI (30 mg/kg) treated groups. The effectiveness of the PHI treatment was determined by looking at the histological scores of the pancreas, the levels of serum amylase and lipase, pathological changes and myeloperoxidase (MPO) activity. The affinity between PHI and the TLR4/NF-κB proteins were predicted using molecular docking. Rat pancreatic AR42J cells were divided into control, cerulein plus LPS and PHI-treated groups (12.5, 25 and 50 μg/mL). In vivo and vitro experiments, the effect of PHI on the release of TNF-α, IL-6, and IL-1β were measured by ELISA. The Western blot and immunohistochemistry were used to uncover the underlying molecular mechanisms targeting on TLR4/NF-κB.

Results: PHI treatment significantly improved pancreatic pathology ($P < 0.001$), decreased the levels of serum amylase and lipase ($P < 0.05$), reduced pancreatic MPO activity ($P < 0.01$). Additionally, PHI reduced the release of TNF-α ($P < 0.01$), IL-6 ($P < 0.05$), and IL-1β ($P < 0.01$) in rats. Furthermore, PHI exhibited strong binding ability to both TLR4 and NF-κB. The protein expression of TLR4, NF-κB, and phospho-NF-κB proteins in vivo and in vitro models were downregulated by PHI ($P < 0.05$). The transfer of phospho-NF-κB to the nucleus was also reduced in the rat pancreas ($P < 0.05$).

Conclusion: PHI exhibits a protective effect against SAP. It can alleviate SAP in rats by inhibiting TLR4/NF-κB pathway. Therefore, PHI may be considered as a novel therapeutic agent against SAP.

Keywords: phillygenin, severe acute pancreatitis, inflammation, toll-like receptor 4, nuclear factor-κB

Introduction

The frequency of acute pancreatitis (AP), a common gastrointestinal ailment, has been rising recently.[1] Roughly 20–30% of patients experience the onset of severe acute pancreatitis (SAP).[2] SAP advances swiftly and has the potential to be life-threatening, resulting in a fatality rate of 15–20%.[3] Regrettably, the existing clinical treatments for SAP are limited and unsatisfactory.[4] Therefore, it is imperative to develop novel medications to offer patients with more extensive and efficacious therapeutic options.

While the precise etiology of SAP remains incompletely comprehended, accumulating data indicates that the inflammatory response is pivotal in its pathogenesis.[5] During SAP, the damaged pancreas releases inflammatory substances that exacerbate the inflammation in the pancreas and triggers a widespread inflammatory response in the body, resulting in the advancement of systemic inflammatory response syndrome (SIRS). The development of multiple organ dysfunction (MODS) may potentially occur in more serious instances of this inflammatory response. These serious consequences may ultimately cause the patient to

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die.[5,6] Therefore, recent studies have focused on effectively controlling the inflammatory response in SAP to alleviate the disease damage.[6,7] The Toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) pathway is a widely recognized pathway that has a crucial role in the body's inflammation development among the numerous pathways linked to inflammation.[7] It has been reported that this pathway likewise promotes the development of SAP, whereas inhibition of its activation attenuates pancreatic pathologic injury and inflammatory response.[8] This suggests that this pathway could be used as a target for SAP therapy.

Forsythia is a conventional Chinese medicinal plant extensively utilized in Asia and incorporated in the Chinese, Japanese, and Korean compendiums of drugs.[9] Known for its capacity to eliminate heat and toxins, this substance is frequently utilized in clinical practice to treat a range of illnesses including wind-heat, gonorrhea, dengue, ulcers, and carbuncles.[10–12] Phillygenin (PHI) is a naturally occurring chemical derived from the fruit of the forsythia plant. A review on the pharmacological, toxicological, and pharmacokinetic properties of PHI demonstrates that PHI possesses robust anti-inflammatory activity, substantiated by substantial mechanistic and preclinical evidence.[13] Its anti-inflammatory function has received considerable study in recent years and has shown potential in treating ailments such as pneumonia and arthritis.[14,15] Moreover, recent research has indicated that PHI potentially exert a healing impact on colitis and hepatitis by disrupting the TLR4/NF-κB signaling pathway.[16,17] Therefore, we propose the hypothesis that PHI might exert a curative impact by influencing the TLR4/NF-κB pathway in SAP models.

This study aimed to evaluate the effectiveness of PHI in treating SAP and explore the underlying mechanisms that contribute to its efficacy. By clarifying the mechanisms underlying the therapeutic effects of PHI, we hope to provide a valuable rationale for its use as a novel strategy for treating SAP.

Materials and Methods

Materials

Phillygenin (purity> 98%) was provided by Chengdu Must Biotechnology Co., Ltd, located in Chengdu, China. Macklin Biochemical Technology Co., Ltd (Shanghai, China) offered Sodium taurocholate (NaTc) (purity>98%). Solaibao Technology Co., Ltd (Beijing, China) provided cerulein and Lipopolysaccharide(LPS). Procell Life Science&Technology Co.,Ltd (Wuhan, China) provided the AR42J cells. ABclonal technology, located in Wuhan, provided the specific β-actin antibodies. The Anti-TLR4 and Anti-phospho-NF-kB antibodies were obtained from Bioss in Beijing, China, while Boster biological technology Co., Ltd, also located in Wuhan, supplied the anti-NF-κB antibody. The Myeloperoxidase activity assay kit was provided by Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits for tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were provided by Bioswamp Life Science Lab Co., Ltd (Wuhan, China). The sensitivity and variation range of the ELISA kits were as follows: TNF-α kit (sensitivity ≤1.6 pg/mL, variation range 8–640 pg/mL), IL-6 kit (sensitivity ≤1.2 pg/mL, variation range 6–480 pg/mL), and IL-1β kit (sensitivity ≤1 pg/mL, variation range 5–400 pg/mL). The kits for assessing amylase and lipase were purchased from Jiancheng Biotech Co., which is situated in Nanjing, China. Moreover, the Cell counting kit-8 was supplied by Boster Biological Technology Co., Ltd, which is located in Wuhan, China. Enhanced chemiluminescence (ECL) kit was supplied by Seven Innovation Biological Technology Co., Ltd (Beijing, China). Bicinchoninic acid (BCA) protein assay kit was supplied by New Cell Molecular Biotech Co., Ltd (Suzhou, China).

Animals

The SAP model was established by retrograde injection of sodium taurocholate through the pancreaticobiliary duct. Thirty male Sprague-Dawley (SD) rats were acquired from the Laboratory Animal Centre of Shanxi Medical University. Every single rat was in excellent physical condition. The rats exhibited a mean weight of 220±20 g. Rats were acclimatized for one week in an indoor environment at 24°C with a natural light-dark cycle. Rats were given adequate standard feed and water throughout the acclimatization phase. Before the start of the experiment, rats were fasted for 12 hours but allowed to drink water. At the experiment's conclusion, every rat was anesthetized and euthanized with 5% isoflurane (RWD Lifescience, Shenzhen, China). This study was conducted from January 12, 2025 to October 23, 2025 at the Central Laboratory of the Second Hospital of Shanxi Medical University. All experimental processes followed the standard ethical rules, which were planned by the National Guidelines for the Care and Use of Laboratory Animals.

Ethical approval for this study (Approval No. DW2023015) was provided by the ethics committee of the second Hospital of Shanxi Medical University, Taiyuan, China on 8 March 2023. This study was carried out in strict accordance with the ARRIVE guidelines.

Rat Model Preparation

Following a 12-hour period of fasting, a total of thirty rats were randomly allocated into three groups using a random number table, comprising 10 individuals each: the control group (Control), the SAP model group (SAP), and the PHI treatment group (SAP + PHI). Inducing SAP in rats was accomplished through the use of the experimental procedure outlined by Li et al[18] The rats were administered sodium pentobarbital via intraperitoneal injection to induce anesthesia. After preparing and disinfecting the skin, a vertical incision was made in the middle of the abdomen to get access to the abdominal cavity. Following the incision of the abdominal cavity of the rats, the position of the common bile duct was first meticulously located. Afterwards, small blood vessel clamps were used to secure the bile duct and avoid the backflow of medication into the liver. The biliopancreatic duct was accessed by making an angled puncture with a 1 mL syringe needle and then secured with a microvascular clamp. A 5% sodium taurocholate solution (0.1 mL/100 g) was injected into the pancreaticobiliary duct at a rate of 0.3 mL/min using a microinfusion pump. Following the injection, the microvascular clips were extracted and the abdominal incision was sutured. In the Control group, the incision was sutured only after gently twisting the pancreas. Thirty minutes after modeling, rats in the PHI-treated group received 30 mg/kg intraperitoneal PHI. Both the untreated SAP group and the control rats were administered an equivalent amount of saline. The rat pancreas was clipped after 12 hours. After euthanasia, pancreatic body tissue was collected as the experimental sample and divided into two portions: one portion was fixed in 4% paraformaldehyde solution for subsequent histopathological examination and immunohistochemistry; the other portion was snap-frozen and stored at −80°C for subsequent MPO activity assay and Western blot analysis. Then, blood specimens were collected by obliquely puncturing into the inferior vena cava using a blood collection needle. The blood was allowed to stand at room temperature for 30 minutes and then centrifuged at 3000 rpm for about 15 minutes, and then the upper serum layer was collected.

Cell Culture and Treatment

The rat pancreas AR42J cell line was cultured in F12K medium supplemented with 1% antibiotics (penicillin and streptomycin) and 20% fetal bovine serum. The cells were incubated at a temperature of 37°C in a CO2 incubator with a concentration of 5%. In order to attain the necessary concentration, PHI is first dissolved in dimethyl sulfoxide, and then it is continued to be diluted through the culture solution. In the end, the content of dimethyl sulfoxide is maintained at a level lower than 0.1%. The cells were categorized into five groups for the experiment. The control group was cultured normally without intervention. The cerulein plus LPS group's cells were subjected to cerulein (10 nmol/L) plus LPS (10mg/L) for 24 hours. The PHI group received cerulein (10 nmol/L) plus LPS (10mg/L) and PHI (12.5, 25, 50 μg/mL) for 24 hours. After the intervention period had come to an end, the supernatants and cells were collected for the ensuing analytical procedures.

Cell Viability Assay

AR42J cells ($1x10^4$/well) were seeded into a 96-well plate and incubated overnight. On day two, various concentrations of PHI (0, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) were administered for a duration of 24 hours. Then, 10 microliters of CCK-8 solution were introduced into every well and left to incubate for a duration of 2 hours. Subsequently, the absorbance at 450 nm for each well was measured using the microplate reader (Tecan Sunrise, Salzburg, Austria). The cell viability was then determined by calculating it based on the readings.

Histopathological Examination

The pancreatic tissues were then embedded in paraffin and cut into 4 μm slices. After deparaffinization and hydration, HE stained the sections. Two experienced pathologists evaluated sections from each rat, without knowledge of the experimental conditions. Using the scoring criteria outlined by Schmidt et al,[19] five arbitrary fields of view were chosen under intense magnification to evaluate edema, necrosis, inflammatory cell infiltration, and hemorrhage.

Immunohistochemical Staining

Paraffin-embedded tissues were sliced into pieces with a thickness of 3.5 micrometres, after which they were deparaffinized and hydrated. To inactivate catalase, Hydrogen Peroxide 0.3% was used as a blocking agent. Subsequently, the sections were immersed in a boiling solution of citric acid (pH=6.0) for 20 minutes to facilitate antigen restoration. Goat serum was used to seal the sections, and anti-phosphorylated NF-κB antibody (1:300) was then incubated for an overnight period at 4°C. Subsequently, the peroxidase-coupled secondary antibody was put to the sections and left to incubate at room temperature for a duration of 2 hours. The color development was accomplished by incubating the sections with diaminobenzidine (DAB). In each section, five random fields of view were selected in the high-power field, and positive nuclei were counted. The proportion of positive nuclei in each field was determined through quantitative analysis by comparing it to the total number of nuclei.[20]

Biomarkers Estimation

Rat serum was collected and stored in a refrigerator at −80 degrees Celsius, and then the levels of trypsin in serum were detected using an amylase kit (Cat: C016-1-1, Nanjing, China) and a lipase kit (Cat: A054-1-1, Nanjing, China).

Myeloperoxidase Assay

MPO is an inflammatory marker indicating neutrophil infiltration. MPO activity in pancreatic tissue samples was assayed using the MPO Specific Assay Kit (Cat: A044-1-1, Nanjing, China) according to the manufacturer's instructions.

Elisa

Specialized ELISA kits were used to measure the levels of TNF-α, IL-6, and IL-1β in serum and cell supernatants, following the guidelines provided by the manufacturer.

Molecular Docking and Molecular Dynamics Simulations

PHI's structure was derived from PubChem (https://pubchem.ncbi.nlm). The protein crystal structures of TLR4 (Uniprot code: Q9QX05) and NF-κB (Uniprot code: F7F7J5) were retrieved from the Uniprot database (https://www.uniprot.org). Afterwards, the PyMOL program (4.3.0 version) was employed to eliminate water molecules and small molecule ligands from the protein structures. Both the PHI structure and protein structures were imported into AutoDockTools (1.5.6) for the addition of nonpolar hydrogens, charge calculations, charge assignments, and the construction of docking grid boxes. AutoDock Vina (1.1.2) was used for docking research to forecast possible interactions between PHI and TLR4 or NF-κB. Models with the lowest binding energy from the docking results were selected for further analysis. The results were visualized using the PyMOL software.

To assess the dynamic stability of the PHI-protein complexes, 100-ns Molecular dynamics simulations were performed for both PHI-TLR4 and PHI-NF-κB systems. Molecular dynamics simulations were conducted using GROMACS 2022. Force field parameters for the receptor proteins were generated using the AMBER14SB force field, while ligand topologies were prepared using the General AMBER Force Field 2 (GAFF2) with sobtop_1.0(dev3.1). Partial charges for the ligand were assigned using the RESP method to ensure physicochemical accuracy. Each complex was solvated in a cubic TIP3P water box extending at least 1.0 nm from the solute in all directions. The systems were neutralized by adding Na^+ and Cl^- ions to achieve a final concentration of 0.15 M NaCl, mimicking physiological ionic strength. Long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method with a real-space cutoff of 1.0 nm. All bonds involving hydrogen atoms were constrained using the LINCS algorithm.Prior to production runs, each system underwent a comprehensive energy minimization protocol consisting of 3,000 steps of steepest descent followed by 2,000 steps of conjugate gradient optimization. This procedure was executed in three sequential stages: (i) solute-restrained minimization of solvent molecules, (ii) counterion-restrained minimization, and (iii) unrestrained minimization of the entire system. Production simulations were performed in the NPT ensemble at 310 K and 1 bar, maintained using the Nosé–Hoover thermostat and Parrinello–Rahman barostat, respectively. A 2 fs integration time step was employed, and trajectories were recorded every 10 ps over a total simulation time of 100 ns.

System stability and structural dynamics were analyzed using GROMACS built-in tools, including root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and hydrogen bond analysis.

Western Blot

RIPA lysis buffer was added to the sheared pancreatic tissue, homogenized and then incubated on ice for 30 min and centrifuged to obtain protein extracts. AR42J cells were incubated with RIPA lysis buffer on ice for 30 min, followed by centrifugation to obtain proteins. Protein concentrations were determined using a BCA assay kit. Subsequently, the protein samples were denatured by heating at 100°C for 10 min in a metal bath.20 μg of each protein sample was introduced into a 10% SDS-polyacrylamide gel, and electrophoretic migration was carried out. Afterward, the proteins underwent an electrotransfer procedure to be transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were then sealed using either 5% skimmed milk or BSA. Afterwards, the membranes underwent treatment with anti-TLR4 (1:1000), anti-NF-κB (1: 1000), and anti-phosphorylated NF-κB (1:1000) for the duration of the night at a temperature of 4 degrees Celsius. The next day, the membrane was incubated with the secondary antibody (Cat: BA1054, Wuhan, China) treatment for 2 hours at room temperature. Protein bands were visualized using an ultrasensitive ECL kit and detected with a gel imaging system. Band intensities were quantified using Image J software, and statistical comparisons were performed using GraphPad Prism 10.1.2. For normalization, the intensity of each target protein band was divided by the intensity of the corresponding internal reference protein β-actin.

Statistical Analysis

Experimental data were analyzed and plotted using the GraphPad Prism 10.1.2 software. For all data, tests for normal distribution and homogeneity of variance were conducted first. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. All data in this study meet the assumptions of homogeneity of variance and normal distribution. Quantitative data were expressed as mean±standard error (Mean±SEM). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. A two-tailed P-value <0.05 was considered statistically significant.

Results

PHI Reduced Sodium Taurocholate-Induced Damage to the Pancreas in Rats

A comprehensive examination and evaluation of the pancreatic tissues of rats suffering from SAP were performed to evaluate the impact of PHI on the histopathological alterations. The examination showed that the pancreatic tissues of the rats in the control group did not exhibit any notable alterations in their structural integrity. In sharp juxtaposition, the pancreatic tissues of the rats in the SAP group exhibited significant necrotic harm to the acinar cells, prominent interstitial swelling, substantial invasion of inflammatory cells, and evident regions of focal bleeding ($P < 0.001$). Importantly, the introduction of PHI into the treatment regimen showed a pronounced reduction in these severe pathological manifestations ($P < 0.001$), as clearly illustrated in Figure 1A. The beneficial impact of PHI was further substantiated by the reduced pathological scores observed in the SAP group, as depicted in Figure 1B. Furthermore, the administration of PHI was found to effectively suppress the increased levels of blood amylase and lipase ($P < 0.05$), while also decreasing the MPO activity ($P < 0.01$) in the pancreatic tissues of the rats with SAP (Figure 1C–E).

Potential Toxicity of PHI on AR42J Cells

The chemical structure of PHI is shown in the Figure 2A. Cell viability of rat pancreatic AR42J cells was evaluated via a cck-8 kit following treatment with different concentrations of PHI over a duration of 24 hours. The findings are displayed in Figure 2B, revealing that PHI exhibits potential cytotoxicity to cells at concentrations up to 100 ug/mL($P < 0.05$). Therefore, we opted to employ concentrations of 12.5, 25, and 50 ug/mL for subsequent PHI interventions in the cells.

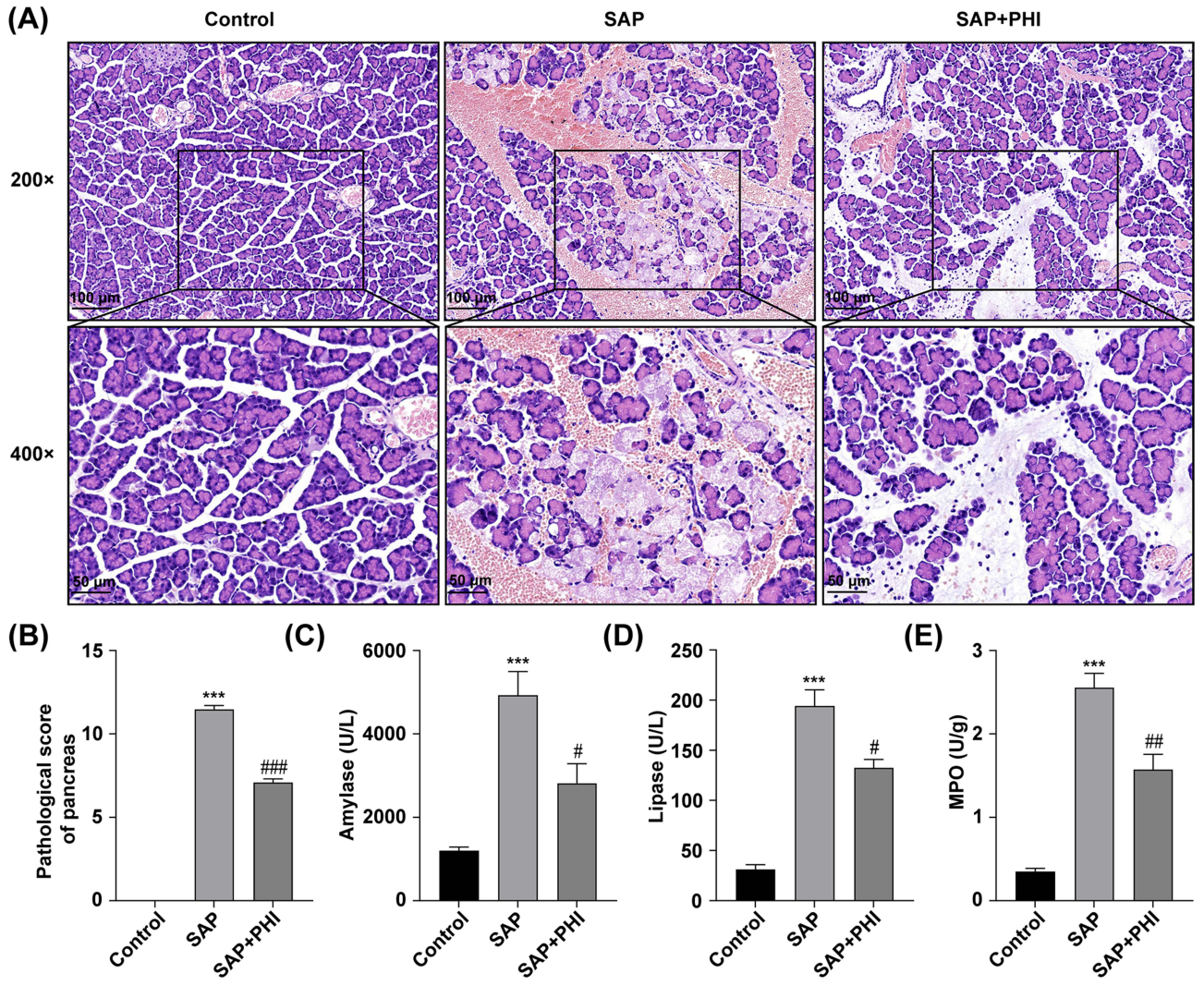

Figure 1 Effects of PHI on pancreatic injury in SAP rats. (**A**) Representative H&E-stained pictures of pancreatic tissues; (**B**) Histological scores of the pancreas; (**C** and **D**) Changes in serum amylase and lipase levels; (**E**) Changes in MPO activity levels in the pancreas. n=10 rats per group.***P < 0.001 vs control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs SAP group.

PHI Inhibited Release of Pro-Inflammatory Cytokines in vivo and in vitro

The development of SAP is dependent on the presence of pro-inflammatory cytokines. To gain a deeper understanding of the potential mechanism behind the beneficial effect of PHI on the pancreas, we examined how PHI affects the concentrations of TNF-α ($P < 0.01$), IL-6 ($P < 0.05$), and IL-1β ($P < 0.01$). The results showed that the levels of these inflammatory factors were significantly increased in the serum of rats in the SAP group. However, PHI administration reduced the release of these inflammatory factors (Figure 2C). In vitro experiments yielded similar findings, with PHI dose-dependently decreasing the production of these inflammatory factors by cerulein plus LPS-stimulated AR42J cells (Figure 2D).

Molecular Docking and Molecular Dynamics Simulation

To investigate whether PHI inhibits inflammatory responses by interacting with TLR4 and NF-κB, we performed molecular docking studies to predict potential binding sites. The affinity between PHI and the proteins was determined by using the binding energy, where binding energies below −5.0 kcal/mol indicated favorable binding activity. Our analysis of the docking model revealed that PHI exhibited strong binding ability to both TLR4 (binding energy: −7.1 kcal/mol) and NF-κB (binding energy: −6.8 kcal/mol). As depicted in the 3D binding model, the interaction of PHI with

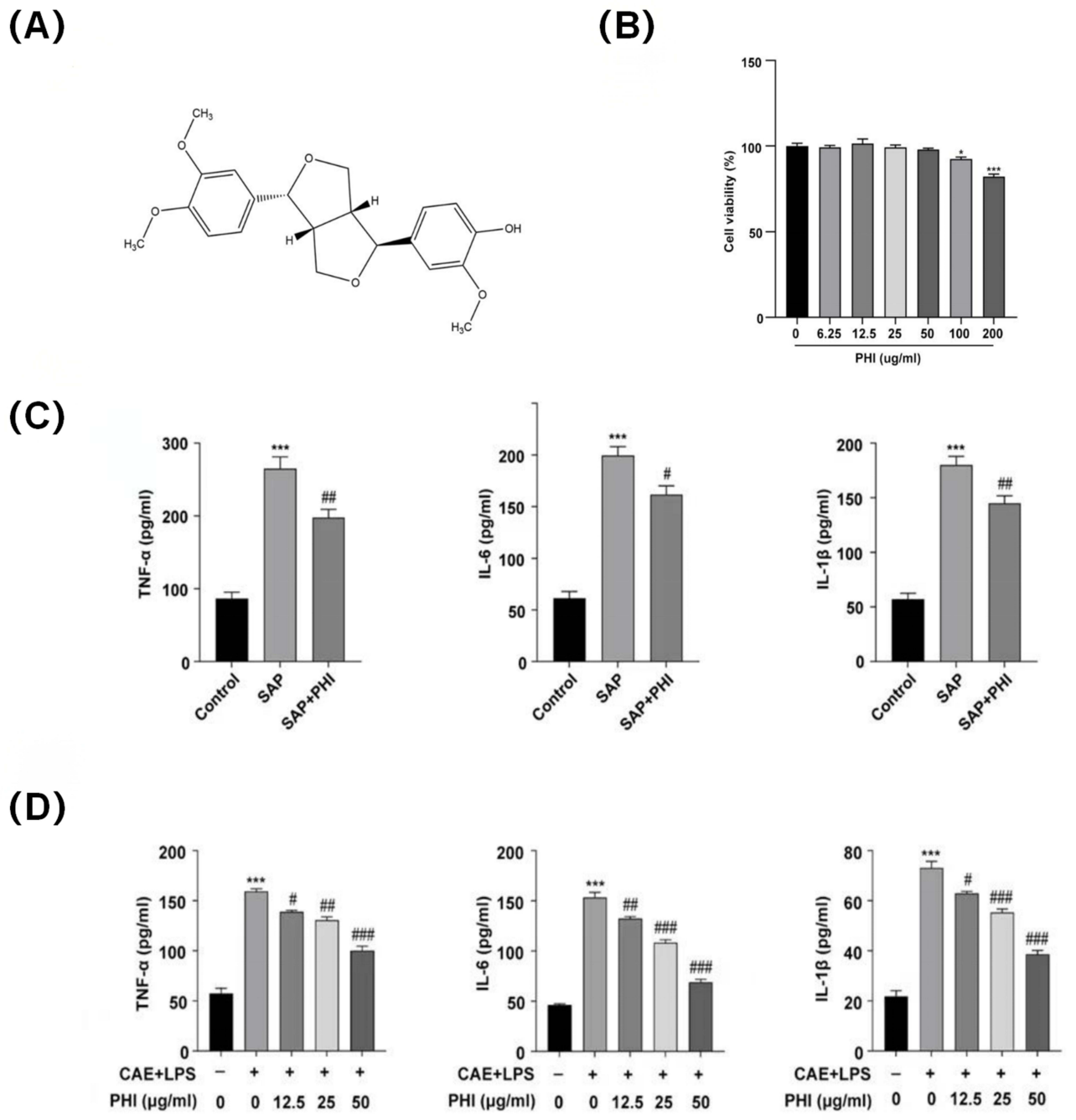

Figure 2 Effect of PHI on the release of pro-inflammatory factors. (**A**) Chemical structure of phillygenin. (**B**) Effect of PHI on AR42J cell viability. (**C**) PHI inhibits the release of TNF-α, IL-6, and IL-1β in rats.n=10 rats per group. (**D**) Effect of PHI on the release of pro-inflammatory factors in vitro. ***P < 0.001 vs control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs SAP group.

TLR4 is facilitated by forming hydrogen bonds with THR-473 and ASN-472, and establishing hydrophobic interactions with LEU-448, TYR-424, TYR-401, and LYS-400 (Figure 3A). In addition, The interaction of PHI with NF-κB is facilitated by forming hydrogen bonds with ASP-160, LYS-86, and SER-283, and establishing hydrophobic interactions with ASP-284, ASP-160, THR-198, and ASP-87 (Figure 3B).

The RMSD of protein backbone atoms relative to the initial structure indicated that the PHI-NF-κB complex equilibrated after approximately 40 ns, fluctuating around 2.1 Å thereafter (Figure 3C). In contrast, the PHI-TLR4 complex exhibited greater initial fluctuations but stabilized after 90 ns with an average RMSD of approximately 5.0 Å, suggesting a more

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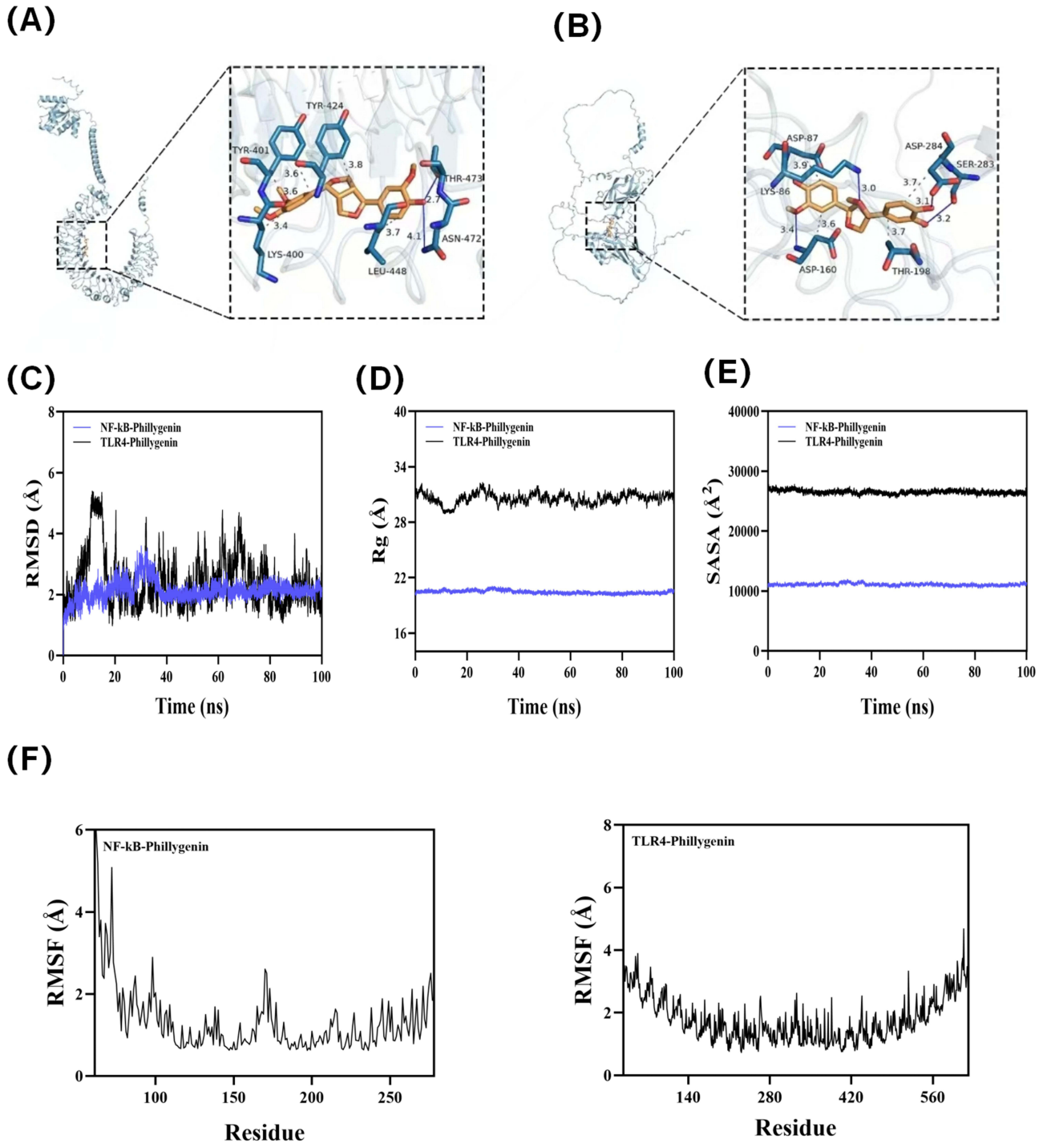

Figure 3 Conformational relationship and molecular dynamics simulation between PHI and TLR4/NF-κB pathway protein molecules. (**A**) 3D schematic of the connection between PHI and TLR4. (**B**) 3D schematic of the connection between PHI and NF-κB. (**C**) The RMSD values of the protein-ligand complex over time. (**D**) The Rg values of the protein-ligand complex over time. (**E**) The SASA values of protein-ligand complexes over time. (**F**) The RMSF value of the protein-ligand complex.

dynamic yet ultimately stable binding mode.Analysis of the radius of gyration (Rg) revealed that the PHI-NF-κB complex maintained a compact and stable conformation throughout the simulation, with minimal structural expansion or contraction (Figure 3D). The PHI-TLR4 complex displayed modest Rg fluctuations, indicating subtle conformational adjustments upon ligand binding.Solvent-accessible surface area (SASA) calculations showed that PHI-NF-κB binding induced minor variations in surface exposure, consistent with localized changes in the binding microenvironment (Figure 3E). Conversely, the

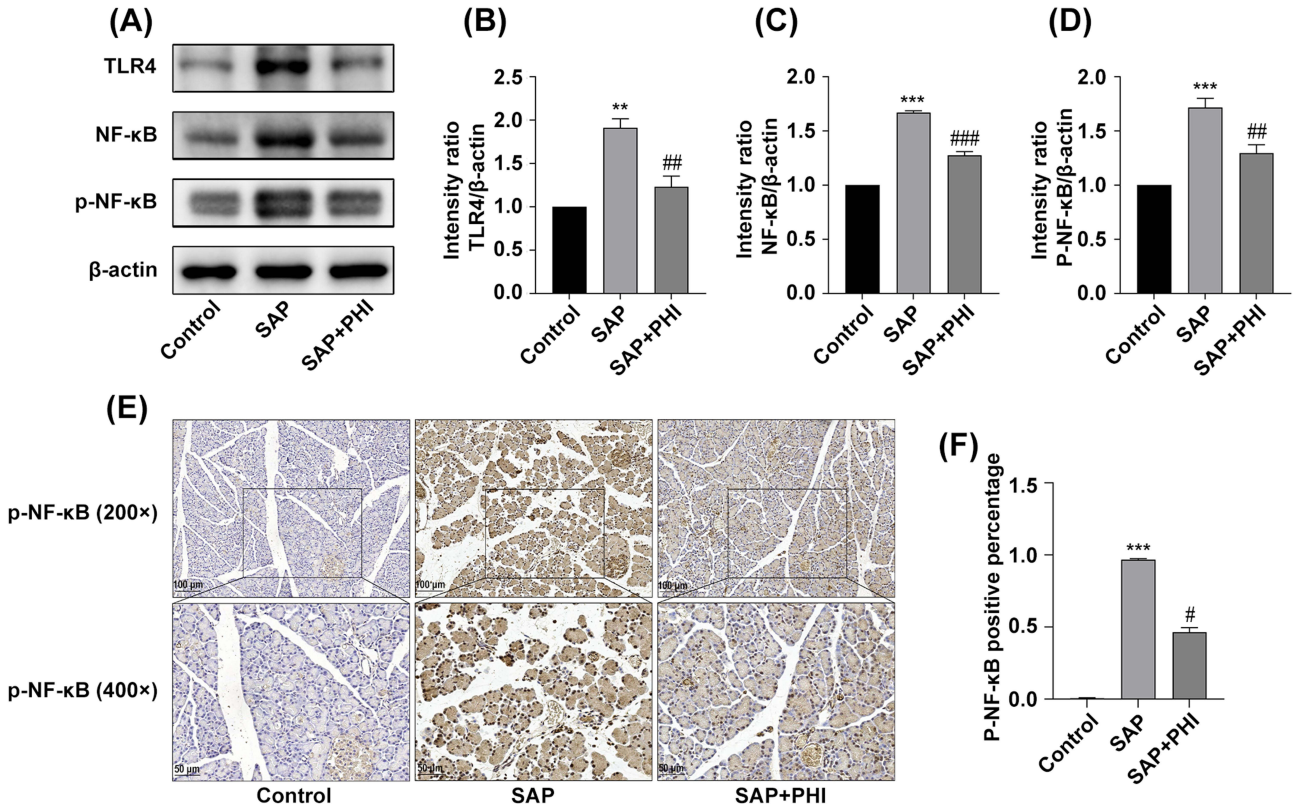

Figure 4 Effect of PHI on the activation of TLR4/NF-κB pathway in rats. (**A**) Expression of TLR4, NF-κB, and phospho-NF-κB in the rat pancreas was detected by protein blotting; (**B**–**D**) quantitative analysis of the gray value of protein bands; (**E**) observation of nuclear translocation of phospho-NF-κB in the rat pancreas by immunohistochemistry; (**F**) quantitative analysis of positive nuclei. n = 10 rats per group for (**B**–**F**); n = 3 for Western blot quantification (**B**–**D**). **P < 0.01, ***P < 0.001 vs control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs SAP group.

PHI-TLR4 complex exhibited negligible SASA variation, further supporting the formation of a stable protein-ligand interface. RMSF analysis demonstrated that both complexes maintained low residue-level flexibility (<4.0 Å mostly), with reduced fluctuations observed in binding pocket residues compared to loop regions (Figure 3F).

PHI Inhibited Activity of TLR4/NF-κB Pathway in vivo and vitro

Protein blotting was employed to assess the presence of TLR4, NF-κB, and phospho-NF-κB in rat pancreatic tissue, based on the results obtained from molecular docking. The data showed a notable increase in the expression of TLR4, NF-κB, and phospho-NF-κB in the pancreatic tissues of the SAP rat group. Nevertheless, the above-mentioned protein expressions were significantly downregulated upon PHI treatment ($P < 0.05$) (Figure 4A–D). Further, immunohistochemical staining was conducted to determine the localization of phospho-NF-κB within pancreatic tissues. The results demonstrated that giving SAP rats PHI considerably reduced the nuclear translocation of phospho-NF-κB (Figure 4E and F). Similarly, the in vitro experiments showed comparable findings, as PHI effectively suppressed the expression of TLR4, NF-κB, and phospho-NF-κB in the cerulein plus LPS-stimulated group in a dose-dependent manner ($P < 0.05$) (Figure 5A–D).

Discussion

In our study, we demonstrated that PHI decreased inflammation and pancreatic damage, while inhibiting the activation of the TLR4/NF-κB pathway in vitro and in vivo models. This suggests that PHI alleviate SAP by affecting the TLR4/NF-κB pathway.

In recent years, the extraction of safe and effective components from natural products for the treatment of diseases has gradually become a research hotspot. A wide variety of herbal extracts have been utilised in the management of various clinical conditions. SAP in patients is characterised by a rapid and perilous disease onset. When accompanied by

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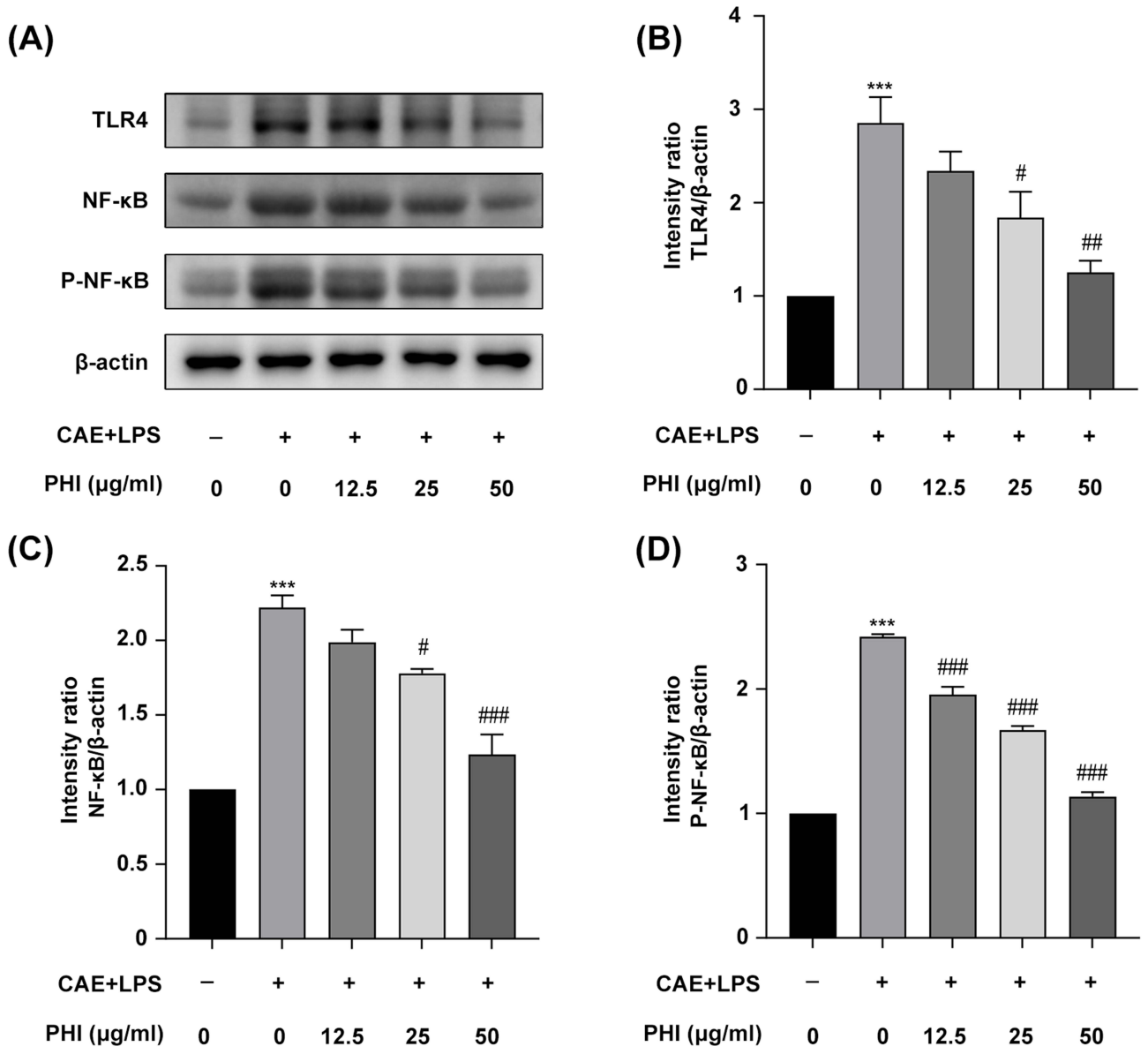

Figure 5 Effect of PHI on TLR4/NF-κB pathway expression in an in vitro cell model. (**A**) Expression of TLR4, NF-κB, and phospho-NF-κB in AR42J cells detected by protein blotting; (**B**–**D**) Quantitative analysis of the grayscale values of protein bands for TLR4, NF-κB, and phospho-NF-κB. n = 3 independent experiments. ***P < 0.001 vs control group. #P < 0.05, ###P < 0.01, ####P < 0.001 vs LPS group.

hazardous complications, the recovery process becomes challenging. Thus, there is a particular requirement for novel and secure medications for adjuvant therapy in these patients. PHI is an active compound discovered in the fruits of *Forsythia suspensa*. Previous studies have indicated that PHI possesses anti-inflammatory, antioxidant, anti-liver fibrosis, and gut microbiota modulatory effects.[21–23] Our previous research has demonstrated that PHI can rescue the impaired autophagic flux by inhibiting the PI3K/Akt/mToR pathway, allowing abnormal autophagic vesicles to complete autophagy to protect the rat.[20] However, it is still unknown whether PHI can exert pancreatic protection in SAP rats through other pathways.

Pancreatitis is primarily characterised by significant damage to the pancreatic tissue, evident through the presence of pancreatic edoema, haemorrhage, and necrosis. Moreover, a significant feature is the considerable invasion of inflammatory cells into the interstitium of the pancreas, exacerbating the damage caused to the pancreatic tissue.[24] We induced SAP in rats by retrograde injection of NaTc via the biliopancreatic duct. This resulted in pathologic changes that closely resembled those observed in human SAP. Significantly, the observed pathological alterations were much reduced following the administration of PHI, indicating that PHI has a protective impact on the pancreas. MPO, an enzyme mostly located in neutrophilic

azurophilic granules, can serve as a marker for the presence of inflammatory cell infiltration.[25,26] Our findings indicate that the administration of PHI effectively decreased MPO levels in the pancreatic tissues of SAP rats, providing further evidence of PHI's ability to suppress the aggregation of inflammatory cells. Furthermore, elevated concentrations of lipase and amylase were seen in the SAP rats' serum. Pancreatic digestive enzymes serve as indicators of pancreatitis and play a crucial role in the destruction of acinar cells and initiation of the inflammatory process in the initial phases of pancreatitis.[27] Notably, the elevation of amylase and lipase was suppressed following PHI administration. Taking all of these findings into consideration, they offer evidence that supports the protective properties of PHI in the rat SAP model, indicating that it may have the potential to be used as a therapeutic drug.

The crucial role of pro-inflammatory cytokines in the progression of SAP is widely recognized. They are responsible for initiating and sustaining the inflammatory response, which leads to the development of SIRS and MODS.[24,28] Among the various cytokines, TNF-α, IL-6, and IL-1β hold significant relevance in the development of SAP.[28] During the initial phases of pancreatitis, injured acinar cells secrete TNF-α and IL-1β, leading to the subsequent release of other inflammatory factors like IL-6. This triggers an inflammatory cascade response.[29–31] Furthermore, the promotion of neutrophil chemotaxis and activation by TNF-α and IL-1β can worsen pancreatic injury by exacerbating inflammatory cell infiltration.[30,32] Previous studies have indicated that the targeted inhibition of TNF-a and IL-1β binding to their receptors reduces the pancreatic damage in SAP rats.[33,34] IL-6 is equally important, as it acts as a major inducer of acute-phase protein responses and serves as a specific and sensitive marker of combined distal organ injury in SAP.[35] Based on the evidence presented above, we can infer that the levels of inflammatory factor directly determine the severity of SAP. Consequently, inhibiting the production of these inflammatory mediators is crucial for SAP treatment. PHI has shown strong anti-inflammatory properties in previous studies.[36] Therefore, we utilised it in the current investigation to assess its ability to alleviate SAP. Through quantifying the levels of inflammatory factors produced in each group, we discovered that PHI successfully suppressed the release of the aforementioned inflammatory factors in both the SAP rat model and the AR42J cell model in vitro. This indicates that PHI has a protective impact on the pancreas by reducing the inflammatory response.

TLRs are a category of receptors within the innate immune system that identify molecular patterns linked to pathogens and injuries, thereby initiating immune responses.[36] This immunological activation is linked to a potent inflammatory cascade response in SAP. TLR4, believed to have a significant pro-inflammatory function in SAP, is crucial for the production and liberation of inflammatory elements.[37] Sawa et al[38] highlighted the importance of TLR4 in SAP pathogenesis and demonstrated that TLR4-deficient mice exhibited a substantial reduction in inflammatory cytokine release after SAP induction compared to wild-type mice. Upon TLR4 activation, the transcription factor NF-κB is further activated in the cytoplasm and then translocated to the nucleus in a phosphorylated state to induce transcription of downstream inflammatory factors. Activated NF-κB has been shown to actively participate in the inflammatory response in SAP by mediating the production of inflammatory cytokines.[39–41] As a result, the therapy of SAP can benefit from the inhibition of the activation of this pathway. During the course of our research, we initially utilised molecular docking models to examine the interaction between PHI molecules and protein molecules in this pathway (TLR4 and NF-κB). Molecular dynamics simulations demonstrate that PHI maintains stable binding with both TLR4 and NF-κB under physiologically relevant conditions, thereby corroborating our docking results and reinforcing the reliability of the proposed binding mechanisms. Our findings revealed that these molecules possessed favourable binding qualities. We verified this result by further experiments. In our model of inflammation, the expression of both TLR4 and NF-κB was elevated and the phosphorylation of NF-κB was similarly increased, suggesting that the pathway was significantly activated. However, the levels of these proteins decreased after treatment with PHI. Furthermore, the application of PHI resulted in a reduction in the transportation of phospho-NF-κB to the nucleus in the pancreatic tissues of rats suffering from SAP. This suggests that PHI reduces the transcriptional activity performed by NF-κB within the nucleus, resulting in a decline in the synthesis of inflammatory mediators. The findings align with previous studies indicating that PHI has a defensive impact on various inflammatory disease models by blocking the TLR4/NF-κB pathway.[16,17] Therefore, this research proposes that PHI could potentially provide therapeutic benefits for SAP by inhibiting the secretion of inflammatory mediators via its disruption of the TLR4/NF-κB pathway.

Based on previous studies,[20] we have found that PHI may exert its anti-SAP effect through multiple pathways in a coordinated manner, providing a research foundation for further in-depth study of the combined regulatory mechanism of PHI. However, this study also has some limitations. In cellular and animal experiments, PHI exhibits significant anti-

inflammatory activity against SAP. However, the effectiveness of PHI in patients with SAP remains to be evaluated. Further studies are required to assess the effectiveness of PHI in patients with SAP.

PHI exhibits poor aqueous solubility, incomplete absorption, and limited permeability, resulting in low oral bioavailability that has impeded its clinical development as a potential therapeutic agent for SAP. Consequently, future research priorities should focus on novel drug delivery systems. For example, exosome-based delivery of PHI could enhance its solubility, bioavailability, and targeting capacity, thereby achieving targeted drug delivery and improving therapeutic efficacy against SAP. Pharmaceutical formulations derived from PHI have been developed and evaluated. Li et al[42] synthesized a PHI derivative through demethylation and hydroxylation to augment its antibacterial activity. Their findings demonstrated that this derivative exhibited superior potency against Helicobacter pylori infections compared to PHI, with activity levels 2–8 fold higher than the parent compound. This advancement suggests that PHI or its congeners could potentially progress under an Investigational New Drug (IND) framework. However, it must be acknowledged that the regulatory pathway would necessitate progression through multiple clinical phases, and currently, no phillygenin-derived agents have received clinical approval. Therefore, Therefore, the clinical translation of PHI or its congeners remains contingent upon further preclinical and clinical investigation. In the future, clinical trials of PHI therapy for SAP are essential, particularly multicenter randomized controlled trials. Moreover, additional research is required to examine whether PHI can also have a pancreatic safeguarding impact through its influence on other pathways, including its regulatory role in the gut microbiota.

Conclusion

In vitro and in vivo experiments, we have demonstrated that PHI has a protective effect against SAP in rats. It can alleviate SAP by inhibiting inflammatory factor and TLR4/NF-κB pathway. This makes it a candidate for the treatment of SAP. Further studies are required to evaluate the effectiveness of PHI in patients with SAP in the future.

Abbreviations

AP, Acute pancreatitis; IL-6, Interleukin-6; IL-1β, Interleukin-1β.MODS, Multiple organ dysfunction. LPS, Lipopolysaccharide.MPO, Myeloperoxidase; NF-κB, Nuclear factor-kappa B;SAP, Severe acute pancreatitis; SIRS, Systemic inflammatory response syndrome; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-α.

Data Sharing Statement

Data available in a publicly accessible repository. The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.26069473.

Ethics Approval Statement

Ethical approval for this study (Approval No. DW2023015) was provided by the ethics committee of The Second Hospital of Shanxi Medical University, Taiyuan, China on 8 March 2023. This study was carried out in strict accordance with the ARRIVE guidelines.

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Author Contributions

Yiwen Sun (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft), Shengze Li (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft), Jiming Duan (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft), Jiaxing Li (Formal analysis, Validation, Visualization, Writing—original draft) and Wenxing Li (Conceptualization, Investigation, Methodology, Resources, Supervision, Writing—review & editing).All

authors gave final approval of the version to be published, have agreed on the journal to which the article has been submitted, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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