Rhein kills Actinobacillus pleuropneumoniae, reduces biofilm formation, and effectively treats bacterial lung infections in mice

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Abstract

Actinobacillus pleuropneumoniae, a member of the Pasteurellaceae family, is known for its highly infectious nature and is the primary causative agent of infectious pleuropneumonia in pigs. This disease poses a considerable threat to the global pig industry and leads to substantial economic losses due to reduced productivity, increased mortality rates, and the need for extensive veterinary care and treatment. Rhein, as an antimicrobial compound, is one of the effective components extracted from Polygonaceae plants which has effects against kinds of bacteria. Here, in this study, we found that Rhein had a good killing effect on *A. pleuropneumoniae* and that the minimum inhibitory concentration (MIC) was 25 μg/mL. After 3h of action, Rhein (4×MIC) completely kills *A. pleuropneumoniae* and Rhein has good stability. In addition, the treatment with Rhein (1×MIC) significantly reduced the formation of bacterial biofilms. Therapeutic evaluation in a murine model showed that Rhein protects mice from *A. pleuropneumoniae* and relieves lung inflammation. Quantitative RT-PCR results showed that Rhein treatment significantly downregulated the expression of the IL-18、TNF-α、p65、p38 gene. This study reports the activity of Rhein against *A. pleuropneumoniae* and its mechanism and reveals, the ability of Rhein to treat *A. pleuropneumoniae* infection in mice, laying the foundation for the development of new drugs for bacterial infections.

Introduction

A. pleuropneumoniae, a significant bacterial pathogen of porcine respiratory tract, responsible for causing pig pleuropneumonia, has led to substantial economic losses globally[1]. *A. pleuropneumoniae* is a Gram-negative bacterium with a coccobacillary morphology. It is non-motile, naturally capable of transformation, and a facultative anaerobe[2]. *A. pleuropneumoniae* is categorized into two biovars, where biovar 1 strains necessitate NAD for growth, whereas biovar 2 strains do not. The bacterium comprises 19 recognized serovars distinguished by their capsule loci[3]. *A. pleuropneumoniae*, a Gram-negative bacterium, is the causative agent of pleuropneumonia in pigs, its sole known natural host. Acute disease is characterized by symptoms such as fever, apathy, and anorexia. Severe lung lesions are caused by the presence of one or both of the ApxI-III toxins. Acutely infected pigs often succumb to the infection within 24 hours. Furthermore, both acute and chronic infections are common occurrences[4]. Pleuropneumonia is marked by intense respiratory distress and a high mortality rate. The condition can lead to various types of pneumonia, including exudative pneumonia, fibrotic pneumonia, hemorrhagic pneumonia, as well as associated pleurisy[5,6]. *A. pleuropneumoniae* is responsible for causing porcine pleuropneumonia, a highly contagious disease for which an effective vaccine is not yet available [7]. Presently, drug-resistant strains of *A. pleuropneumoniae* have emerged as a worldwide issue, making it imperative to urgently explore effective antibiotic alternatives.

Due to its multitarget and potent curative effects, traditional Chinese medicine (TCM) preparation has emerged as a promising area of research for anti-inflammatory mechanisms. Rhein ($C_{15}H_8O_6$), an effective component extracted from Polygonaceae plants such as Senna alexandrina Mill., Rheum

palmatum L., Aloe barbadensis Miller, and Polygonum multiflorum Thunb, is known for its purgative effects in TCM[8,9]. Rhein contains two hydroxyl groups and one carboxyl group, rendering it highly polar and possessing notable electrochemical REDOX properties. Pharmacological studies have revealed that Rhein exhibits various actions, including antibacterial, anti-inflammatory, and anti-tumor activities. Additionally, it has shown potential in improving renal function, reducing blood lipid levels, and aiding in weight loss, among other benefits[10]. As an antimicrobial agent, Rhein exhibits effects against various microorganisms, including Staphylococcus aureus[11,12], Pseudomonas aeruginosa[13], Porphyromonas gingivalis[14], and influenza virus[15]. Its anti-inflammatory properties are attributed to its ability to inhibit NF-κB and NLRP3 inflammasome activation in macrophages[16]. a Moreover, Rhein can also modulate the expression of Nrf2 and MAPK, further contributing to its anti-inflammatory effects[17]. The bactericidal effect of Rhein on *A. pleuropneumoniae* (APP) has been poorly studied, and it remains uncertain whether Rhein exhibits a superior bactericidal effect against APP. Further research and studies are needed to investigate the specific antimicrobial activity of Rhein against APP and to determine its efficacy as a potential treatment option.

This study explored the antibacterial effect of Rhein against the Gram- negative bacterium *A. pleuropneumoniae*. Rhein could also effectively destroy the *A. pleuropneumoniae* cell membrane and significantly inhibit the biofilm formation of *A. pleuropneumoniae*. The therapeutic effect on a mouse model of *A. pleuropneumoniae* infection showed that Rhein reduced the expression of inflammatory factors, significantly alleviated lung pathological damage and reduced congestion symptoms. The above results indicate that Rhein has a good killing effect on *A. pleuropneumoniae* and is expected to become a new drug for treating *A. pleuropneumoniae* infections.

Result

The Rhein Shows Bactericidal Activity Against A. pleuropneumoniae

The antibacterial activity of Rhein against *A. pleuropneumoniae* was detected by the agar diffusion method. The research findings indicate that at 800 μg/mL, Rhein exhibits an inhibition zone against *A. pleuropneumoniae*, while the control group shows no inhibition zone. This suggests that Rhein antibacterial activity against *A. pleuropneumoniae*. Furthermore, the Minimum Inhibitory Concentration (MIC) of Rhein against *A. pleuropneumoniae* is 25 μg/mL. In Fig. 1A, the optical density at 600 nm (OD_{600}) of the bacterial solution in the Rhein(1×MIC) treatment group was lower than that of the control group, indicating that Rhein at 1×MIC concentration inhibited the growth of *A. pleuropneumoniae*. Moreover, the OD_{600} value of the bacterial solution in the Rhein(4×MIC) treatment group was even lower than that of the Rhein(1×MIC) treated group, suggesting that a higher concentration of Rhein (4×MIC) further enhanced its bacteriostatic effect. This positive correlation between the bacteriostatic effect and Rhein concentration indicates the dose-dependent nature of Rhein's antibacterial activity against *A. pleuropneumoniae*. In Fig. 1B, it is observed that Rhein at 4×MIC concentration was able to completely eliminate *A. pleuropneumoniae* (APP) within 3 hours in the 5b WT (wild-type) strain. Additionally, it was

noted that as the concentration of Rhein increased, the rate of decline in the number of live APP bacteria accelerated, indicating a strong dose-dependent bactericidal effect of Rhein against *A. pleuropneumoniae*. Then, in order to further evaluate the effect of Rhein, the morphological changes of *A. pleuropneumoniae* after exposure to Rhein were examined using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The results from SEM showed (Fig. 1C) that after the action of Rhein, there was a significant decrease in the number of *A. pleuropneumoniae* bacteria. Additionally, the SEM images revealed that the cell membranes of the bacteria underwent contraction, and there was evidence of cell contents leakage. This indicates that Rhein treatment resulted in damage to the cell membranes and disruption of the bacterial structure. On the other hand, the blank control group (without Rhein treatment) exhibited smooth cell surfaces and intact structures. The results from TEM further support the observations from SEM. The surface of *A. pleuropneumoniae* without the action of Rhein appeared smooth and bright, with a complete and intact structure. However, after treatment with Rhein, the cell membrane of *A. pleuropneumoniae* was significantly damaged, and the overall structure of the bacteria became incomplete(Fig. 1D). The above experimental results show that Rhein has good bactericidal activity against *A. pleuropneumoniae.*

Rhein has stable bacteriostatic effect

The study investigated the antibacterial activity of Rhein against *A. pleuropneumoniae* under various conditions. Firstly, the impact of repeated freeze-thaw cycles on the antibacterial activity of Rhein was examined (Fig. 2A). The results showed that after undergoing multiple freeze-thaw cycles (0–12 times), the antibacterial activity of Rhein decreased significantly, indicating that its efficacy was affected by repeated freeze-thaw processes. Next, the effect of different temperatures on the antibacterial activity of Rhein was assessed (Fig. 2B). It was found that the inhibition zone gradually increased with higher temperatures within the range of 25 ~ 100℃, suggesting that elevating the temperature improved the antibacterial activity of Rhein.

To understand the influence of monovalent cations (Na^+ and K^+) on Rhein's antibacterial activity, the Minimum Inhibitory Concentration (MIC) of Rhein was determined in the presence of different concentrations of NaCl and KCl (50, 100, 150, and 200 mmol/mL) (Fig. 2C and 2D). The results indicated that Na^+ had a positive impact on the antibacterial activity of Rhein, as the diameter of the inhibitory zone increased significantly with higher NaCl concentrations. However, K^+ had minimal effect on Rhein's antibacterial activity, even at increasing concentrations. This indicated that Rhein was stable in the presence of Na^+ and K^+.

Furthermore, the effects of divalent cations (Ca^{2+} and Mg^{2+}) on Rhein's antibacterial activity were investigated by incubating *A. pleuropneumoniae* with different concentrations of $CaCl_2$ and $MgCl_2$ solutions (50, 100, 150, and 200 mmol/mL) (Figs. 2E and 2F). It was found that both Ca^{2+} and Mg^{2+} had a significant impact on the antibacterial activity of Rhein, showing a notable inhibition of Rhein's antimicrobial activity against *A. pleuropneumoniae* in the presence of higher concentrations of these

divalent ions. It was hypothesized that the presence of Ca^{2+} and Mg^{2+} might induce changes in the secondary structure of Rhein, thus affecting its antibacterial activity.

In summary, the study revealed that the antibacterial activity of Rhein against *A. pleuropneumoniae* is influenced by repeated freeze-thaw cycles and the presence of divalent cations (Ca^{2+} and Mg^{2+}), while temperature and monovalent cations (Na^{+} and K^{+}) had minimal effects on its efficacy.

Rhein Destroys the Integrity of the Bacterial Cell Membrane, Changes the Membrane Potential, and Releases Cell Contents.

Multidrug-resistant pumps are membrane proteins located on the bacterial cell membrane that use ATP hydrolysis or proton movement to expel antibiotics, dyes, detergents, disinfectants, and some cellular metabolites that enter the cell. DiSC3(5) is a fluorescent dye that accumulates in the cytoplasmic membrane in response to the proton component (Δψ) of the proton motive force and self-quenches its fluorescence. When the pH gradient (ΔpH) is disrupted, cells increase Δψ as a compensatory mechanism, leading to enhanced uptake of DiSC3(5) by the cytoplasmic membrane, resulting in a reduction of DiSC3(5) fluorescence. In the study, DiSC3(5) uptake was used to evaluate the effect of Rhein on APP 5b WT. Figure 3A demonstrates that Rhein acid at different concentrations (1MIC, 2MIC, 4MIC) was applied to *A. pleuropneumoniae* at different time points (0s, 20s, 40s, 60s, 80s, 100s). The fluorescence intensity of each well solution was measured, and it was observed that the fluorescence intensity decreased rapidly with the increase in the concentration of Rhein.

To investigate the impact of Rhein on the integrity of bacterial membranes in *A. pleuropneumoniae*, we evaluated the membrane disruption mechanism using NPN probes, a hydrophobic fluorescent reagent that emits strong fluorescence in hydrophobic environments. When cell membranes are disrupted, NPNs enter the hydrophobic environment and release intense fluorescence. As depicted in Fig. 3B, we subjected *A. pleuropneumoniae* to different concentrations of Rhein (1×MIC, 2×MIC, 4×MIC) for varying durations (0, 20, 40, 60, 80, and 100 seconds), followed by measuring the NPN fluorescence intensity. Our findings clearly demonstrated that Rhein rapidly and concentration-dependently disrupted the bacterial membrane of *A. pleuropneumoniae*. Remarkably, even at a concentration of 1×MIC, it substantially compromised the integrity of the bacterial membrane, resulting in significantly higher fluorescence intensity compared to the control group. These results underscore the potent membrane-disrupting capability of Rhein against *A. pleuropneumoniae*.

In order to further explore the killing mechanism of *A. pleuropneumoniae*, the effect of Chrustine on the cell membrane structure of *A. pleuropneumoniae* was studied. Determination of changes in total protein content in bacterial supernatant after reaction of Rhein using the BCA Protein Quantitation Kit. The findings are presented in Fig. 3C. After exposing *A. pleuropneumoniae* to Rhein for 5 minutes, the total protein content in the supernatant of bacterial culture was found to be significantly higher than that of the control group. This increase in total protein content indicates that Rhein treatment leads to the disruption of the bacterial cell membrane, causing the release of cellular contents into the surrounding

environment. The membrane damage caused by Rhein results in the leakage of intracellular proteins, which is reflected by the higher total protein concentration in the supernatant.

The study demonstrated that PI (propidium iodide) is unable to stain live bacterial cells. However, when the bacterial cell membranes are disrupted, PI can penetrate the membrane and stain the nuclei. The results of this staining are depicted in Fig. 3D. After subjecting *A. pleuropneumoniae* to Rhein treatment at a concentration of 1×MIC for 5 minutes, the number of bacteria stained with PI substantially increased compared to the blank control group. This indicates that Rhein severely damages the bacterial cell membrane, leading to a significant influx of PI into the bacteria. Consequently, the number of dead bacteria increased notably.

Rhein Reduces the Formation of Bacterial Biofilms

Bacterial biofilms play a crucial role in bacterial adaptability and the development of resistance to antibacterial drugs. To investigate the impact of Rhein on *A. pleuropneumoniae* biofilms, different concentrations of Rhein (0.25×MIC, 0.5×MIC, 1×MIC) were exposed to *A. pleuropneumoniae* for 24 hours. Crystal violet staining was used to observe the effects on biofilm formation (Fig. 4A). The results revealed that Rhein effectively inhibited the formation of *A. pleuropneumoniae* biofilms. After dissolving the crystal violet with 70% ethanol, the OD_{570} measurements further demonstrated that Rhein at 1×MIC significantly reduced *A. pleuropneumoniae* biofilm formation (statistical significance indicated as $**$, $p < 0.01$, Fig. 4B). The impact of Rhein on *A. pleuropneumoniae* biofilm formation was also assessed using scanning electron microscopy (SEM). *A. pleuropneumoniae* was incubated with Rhein at 1×MIC for 24 hours, and the results are displayed in Fig. 4C. In the blank control, the *A. pleuropneumoniae* biofilm appeared dense with small gaps between bacterial cells. However, after treatment with Rhein (1×MIC), the biofilm formation was significantly reduced, displaying a blurred boundary, loss of original shape, and a looser structure (Fig. 4C).

Rhein protects mice against infection with A. pleuropneumoniae

We developed a mouse model of *A. pleuropneumoniae* infection to assess the antimicrobial efficacy of Rhein. Additionally, to evaluate the anti-inflammatory properties of Rhein, we analyzed the expression levels of macrophage markers (F4/80, Ly6G, and p-p38) in mouse lung tissues using immunohistochemistry. The results depicted in Fig. 5 revealed that following infection with *A. pleuropneumoniae*, the expression levels of lung macrophage markers in mice were significantly higher compared to the blank control group. However, upon treatment with Rhein, the expression levels of these macrophage markers decreased, suggesting a potential anti-inflammatory effect of Rhein.

In the 8-hour treatment group, the lungs of mice were stained with HE staining. Figure 6A illustrates that the treatment significantly alleviated lung pathological damage, leading to reduced congestion and bleeding symptoms, diminished inflammatory cell infiltration in alveoli, and decreased alveolar damage and alveolar septal thickening. Conversely, in untreated mice infected with APP 5b WT, the symptoms

were more severe, including hyperemia, hemorrhage, infiltration of fibrin and inflammatory cells, and typical pneumonia symptoms. To further investigate the verification of transcriptional expression levels of genes related to inflammatory factors in the lungs of mice during *A. pleuropneumoniae* infection, changes in these genes were quantitatively detected using fluorescence. As shown in Fig. 6B-I, the mRNA expression of IL-18 and IL-1β in mouse lungs increased after 2 hours of infection with APP 5b WT. However, after 8 hours of treatment with Rhein, the expression of IL-18, TNF-α, and IL-1β mRNA in the lungs of mice infected with APP 5b WT was down-regulated. These results indicate that Rhein treatment has a potential protective effect on the lungs of mice during *A. pleuropneumoniae* infection. It appears to reduce the inflammatory response by down-regulating the expression of certain inflammatory factors, which may contribute to the observed alleviation of lung pathological damage and the amelioration of disease symptoms.

Discussion

To date, numerous studies have investigated the antibacterial properties of plant extracts, specifically phenols and flavonoids, and have found them to be effective[18]. Rhein, a compound known for its various pharmacological effects, such as hepatoprotection, nephroprotection, anti-inflammatory, antioxidant, anticancer, and antimicrobial activities, exhibits excellent thermal stability and monovalent salt ion stability. Therefore, Rhein holds great potential as a therapeutic agent. For the first time, we report that Rhein possesses potent bactericidal activity against *A. pleuropneumoniae*, which is attributed to its ability to increase cell membrane permeability, alter bacterial morphology, and significantly reduce *A. pleuropneumoniae* biofilm formation. In addition, Rhein reduced the expression of inflammatory factors, lessened lung histological damage, and protected mice from fatal dosages of *A. pleuropneumoniae* mycoplasma infection. This comprehensive investigation systematically assessed the antibacterial and anti-inflammatory properties of Rhein in vivo and invitro, thereby establishing a fundamental basis for the utilization of Rhein as a therapeutic agent for *A. pleuropneumoniae* infections.

In a previous study, it was reported that the majority of Canadian isolates were susceptible to most tested antimicrobials, with the exception of a high level of resistance observed for chlortetracycline (88.4%) and oxytetracycline (90.7%)[19]. It should be emphasized that the serovars implicated in clinical cases have undergone significant changes over time in Canada[20]. More recently, plasmids conferring resistance to florfenicol and chloramphenicol were discovered from App clinical isolates from Greece and Brazil[21,22], and enrofloxacin resistant strains have been reported in Taiwan and the UK[23,24]. The risk of drug-resistant strains of App developing is generally increased due to the use of antibiotics[25]. The fundamental factor causing bacterial resistance to β-lactamide antibiotics is bacterial synthesis of β-lactamases[26]. It has been found that Shuanghuanglian and Qingkailing, two antipyretic and detoxifying TCM compounds, can eliminate destroy the drug-resistant plasmid of extended-spectrum β-lactamases (ESBLs) and inhibit the activity of β-lactamase. The activity of ESBLs can be inhibited by HuanglianJiedu decoction, Sanhuang decoction, and Wuwei disinfecting decoction, which is an important mechanism for

reducing bacterial resistance. Therefore, in light of the current challenge posed by the increasingly severe bacterial drug resistance, TCM can be considered for the treatment bacterial infections.

Biofilms are microbial aggregates that adhere to biotic or abiotic surfaces and grow in an organized manner[27]. According to one investigation, App may employ biofilm formation as a survival tactic in drinking water on swine farms[28]. Because low antibiotic penetration, reduced metabolic and growth rates, induction of adaptive stress responses, and production of persisted cells in the biofilm milieu make bacteria in biofilms more resistant to antimicrobial agents than planktonic cells[29,30]. Importantly, approximate 80% of chronic infections are associated with biofilm formation, which means that disruption of biofilm is critical for controlling persistent bacterial infections[31]. According to a study by Wang et al. sodium houttuyfonate was able to penetrate P. aeruginosa biofilm and suppress biofilm dispersion by inhibiting the expression of the key biofilm regulator BdlA[32]. According to Fu et al., the water extract of Herba patriniae was able to disrupt the biofilm formation and alter the biofilm structure of P. aeruginosa PAO1 through inhibition of exopolysaccharide production and biofilm-associated genes algU, pslM, pelA, algA, and bdlA[33]. Numerous studies[34,35] have demonstrated the ability of monomers such as berberine, allicin, TCM extracts like those of Euphorbia burnet, Tanreqing compound[36], and HuanglianJiedu decoction to prevent the development of bacterial biofilm, and ultimately reverse drug resistance. Therefore, Traditional Chinese Medicines can be utilized as a novel method of treating biofilm-related infections. Rhein has the potential to be developed into an antibacterial medication because it dramatically reduced *A. pleuropneumoniae* biofilm formation at 1×MIC (4 μg/mL), and Rhein could provide references for the development of other antibiofilm formulations.

Our in vitro studies revealed that Rhein has the ability to kill bacteria by increasing membrane permeability and disrupting biofilm formation. Protein content of bacterial culture supernatant was significantly increased compared with a control group, indicating that the cell membrane has been disrupted. Regarding in vivo studies, we discovered that Rhein decreased the levels of Ly6G and F4/80, alleviated pneumonia symptoms, and eventually protected mice from lethal doses of APP infections.

METHODS

Ethics statement. Thirty-six BALB/c mice (6–8 weeks old, weight 18–20 g, female mice) were purchased from Zhengzhou University Animal Center (No. 41003100024648). All animal studies were conducted in accordance with the experimental practices and standards of the Animal Welfare and Research Ethics Committee of Henan Institute of Science and Technology.

Reagents. A BCA protein kit was purchased from Solaibao Technology. PBS, LB medium, crystal violet, NaCl, KCl, $CaCl_2$, $MgCl_2$, 96-well culture plates, N-phenyl-1-naphthylamine (NPN), and 3,3'-dipropylthiadicarbocyanine iodide (DiSC3-5) were purchased from Zhibao Biotechnology Co., Ltd

Chinese herbs Synthesis. The Rhein was acquired as a standard from Beijing Solarbio and then diluted to a concentration of 800 μg/mL using a DMSO solution. For this study, the *A. pleuropneumoniae* serovar

5b wild-type strain was employed.

Antibacterial activity. The agar diffusion test was employed to assess the antibacterial effect of Rhein on *A. pleuropneumoniae*. *A. pleuropneumoniae* was cultured overnight, and the supernatant was removed by centrifugation. The bacteria were then washed with sterile PBS and resuspended in a smaller volume to concentrate the cells. Subsequently, 50 μL of the bacterial solution was pipetted into 15 mL of agar and gently shaken to ensure even distribution. Once the agar medium solidified, holes were made using a puncher, and 20 μL of 1 mg/mL Rhein was added to the wells. DMSO water served as the control. The plates were incubated at 37℃ overnight, and the results were observed.

To assess the influence of salt ions, temperature, and repeated freezing and thawing on the antibacterial activity of Rhein, Rhein solutions were incubated with various concentrations of NaCl, KCl, $CaCl_2$, and $MgCl_2$ (0, 50, 100, 150, and 200 mmol/mL) for 30 minutes. Additionally, Rhein solutions were subjected to different temperatures (0, 25, 50, 75, and 100℃) for 30 minutes. Furthermore, a 800 μg/mL Rhein solution underwent a series of repeated freezing and thawing cycles, specifically 0, 2, 4, 6, 8, 10, and 12 times.

After each treatment, twenty microliters of the Rhein solutions were collected and assessed for changes in antimicrobial activity using the agar diffusion assay. Subsequently, 20 μL of Rhein treated under different conditions (repeated freezing and thawing, temperatures, and salt concentrations) was added to a plate containing *A. pleuropneumoniae*. The plate was then incubated overnight at 37℃ in an incubator, and the effects of the different treatments on the antibacterial activity of Rhein were observed.

Killing Curve. *A. pleuropneumoniae* was cultured in BHI + NAD liquid medium until it reached the log phase (OD_{600} = 1.0). Rhein was then added to the bacterial solution at final concentrations of 1×MIC and 4×MIC. DMSO was used as the negative control. The cultures were incubated with shaking at 37℃. The OD_{600} value of the bacterial solution was measured at 0, 15, 30, 45, 60, 120, and 180 minutes. At the same time points, plate counting was performed to obtain the antibacterial time-killing curve of Rhein against *A. pleuropneumoniae*.

Stability. To assess the influence of salt ions, temperature, and repeated freezing and thawing on the antibacterial activity of Rhein, Rhein solutions were incubated with various concentrations of NaCl, KCl, $CaCl_2$, and $MgCl_2$ (0, 50, 100, 150, and 200 mmol/mL) for 30 minutes. Additionally, Rhein solutions were subjected to different temperatures (0, 25, 50, 75, and 100℃) for 30 minutes. Furthermore, a 800 μg/mL Rhein solution underwent a series of repeated freezing and thawing cycles, 0, 2, 4, 6, 8, 10, and 12 times.

After each treatment, twenty microliters of the Rhein solutions were collected and assessed for changes in antimicrobial activity using the agar diffusion assay. Subsequently, 20 μL of Rhein treated under different conditions (repeated freezing and thawing, temperatures, and salt concentrations) was added to a plate containing *A. pleuropneumoniae*. The plate was then incubated overnight at 37℃ in an incubator, and the effects of the different treatments on the antibacterial activity of Rhein were observed.

BCA protein assay. *A. pleuropneumoniae* was cultured overnight and subsequently washed and resuspended in sterile PBS. To this bacterial solution, Rhein was added with a final concentration of 1×MIC and 4×MIC, while DMSO was added as a control. After allowing the mixture to stand for 5 minutes, the supernatant was collected following centrifugation. The impact of Rhein on the permeability of *A. pleuropneumoniae* for protein was analyzed using the BCA kit, following the provided instructions.

Propidium Iodide Uptake. *A. pleuropneumoniae* was centrifuged, washed, and resuspended in a sterile saline solution. Rhein at a concentration of 1×MIC was added to the bacterial solution. The bacteria were cultured with shaking at 37℃. After culturing, the bacteria were centrifuged again and resuspended in a PBS solution. Subsequently, the bacterial cells were incubated with propidium iodide (PI) stain for 10 minutes in the dark. The stained bacterial suspension was fixed onto a clean glass slide and then observed using a fluorescence microscope.

Crystal Violet Assay. An overnight culture of *A. pleuropneumoniae* was inoculated into a 96-well polystyrene microtiter plate at a 1% ratio. Different concentrations of Rhein (0.25×MIC, 0.5×MIC, and 1×MIC) were added to separate wells, while DMSO was used as a control. The microplates were then incubated at 37℃ for 24 hours. After incubation, the microplates were washed three times with sterile PBS, and the cells were fixed with 70% methanol for 30 minutes. Subsequently, the cells were stained with 1% Hucker crystal violet staining solution at room temperature for 5 minutes. The excess dyeing solution was aspirated, and the cells were rinsed with water until no color remained. Images were taken after drying. To dissolve the residual dye, 100 μL of a 70% ethanol solution was added to each well, and the plate was vortexed to mix the contents. The absorbance at OD_{570} nm was then measured using a microplate reader.

N-phenyl-1-naphthylamine uptake. *A. pleuropneumoniae* was cultured overnight in a 96-well plate. Next, an NPN (N-phenyl-1-naphthylamine) solution was added to each well, achieving a final concentration of 10 μM. The solutions were thoroughly mixed and incubated for 20 minutes. Subsequently, Rhein at different concentrations (1×MIC, 2×MIC, and 4×MIC) was added to separate wells, while DMSO was used as the control. The microplate was then subjected to fluorescence measurement using a microplate reader, with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The fluorescence intensity was recorded for further analysis.

DiSC3(5) **Assay for Detection of Membrane Potential.** Overnight-cultured *A. pleuropneumoniae* was centrifuged and then resuspended in sterile PBS. Next, a DiSC3-5 (3,3'-dipropylthiadicarbocyanine iodide) solution was added to each well, achieving a final concentration of 1 μM. The cells were incubated in the dark for 20 minutes to allow for proper staining. Afterward, Rhein was added at different concentrations (1×MIC, 2×MIC, and 4×MIC) to separate wells, while DMSO was used as the control. The microplate was then subjected to fluorescence measurement using a microplate reader, with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The fluorescence intensity was recorded for further analysis.

Scanning Electron Microscopy. A sterile slide was placed in each of the 6-well cell culture plates, and the bacteria were allowed to naturally grow on the slides, forming a biofilm. The biofilm was established by adding overnight culture broth and fresh broth at a volume ratio of 1:100, followed by incubation at 37℃ for 24 hours. After the incubation period, *A. pleuropneumoniae* was washed three times with sterile physiological saline to remove any floating bacteria. Subsequently, Rhein was added to achieve a final concentration of 1×MIC, and the incubation was continued. To prepare the samples for scanning electron microscopy (SEM), a 2.5% glutaraldehyde solution was added to each well, and the cells were fixed at room temperature for 30 minutes. The plate was then rinsed three times with PBS for 10 minutes each time. To facilitate sample preparation for SEM, gradient dehydration was performed using an increasing series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100%). After completing the dehydration process, the samples were ion-sputtered with gold coating. Finally, the morphological changes of *A. pleuropneumoniae* biofilm were observed using scanning electron microscopy (SEM).

Mouse model of A. pleuropneumoniae infection. The *A. pleuropneumoniae* serotype 5b L20 strain was cultured until it reached the logarithmic growth phase. Subsequently, it was centrifuged at 8000 rpm for 5 minutes and washed three times with phosphate buffer at pH 7.4. The mice were then divided into three groups. Each mouse in all groups received a nasal drop containing a total of 1×10^8 CFU (colony-forming units) of *A. pleuropneumoniae* for infection. Two hours after bacterial infection, the second group of mice received treatment with Rhein through intraperitoneal injection. The Rhein dosage administered was 100 μL, and the drug concentration was 800 μg/mL. The third group served as the untreated blank control.

After being treated for 8 hours, 24 hours, and 32 hours, the mice were anesthetized and euthanized. During the experimental process, we employed ether anesthesia to ensure that the experimental animals were in a painless state. Throughout the anesthesia procedures, we strictly adhered to international and local ethical guidelines, making every effort to minimize the suffering of the experimental animals. All methods in this study were conducted in accordance with applicable ethical guidelines and regulations. The study obtained approval from the Ethics Committee of HenanInstitute of Science and Technology, and all participants signed an informed consent form prior to the experiment. The lungs were then extracted and photographed for observation.

During euthanasia, we ensured that animals did not experience undue suffering and made efforts to minimize any potential discomfort. After euthanasia, we harvested lungs for further analysis. All euthanasia procedures followed the laboratory's standard operating procedure res and ethical guidelines to ensure animal welfare and experimental compliance.

For the 8-hour treatment group, the lungs were fixed with 4% paraformaldehyde, followed by paraffin embedding, sectioning, and HE staining to observe the pathological changes. Subsequently, the lung samples were examined under an electron microscope to study the histological alterations.**Immunohistochemical analysis.** The lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 5 μm thick slices. After deparaffinization, rehydration, and

antigen retrieval, the endogenous peroxidase activity was blocked using 3% H_2O_2. Primary antibodies targeting F4/80, Ly6G, and p-p38 were incubated with the tissue sections at 4℃ overnight. Following washing, the slides were incubated with biotin-labeled secondary antibodies specific to *A. pleuropneumoniae*, followed by staining with 3, 3'-diaminobenzidine (DAB). Hematoxylin was used for counter-staining. The stained slides were examined under a light microscope for analysis.

Real-time polymerase chain reaction (PCR) analysis. Total RNA was extracted from various tissues using TRIzol reagent (Life Technologies, Carlsbad, CA), and cDNA was synthesized using the PrimeScript RT reagent kit with gDNA eraser (TakaRa, Dalian, China), following the established protocol. The GADPH gene was employed as an internal standard for normalization. The efficiency of Inflammatory Genes and GADPH was evaluated using a standard curve. qRT-PCR was conducted with SYBR Premix Ex TaqII (Takara, RR820A, Dalian, China) according to the manufacturer's instructions. Each quantification was performed in triplicate. Following PCR, the data were analyzed using the QuantStudio 5 system (Applied Biosystems, Thermo Fisher Scientific, Marsiling, Singapore) with the $2^{-\triangle\triangle Ct}$ method.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism software (version 8.0, La Jolla, CA, USA). The results are presented as mean ± standard deviation. Group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance is represented as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Declarations

Ethics declarations. This study was designed and reported following the guidelines of the ARRIVE (Animal Research: Reporting of In Vivo Experiments) to ensure transparency and ethical compliance in animal research. Throughout the experimental process, appropriate sample sizes, randomization, and blinding measures were taken, and the welfare and ethical approval of the experimental animals were ensured.

Data Availability. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

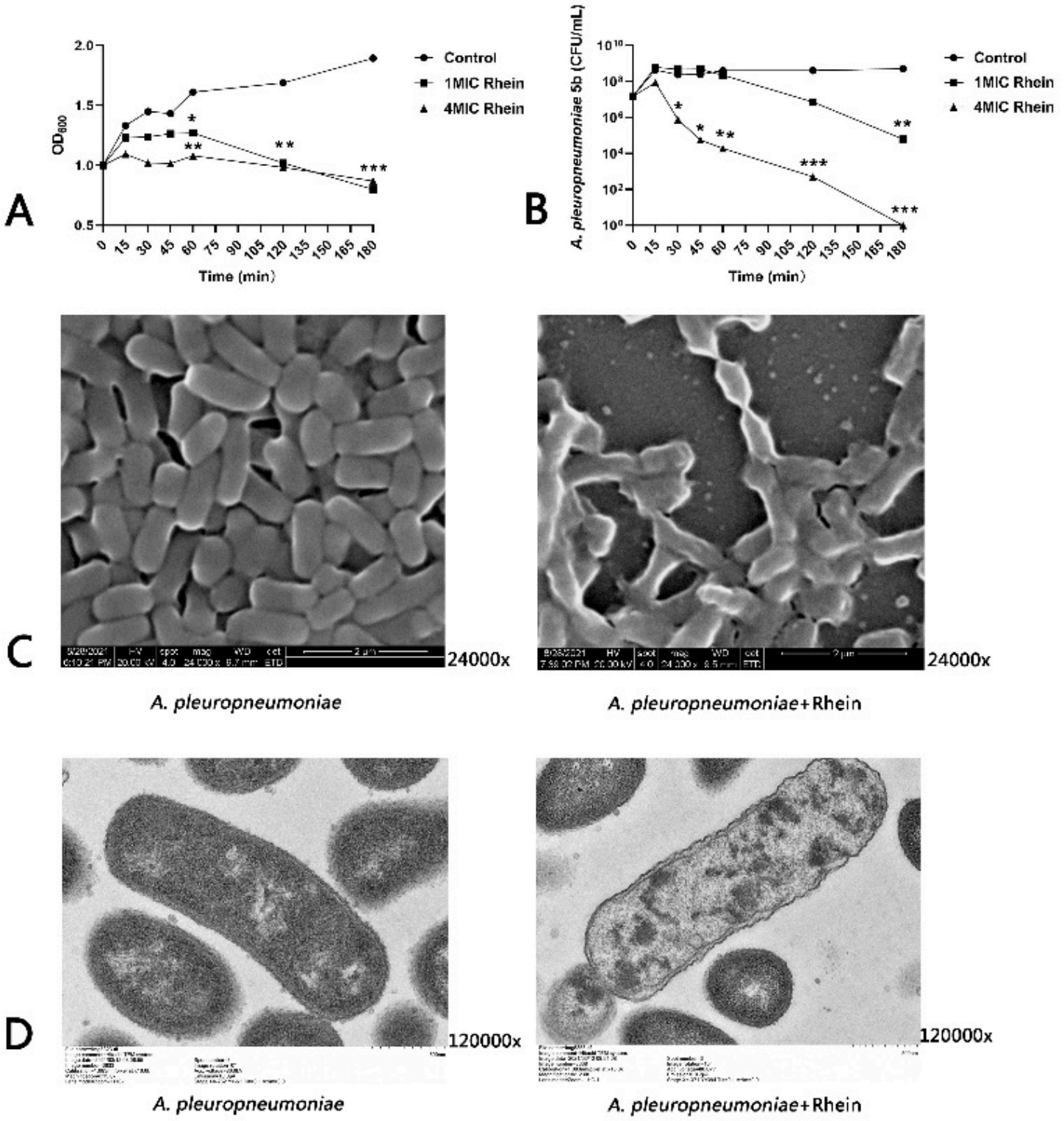

Figure 1

Detection of the bactericidal activity of Rhein.

(**A**) OD_{600} measurement of bacterial culture after the action of Rhein for different times. (**B**) The determination of the number of viable bacteria after the action of Rhein for different times. (**C**) Scanning electron microscopy observation of the effect of Rhein on *A. pleuropneumoniae*. The left picture shows the normal morphology of *A. pleuropneumoniae*, and the right picture shows the bacterial morphology of *A. pleuropneumoniae* after Rhein (1×MIC) exposure. (**D**) Transmission electron microscopy observation of the effect of Rhein on *A. pleuropneumoniae.* The left picture shows the normal morphology of *A. pleuropneumoniae*, and the right picture shows the bacterial morphology of *A. pleuropneumoniae* after Rhein (1×MIC) exposure. Error bars indicate the mean ± SEM, n = 3. Statistical significance was defined as **p < 0.01 and ***p < 0.001.

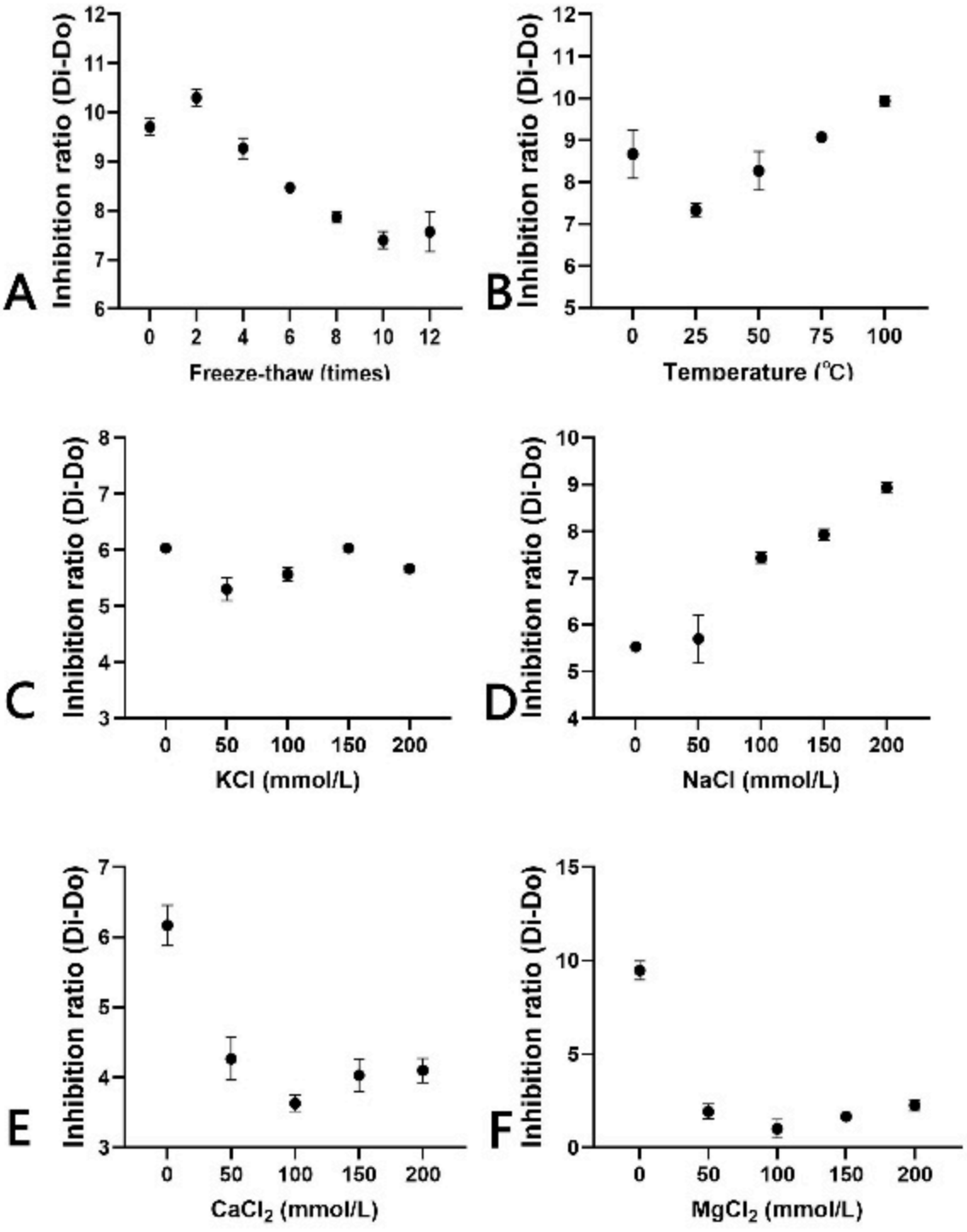

Figure 2

Stability test of Rhein. (A) The effect of repeated freezing and thawing on the antibacterial activity of Rhein. (B) The effect of different temperatures on the antibacterial activity of Rhein. (C–F) The effect of different concentrations of NaCl, KCl, $CaCl_2$ and $MgCl_2$ on the antibacterial activity of Rhein. Error bars indicate the mean ± SEM, n = 3.

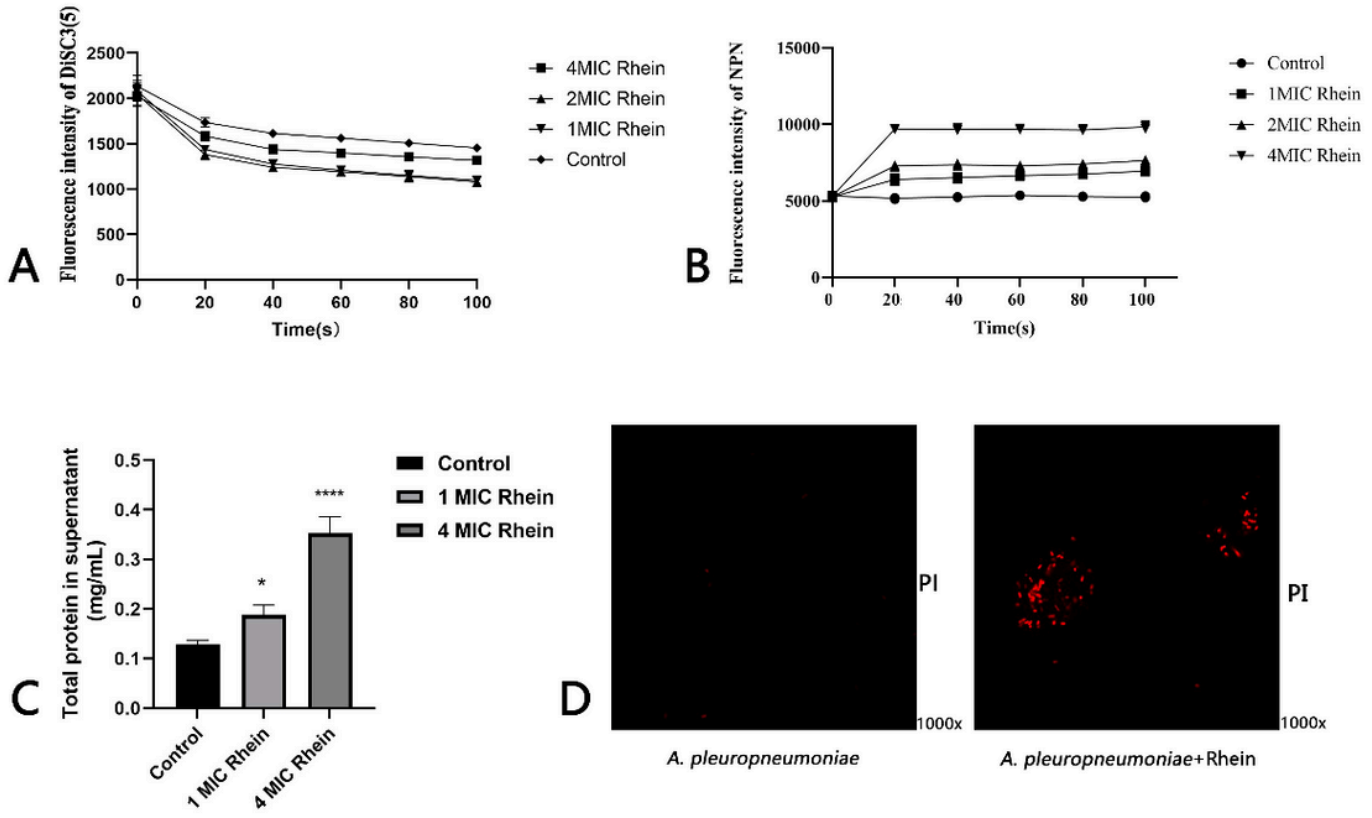

Figure 3

The mechanism of action of Rhein activity on *A. pleuropneumoniae*. (A) DiSC3(5) was used to detect changes in the membrane potential of *A. pleuropneumoniae* after Rhein treatment. (B) Uptake of NPN by *A. pleuropneumoniae* treated with Rhein. (C) The BCA kit was used to detect the results of total protein content in bacterial supernatant. (D)Fluorescence microscopy observation of PI staining after Rhein treatment. Error bars indicate the mean ± SEM, n = 3. Statistical significance was defined as **p < 0.01.

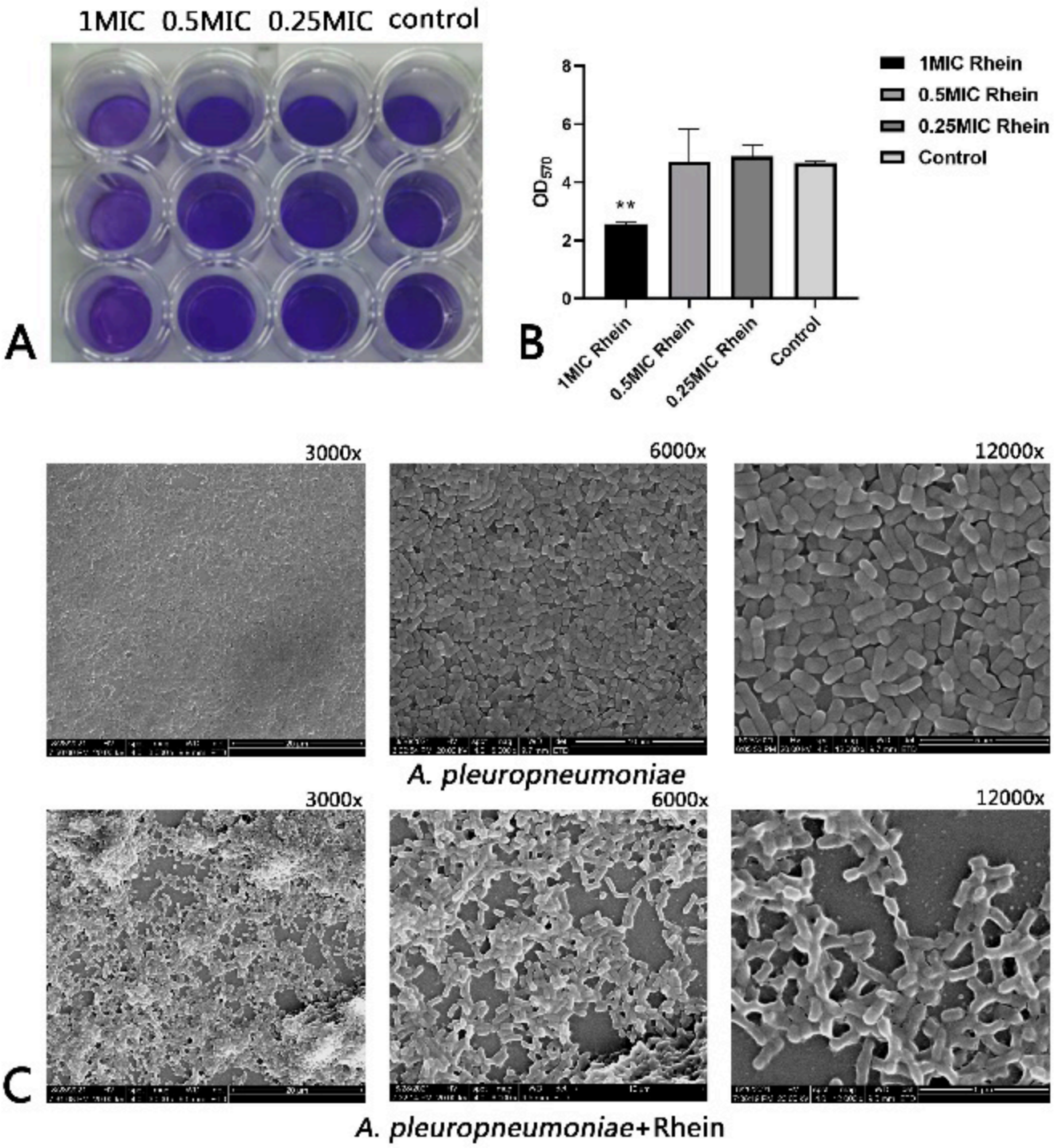

Figure 4

Rhein inhibits biofilm formation. (A) The results of crystal violet staining of biofilms after different concentrations of Rhein were applied to *A. pleuropneumoniae*. (B) OD_{570} measurement after crystal violet was dissolved in 70% ethanol. (C) After exposure of *A. pleuropneumoniae* to Rhein (1×MIC), scanning electron microscopy was used to observe the biofilm. Error bars indicate the mean ±SEM, n = 3. Statistical significance was defined as follows: **p < 0.01; ***P < 0.001; ns P > 0.05.

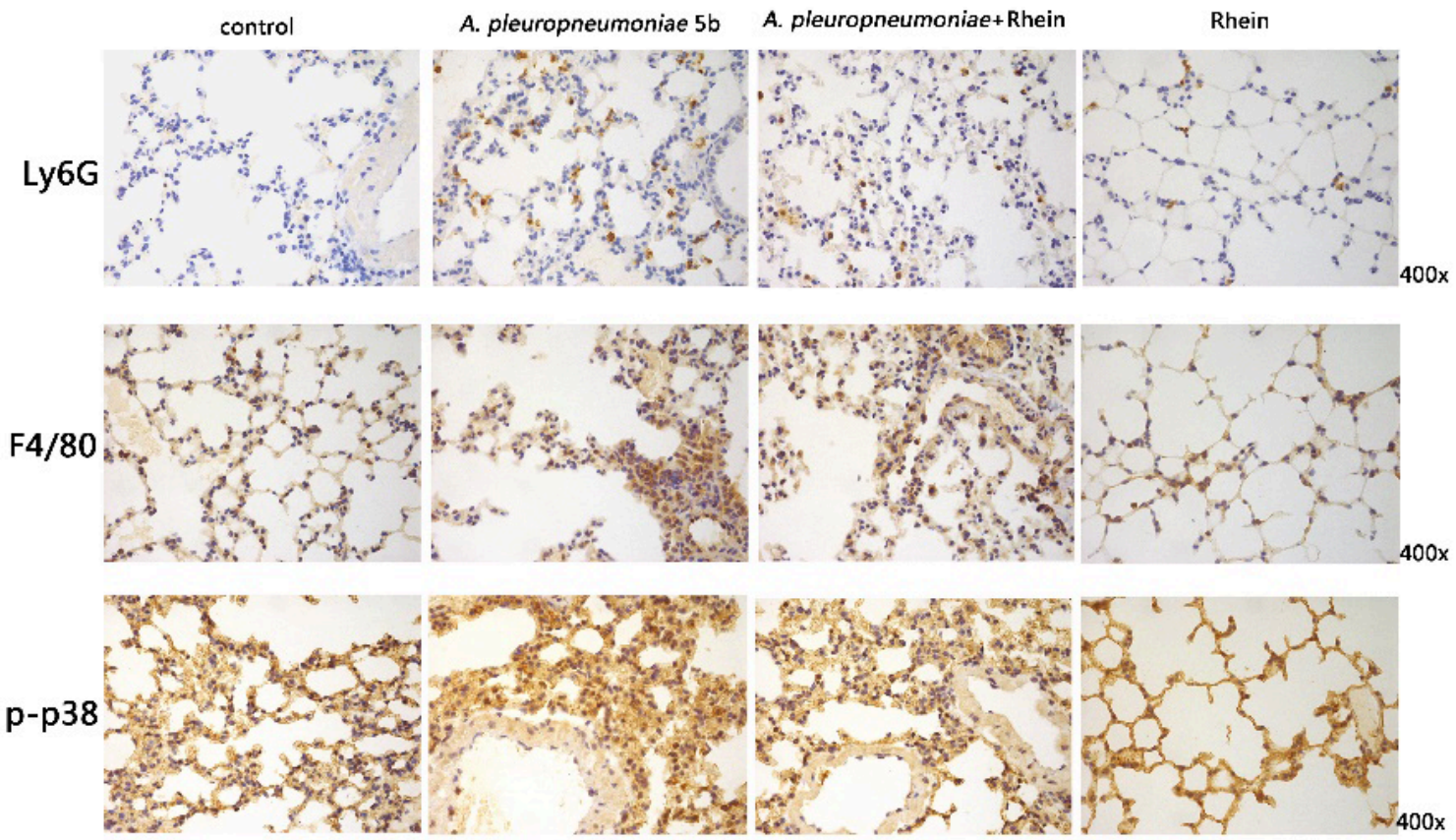

Figure 5

Rhein attenuated lung inflammation in mice infected with *A. pleuropneumoniae*. Mice in each group were infected with *A. pleuropneumoniae* without treatment (APP) or with treatment with Rhein (*A. pleuropneumoniae* +Rhein), Rhein treatment group, and mice in an uninfected group did not receive any treatment(Control). Immunohistochemical observation of Ly6G、F4/80 and p-p38 expression in lung infection with *A. pleuropneumoniae*.

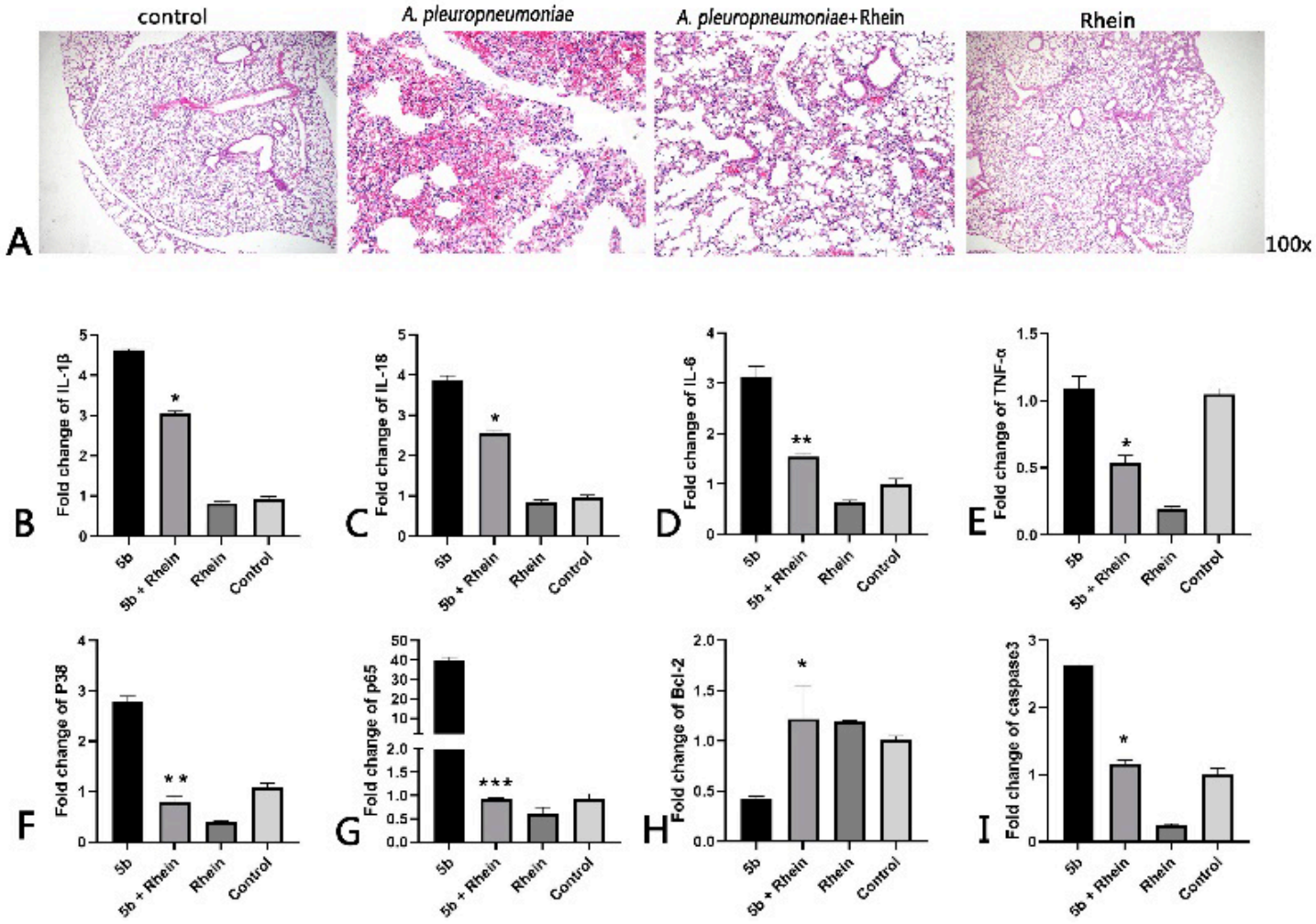

Figure 6

Rhein can reduce lung damage and reduce inflammation. Mice in each group were infected with *A. pleuropneumoniae*without treatment (APP) or with treatment with Rhein (*A. pleuropneumoniae*+Rhein), Rhein treatment group, and mice in an uninfected group did not receive any treatment (Control). (A) The results of HE staining of the wounded skin on the 8h after infection with *A. pleuropneumoniae*. (B-I) Results of transcriptional expression levels of genes related to lung inflammatory factors in mice in the 8h treatment group after *A. pleuropneumoniae* infection.