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Efficacy of Ambroxol Combined with Loquat Syrup on Bacterial Pneumonia in Mice

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Purpose: Bacterial pneumonia is a prevalent respiratory disease and a primary cause of death among hospitalized patients. Ambroxol and loquat syrup are widely utilized pharmaceuticals for managing respiratory infections in China. This study investigates the potential application and efficacy of combining ambroxol with loquat syrup for treating bacterial pneumonia.

Methods: In this study, mice with P. aeruginosa-induced bacterial pneumonia were used to evaluate the therapeutic effects of ambroxol, loquat syrup, and their combination. A bacterial plate coating assay was performed to measure the P. aeruginosa content in saliva, lung tissue, and bronchoalveolar lavage fluid (BALF). A plate colony counting assay was conducted to assess the antibacterial activity of ambroxol and loquat syrup. Serum, BALF, and lung tissues were analyzed using qPCR, ELISA, immunohistochemistry, and hematoxylin-eosin staining to evaluate disease severity.

Results: In this study, the experimental results demonstrate that, compared to treatment with ambroxol and/or loquat syrup alone, the combined administration of ambroxol and loquat syrup significantly increases the volume of saliva expectorated by mice infected with bacteria, concurrently augmenting bacterial presence in saliva while diminishing bacterial burden in the lungs, with significant differences observed ($p<0.05$). Furthermore, the combined therapy of ambroxol and loquat syrup achieved better therapeutic effects on P. aeruginosa pneumonia compared to ambroxol and/or loquat syrup alone ($p<0.05$), as evidenced by significantly reduced P. aeruginosa-induced lung injury, improved lung permeability, decreased inflammatory cell infiltration, and lower expression of inflammatory cytokines.

Conclusion: These findings suggest that the combination therapy of ambroxol and loquat syrup presents a safe and feasible new treatment strategy for bacterial pneumonia, offering promising benefits for ameliorating lung tissue damage and inflammation.

Keywords: bacterial pneumonia, ambroxol, loquat syrup, combination therapy, inflammation, treatment strategy

Introduction

Community-acquired pneumonia (CAP) is a common respiratory infection with an incidence of approximately 2% in adults worldwide, with at least half of CAP cases being attributed to bacterial pneumonia.[1,2] Evidence suggests that the severity of bacterial pneumonia varies based on the pathogen type and the host's immune status.[1,2] To date, antibiotics, such as penicillins, cephalosporins, macrolides, and fluoroquinolones, remain the primary treatment for bacterial pneumonia.[3] However, the emergence of antibiotic resistance,[4] particularly multidrug-resistance (MDR), significantly limits the clinical efficacy and options of antibiotics.[5] For example, β-lactam antibiotics are ineffective against methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin fails to treat heteroresistant Staphylococcus aureus (hVISA) and vancomycin-intermediate Staphylococcus aureus (VISA).[4,5] Projections suggest that failure to overcome resistance in MDR Gram-negative bacteria-specific drugs could result in up to 10 million annual deaths due to antimicrobial resistance by 2050.[6,7] Consequently, guidelines emphasize the importance of drug susceptibility testing and, if necessary, combination antibiotic therapies, such as ceftolozane-tazobactam, ceftazidime-avibactam, and meropenem-vaborbactam.[8] Moreover, antibiotic treatment often entails numerous adverse effects, including an increased risk of diarrhea and colitis, as well as anaphylactic reactions and nephrotoxicity.[9,10] These challenges markedly heighten the

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complexity of treating bacterial pneumonia, underscoring the urgent need for innovative therapeutic approaches to address this pressing issue.

Evidences suggest that traditional Chinese medicine exhibits a synergistic effect with conventional pneumonia therapy.[11,12] For example, certain plant extracts–such as epigallocatechin gallate from *Camellia sinensis*,[13] resveratrol primarily from *Vitis vinifera*,[14] and curcumin from *Curcuma longa*[15]–have been shown to enhance the antibacterial activity of antibiotics,[16] highlighting the feasibility and necessity of combination therapeutic for bacterial pneumonia treatment. Adjunct medications, including expectorants like ambroxol[17] and/or Chinese patent cough and expectorant medicines, such as shedan-chuanbei,[18] demonstrate a unique therapeutic effect in promoting sputum discharge and reducing cough, when used alongside antibiotics, may play a significant role in the clinical management of bacterial pneumonia. Ambroxol, known for its ability to enhance lung fluid secretion and possessing a strong mucoactive function, is commonly used as an over-the-counter mucolytic agent in respiratory diseases treatment.[17,19,20] Evidences suggested that ambroxol has anti-inflammatory, antioxidant, antiviral, and antibacterial properties, and exhibits a complementary synergistic effect with other drugs, such as bromhexine, azithromycin, and budesonide, in the treatment of pneumonia.[21–23] Loquat (*Eriobotrya japonica*) leaf extracts, primarily containing triterpenoids, polyphenols, and flavonoids, possess multiple pharmacological effects including antibacterial, anti-inflammatory, and anti-obesity properties,[24–26] have been integral to traditional Chinese medicine for centuries.[27–29] Loquat syrup, containing loquat leaf extract as a primary component, is renowned in traditional Chinese medicine for its ability to soothe the lungs, alleviate cough, and expel phlegm. However, the application and efficacy of their combined use in bacterial pneumonia treatment remain inadequately explored.

This study aimed to evaluate the potential application and efficacy of ambroxol combined with loquat syrup in the treatment of bacterial pneumonia. To this end, a mouse model of P. aeruginosa-induced pneumonia was established and treated with ambroxol, loquat syrup, or a combination of both. Saliva, bronchoalveolar lavage fluid (BALF), lung tissues, and serum from the infected mice were analyzed to assess P. aeruginosa quantity and activity, as well as disease severity. We determined that the combination of ambroxol and loquat syrup achieved a stronger alleviating effect on lung damage caused by bacterial pneumonia by promoting faster bacterial clearance from the lungs and reducing inflammation in lung tissue, thereby providing a potentially effective treatment strategy for bacterial pneumonia.

Methods and Materials

Bacterial Pneumonia Mice Model Establishment and Drug Administration

Male Institute of Cancer Research (ICR) mice aged 6–8 weeks were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., and housed in both temperature-controlled (22 °C ± 1 ◦C) and light-controlled pathogen-free animal facilities. The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. WYYY-AEC-YS-2024-0317) and conducted in compliance with the ethical guidelines established by the US National Institutes of Health for the care and use of laboratory animals. After one week of acclimatization, the mice were randomly divided into five groups, with 7 mice per group, consisting of the control group (PBS), the Pseudomonas aeruginosa infection group (P. aeruginosa), the group treated with loquat syrup (Chinese name: Qiangli Pipalu, purchased from Chinese pharmacies, 3 mL/kg) alone, the groups treated with ambroxol (purchased from Chinese pharmacies, 6 mg/kg) alone, and the treatment groups receiving ambroxol (6 mg/kg) + loquat syrup (3 mL/kg). Anesthesia was induced by intraperitoneal injection of 1% pentobarbital sodium solution at a dose of 0.1 mL/10g body weight. The trachea was exposed, and mice in the control group were intratracheally instilled with 50 μL of 1× PBS solution, whereas mice in the infection and treatment groups received 50 μL of P. aeruginosa bacterial suspension (1×10^6 CFU/mL, suspended in 50 μL of 1× PBS). Mice were held upright for 2 min to facilitate bacterial entry into the lungs before closing the wound. After 2 h, mice in the treatment groups were orally administered the respective drugs. Saliva was collected 24 h after bacterial infection. Subsequently, all mice were euthanized under anesthesia. Serum, bronchoalveolar lavage fluid, and lung tissues were collected and stored at −80°C for subsequent analysis.

Bronchoalveolar Lavage Fluid (BALF) Collection

Following euthanasia of mice in the control group, bacterial pneumonia model, and drug administration model, the thoracic cavities of all mice were fully exposed. The right lung was clamped with hemostatic forceps, and the left lung was lavaged by instilling a pre-cooled PBS buffer through the trachea three times to collect 200 μL of lavage fluid into 1.5 mL centrifuge tubes, repeated four times.

Determination of Total Cells in BALF

Centrifuge the collected BALF at 3000 rpm for 15 min. Transfer the supernatant to a new 1.5 mL centrifuge tube and label it appropriately. Next, add 1 mL of red blood cell lysis buffer to resuspend the cell pellet and remove red blood cells. Centrifuge again at 3000 rpm for 15 min. Discard the supernatant, resuspend the cells in 50 μL of PBS buffer, and use 10 μL to perform cell counting using a hemocytometer.

Determination of Protein Concentration in BALF

Centrifuge the collected BALF at 3000 rpm for 15 min. Transfer the supernatant to a new 1.5 mL centrifuge tube and label it appropriately. Utilize the Bradford assay to measure the protein concentration in the lavage fluid. Briefly, mix 20 μL of PBS buffer, 2 μL of supernatant, and 200 μL of Bradford reagent. Measure the optical density (OD) value at 595 nm using a spectrophotometer and calculate the protein concentration using a standard curve.

Measurement of Lung Wet/Dry Weight Ratio

After euthanizing male ICR mice under anesthesia, remove the first lobe of the right lung and weigh it, and record this weight as the wet weight. Place the lung lobe in a 65 °C oven for 48 h to dry, then remove it from the oven and weigh it again, recording this weight as the dry weight. Calculate the lung wet/dry weight ratio by dividing the wet weight by the dry weight.

Determination of Bacterial Content in Saliva or Lung Tissues or BALF

Bacterial content determination in saliva, lung tissues, or BALF was performed according to a previously reported method.[30] Briefly, 30 mg of lung tissue was placed into a 1.5 mL centrifuge tube, followed by the addition of 1 mL of PBS buffer for tissue homogenization. After homogenization, a gradient dilution with PBS buffer was performed. Then, 40 μL was taken and spread onto Luria-Bertani (LB) agar plates using an L-shaped spreader for bacterial culture. Alternatively, 40 μL of fresh saliva or BALF was spread onto LB agar plates using an L-shaped spreader for bacterial culture. After incubating the plates inverted at 37°C for 12 h, they were removed, and the bacterial colonies were counted.

Bacterial Drop Plate Assay

In a 96-well plate, mix 1.2 μL of a 1mg/mL drug solution with 198.8 μL of bacteria diluted to 7.5×10^5 CFU/mL by pipetting and vortexing. Perform a gradient dilution using 100 μL of PBS to obtain drug solutions with indicated drug administration strategy: ambroxol (6, 3, 1.5, 0.75, 0.375 μg/mL) or loquat syrup (3, 1.5, 0.75, 0.375, and 0.1875 μg/mL), respectively. Incubate the 96-well plate in a 37°C incubator for 4 h. For each well, take 20 μL of the drug/bacteria mixture and dilute it with 980 μL of PBS. Then, take 10 μL of the diluted mixture and drop it onto LB agar plates. Invert the plates with the drops and incubate them in a 37°C incubator for 12 h. Afterward, remove the plates and photograph them.

Hematoxylin and Eosin Staining (H&E)

The H&E assay was performed according to a previously reported method.[30] Briefly, cut the paraffin-embedded lung tissue sections into 4 μm thickness and mount them onto slides. Remove the dewaxed slides from PBS and pat them dry. Stain the nuclei with filtered hematoxylin for 5 min. Rinse slides twice with distilled water for 30s each time. Then, rinse them under running water for 10 min. Allow the slides to air dry. Stain the cytoplasm with eosin for 1 min. Rinse slides twice with distilled water for 30s each time. Dehydrate the slides according to the following steps: 5 min each in 80%

ethanol, 95% ethanol, and 100% ethanol, followed by immersion in xylene I for 5 min and xylene II for 5 min. Air dry the slides in a fume hood, then seal them with neutral resin. Finally, examine and capture images under a light microscope.

Immunohistochemistry (IHC)

The IHC assay was carried out according to the previously reported method.[30] Briefly, cut the paraffin-embedded lung tissue sections into 4 μm thickness and mount them onto slides. Retrieve the dewaxed and rehydrated slides from PBS. Then, retrieve the antigen by immersing the sections in preheated citrate buffer and heating them over a low flame for 15 min. After the slides cooled to room temperature, block the endogenous peroxidase activity using a 3% hydrogen peroxide solution in the dark for 30 min. Then, wash the slides in PBS three times, 5 min each time. Next, block all slides as follows: Outline tissues with a hydrophobic barrier pen, then incubate the slides horizontally on the benchtop and add 5% Bovine serum albumin (BSA) to tissues for 30 min at room temperature to block nonspecific protein binding. Retrieve the slides the next day and allow them to equilibrate to room temperature in a 37°C oven for 2 h. Blot excess blocking solution and apply the diluted primary antibody (diluted in 5% BSA). Incubate overnight at 4°C in a humidified chamber. Wash the slides in PBS three times, 5 min each time. Blot excess moisture and apply the horseradish peroxidase-conjugated secondary antibody. Incubate at room temperature for 1 h. Before DAB staining, wash all slides in PBS three times, 5 min each time. Blot the excess moisture and apply the DAB staining solution for 5 min. Observe staining intensity under a microscope. Rinse the slides twice in distilled water for 30s each time. Then counterstain nuclei with hematoxylin for 3 min, followed by rinsing under running water for 10 min. Allow the slides to air dry, re-dehydrate, mount them with neutral resin, and observe and capture images under a light microscope. Perform quantitative analysis using Image J software. Select "RGB stack" in the image type and adjust the threshold to change the selected area in the image. Finally, analyze the mean values of the data obtained.

RNA Extraction and Real-Time Fluorescent Quantitative PCR (RT-qPCR) Assay

RNA extraction and RT-qPCR assay were carried out according to the previously reported methods.[30] Briefly, take 30 mg of lung tissue from each of the 7 mice per group. Add 800 μL of Trizol to each tissue, homogenize, transfer to a new 1.5 mL centrifuge tube, add 200 μL of chloroform, vigorously vortex for 15 seconds, incubate on ice for 2 min, centrifuge at 4°C, 12000 g for 15 min. Carefully transfer the upper aqueous phase (three layers: colorless, white, and red) to another labeled 1.5 mL centrifuge tube, add an equal volume of isopropanol, mix, invert 10 times, incubate on ice for 10 min, centrifuge at 4°C, 12000 g for 15 min. Discard the supernatant (first pipette out the upper layer, then the lower layer, or invert and place on a paper towel), add 1 mL of ice-cold ethanol, gently shake, mix by inverting, centrifuge at 4°C, 7500 g for 10 min. Discard the supernatant, blot dry with absorbent paper, invert, and air-dry the pellet on absorbent paper for approximately 2 h. Then, add 30 μL of DEPC-H_2O to dissolve RNA, measure the concentration and purity of RNA using NANO-Drop, and record the results. The extracted RNA was used for RNA reverse transcription assay to obtain cDNA, following the instructions provided by the reverse transcription kit. Finally, the abundance of RNA was detected by adding a certain amount of primers, TB Green, cDNA, and DEPC-H_2O for qRT-PCR detection. Reverse transcription and quantitative PCR were performed using Power SYBR Green qPCR master mix (Invitrogen) in a LightCycler 480 (Roche) with the following specific primers (Thermo Fisher): GAPDH, 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′- TGGTGAAGACGCCAGTGGA-3′ (reverse); TNF-α, 5′-CAGGGGCCACCACGCTCTTC-3′ (forward) and 5′- TTTGTGAGTGTGAGGGTCTGG-3′ (reverse); IL-6, 5′-GAGGATACCACTCCCAACAGACC-3′ (forward) and 5′- AAGTGCATCATCGTTGTTCATACA-3′ (reverse). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The remaining data in different processing groups are compared with the data anterior inner region (α) for normalization processing.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assay was carried out according to the previously reported method.[30] Firstly, coat the plate with a mixture of diluted anti-mouse TNF-α (sc-52746) and IL-6 (Cat No. 26404-1-AP) capture antibodies (prepared by diluting them with 10× coating buffer and ddH_2O to 1×, then adding 100 μL per well to the ELISA plate), cover it with a sealing film, and

incubate overnight at 4°C on a shaker. Then, wash the plate three times with 250 μL per well of 1× Phosphate Buffered Saline (PBS) solution with the detergent Tween 20 (PBST), pat dry after the final wash, and add 200 μL per well of 1× Assay Diluent (1× A). Incubate at room temperature on a shaker for 1.5 h. Afterward, wash the plate three times with 250 μL per well of 1× PBST, pat dry after the final wash, and add prepared standard proteins of TNF-α and IL-6 at 8 different concentrations to establish a standard curve. Following this, add the test samples (serum or BALF) to the sample wells and incubate on a shaker at room temperature for 2 h. Subsequently, wash the plate three times with 250 μL per well of 1× PBST, pat dry after the final wash, and add 100 μL per well of the corresponding detection antibodies for anti-mouse TNF-α and IL-6 diluted in 1× AD. Incubate at room temperature on a shaker for 1 h. Next, wash the plate three times with 250 μL per well of 1× PBST, pat dry after the final wash, and add 100 μL per well of 1× HRP. Incubate at room temperature on a shaker for 30 min. Then, wash the plate five times with 250 μL per well of 1× PBST, pat dry after the final wash, and add 100 μL per well of 3,3',5,5"-tetramethylbenzidine (TMB) substrate for color development. Incubate at room temperature in the dark. Promptly stop the reaction with 50 μL per well of 2 M sulfuric acid to prevent over-coloration. Measure the OD value at 450 nm wavelength using a microplate reader, plot the standard curve, and calculate the concentration of samples based on their OD values.

Statistical Analysis

Statistical analyses were performed with GraphPad (GraphPad Prism) using one-way analysis of variance (ANOVA). Values are presented as mean ± standard deviation (SD). p values were calculated using GraphPad 9. p values <0.05 were considered statistically significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Results

Bacterial Content in Saliva, BALF, and Lung Tissue of Bacterial Pneumonia Mice

The results showed that compared to control group (normal mice), P. aeruginosa infection significantly increased bacteria levels in the saliva, BALF, and lung tissue of mice (Figure 1). Treatment with ambroxol or loquat syrup increased bacterial levels in saliva, and their combination further increased these levels (Figure 1A), indicating that the combined administration of ambroxol and loquat syrup has a stronger effect on promoting bacterial excretion compared to their individual administration. Furthermore, treatment with ambroxol or loquat syrup significantly reduced bacterial levels in the lungs, and their combination exhibited an enhanced ability to reduce bacterial levels in the lungs (Figure 1B), suggesting that the combination of ambroxol and loquat syrup has a greater capacity for lung bacterial clearance. However, neither ambroxol nor loquat syrup, nor their combination, affected the bacterial content in BALF (Figure 1C). Moreover, the results of the bacterial coating plate experiment indicated that neither ambroxol nor loquat syrup affected P. aeruginosa growth at the therapeutic doses used in this study (Figure 1D and E). Taken together, the results suggested that the decrease in bacteria levels in the lungs is primarily due to increased bacteria excretion following drug administration, particularly with the combination of ambroxol and loquat syrup.

In vivo Therapy of P. aeruginosa-Induced Bacterial Pneumonia

We subsequently conducted an in vivo validation of the protective efficacy of ambroxol, loquat syrup, or their combination in mitigating P. aeruginosa-induced lung injury of bacterial pneumonia. Lung injury severity was assessed using established scoring criteria (Figure 2A). Notably, P. aeruginosa infection led to a significant elevation in lung injury scores (Figure 2B). Administration of ambroxol markedly attenuated lung injury, whereas the administration of loquat syrup did not yield a discernible improvement in lung injury scores (Figure 2B). Intriguingly, compared to ambroxol or loquat syrup administration alone, the combined administration of ambroxol and loquat syrup resulted in a lower lung injury score (Figure 2B).

Moreover, P. aeruginosa increased the concentration of proteins in the BALF (Figure 3A), indicating abnormal permeability of the pulmonary vasculature. Administration of ambroxol, loquat syrup, and their combination significantly mitigated protein leakage; however, the combined administration demonstrated the most pronounced effect (Figure 3A). Conformably, the total cell counts in the BALF increased after P. aeruginosa infection, which was significantly reversed

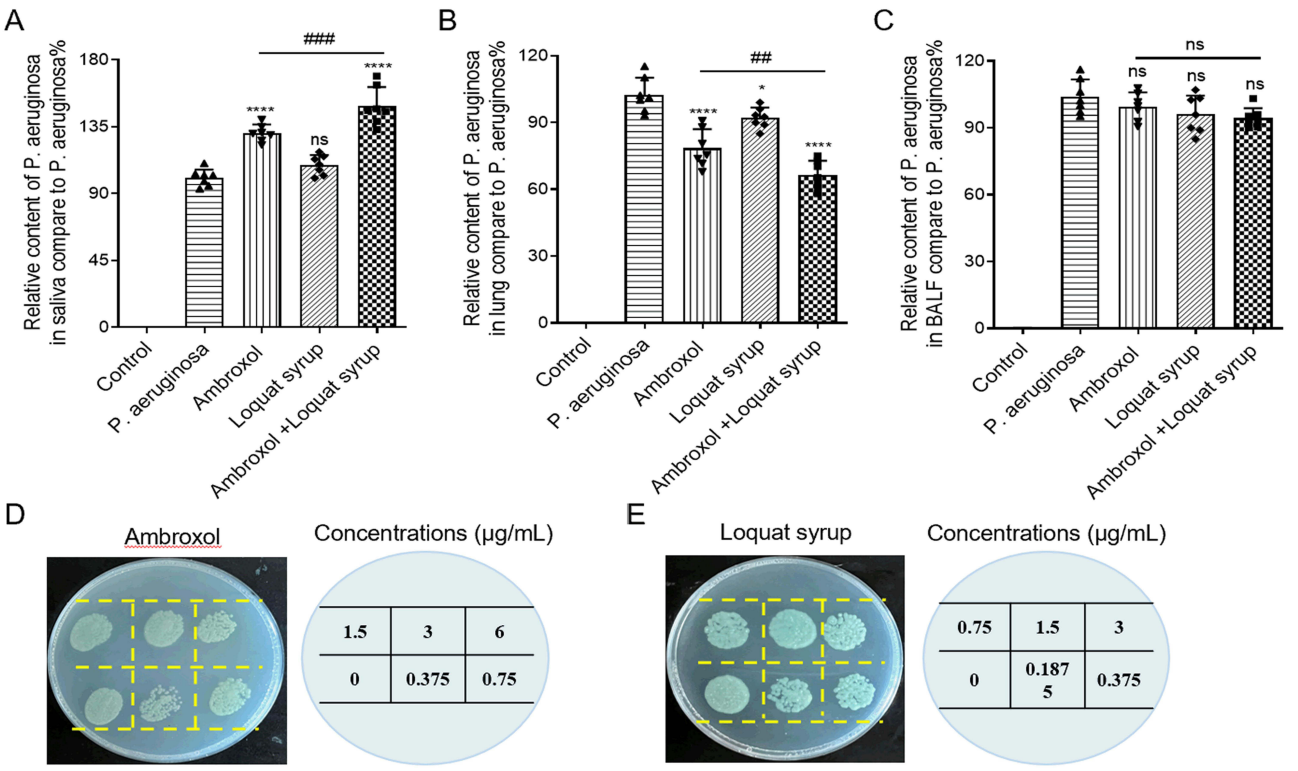

Figure 1 The determination of bacterial content in saliva, bronchoalveolar lavage fluid (BALF) and lung tissue of bacterial pneumonia mice. A–C, The bacterial content in saliva (**A**), BALF (**B**) and lung tissue (**C**) of P. aeruginosa-induced bacterial pneumonia mice, respectively. Mice randomly divided into five groups (n=7 per group), except control group, all mice were infected with 50 μL of P. aeruginosa bacterial suspension (1×10^6 CFU/mL, suspended in 50 μL of 1× PBS). After 2h of P. aeruginosa infection, mice were then treated with PBS, ambroxol (6mg/kg) alone, loquat syrup (3mL/kg) alone, or ambroxol (6mg/kg) + loquat syrup (3mL/kg) for another 22 h, respectively. Finally, the saliva, BALF and lung tissues were collected. D and E, The representative images of the antibacterial activity of ambroxol (**D**) and loquat syrup (**E**) by coated plate experiments, respectively. P. aeruginosa were treated with the indicated concentrations of ambroxol and loquat syrup for 24 h, and the images were took. Data are represented as mean ± SD. ns means not significant with $p>0.05$, *$p<0.05$, ****$p<0.0001$ vs P. aeruginosa group; $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.001$.

A

Score	Lung injury level
0	No inflammatory cell infiltration, interstitial edema, alveolar hemorrhage or alveolar edema
1	Alveolar hyperemia and inflammatory cell infiltration, pulmonary interstitial and alveolar edema
2	The alveolar was moderately hyperemia, and there were more inflammatory cell infiltration such as white blood cells and red blood cells in the alveolar, and the interstitial edema was obvious
3	Severe alveolar congestion, a large number of inflammatory cell infiltration, pulmonary interstitial and alveolar edema

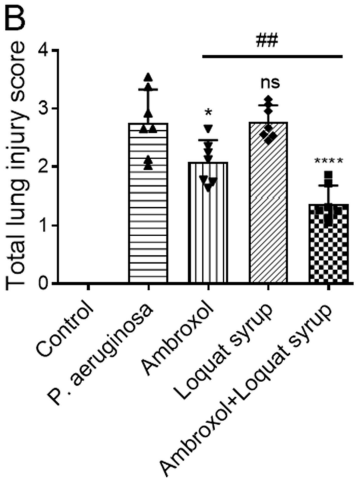

Figure 2 Ambroxol combined with loquat syrup administration reduced the total lung injury score of P. aeruginosa-induced in mice. (**A**), The scoring criteria for lung injury. (**B**), Quantification of the lung injury scores. Mice randomly divided into five groups (n=7 per group), except control group, all mice were infected with 50 μL of P. aeruginosa bacterial suspension (1×10^6 CFU/mL, suspended in 50 μL of 1× PBS). After 2h of P. aeruginosa infection, mice were then treated with PBS, ambroxol (6mg/kg) alone, loquat syrup (3mL/kg) alone, or ambroxol (6mg/kg) + loquat syrup (3mL/kg) for another 22 h, respectively. Data are represented as mean ± SD. ns means not significant with $p>0.05$, *$p<0.05$, ****$p<0.0001$ vs P. aeruginosa group; $^{\#\#}p<0.01$.

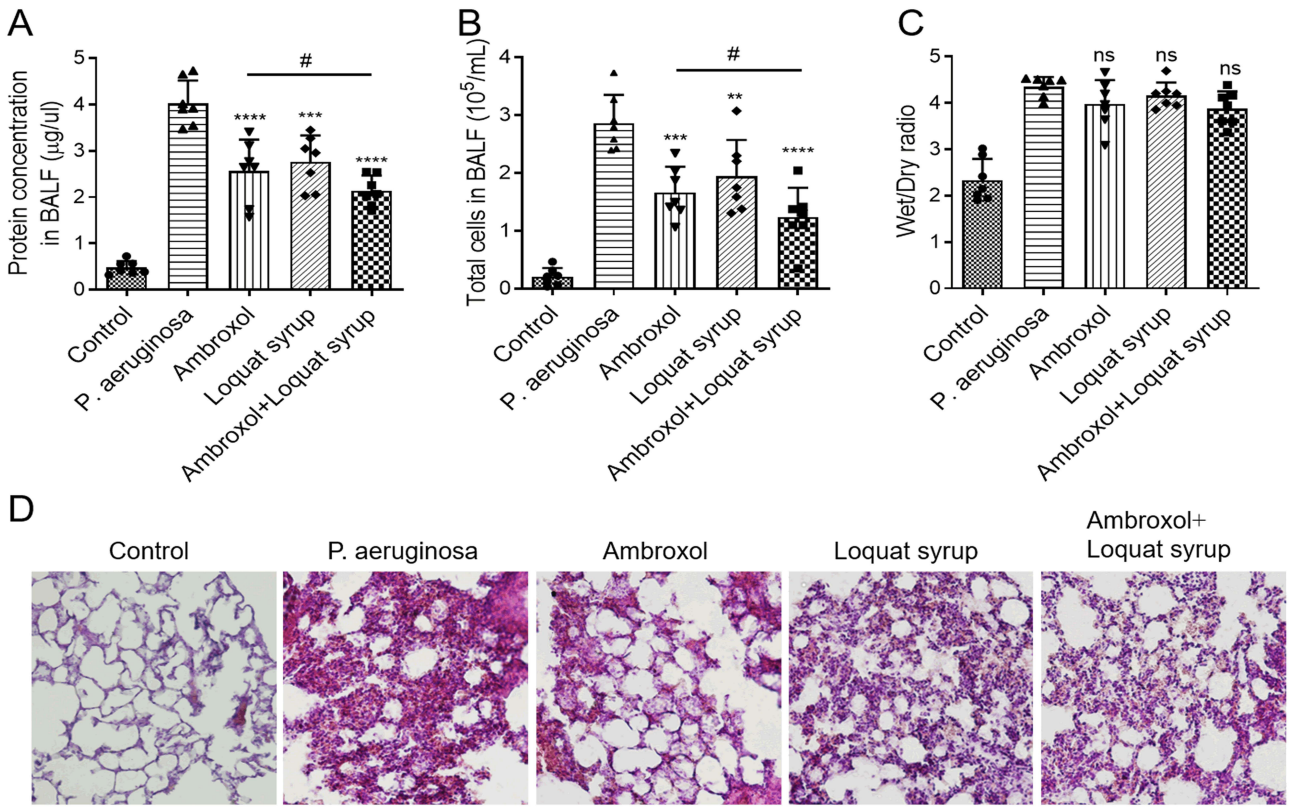

Figure 3 Ambroxol combined with loquat syrup administration reduced P. aeruginosa-induced lung injury in mice. A and B, The total protein concentration (**A**) and cells (**B**) in bronchoalveolar lavage fluid (BALF) samples were measured. (**C**), Lung wet/dry weight ratio. (**D**), Representative H&E-stained lung tissue. Mice randomly divided into five groups (n=7 per group), except control group, all mice were infected with 50 μL of P. aeruginosa bacterial suspension (1×10^6 CFU/mL, suspended in 50 μL of 1× PBS). After 2h of P. aeruginosa infection, mice were then treated with PBS, ambroxol (6mg/kg) alone, loquat syrup (3mL/kg) alone, or ambroxol (6mg/kg) + loquat syrup (3mL/kg) for another 22 h, respectively. Data are represented as mean ± SD. ns means not significant with $p> 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, $^{****}p< 0.0001$ vs P. aeruginosa group; $^{\#}p< 0.05$.

by administration of ambroxol, loquat syrup, or their combination (Figure 3B). Furthermore, the lung wet-to-dry ratio increased following P. aeruginosa infection, with marginal regain observed after administration of ambroxol, loquat syrup, or their combination (Figure 3C). Moreover, histopathological examination via H&E staining revealed elevated levels of pulmonary edema, leukocytic infiltration, and increased thickness of the alveolar wall following P. aeruginosa infection (Figure 3D). Ambroxol and loquat syrup attenuated these lung injuries, with the combination exhibiting the most pronounced improvement (Figure 3D). Taken together, these findings indicated that the protective efficacy of ambroxol combined with loquat syrup against P. aeruginosa-induced lung injury in mice was stronger than that of ambroxol or loquat syrup administration alone.

Inflammation Response in P. aeruginosa-Induced Bacterial Pneumonia in Mice

The qPCR results indicated a significant increase in TNF-α and IL-6 mRNA transcription in both BALF and serum following P. aeruginosa infection, which was markedly attenuated by treatment with ambroxol, loquat syrup, or their combination (Figure 4). Furthermore, the findings suggested that ambroxol combined with loquat syrup administration exerted a more potent inhibitory effect on the transcription of inflammatory factors TNF-α and IL-6 compared to the administration of ambroxol or loquat syrup alone (Figure 4).

Additionally, the results from ELISA assay showed that P. aeruginosa significantly increased TNF-α and IL-6 secretion in BALF and serum, which was significantly inhibited after ambroxol or loquat syrup or ambroxol combined with loquat syrup pretreatment (Figure 5). Consistently, ambroxol combined with loquat syrup pretreatment presented a similar trend as in the qPCR assay (Figure 5).

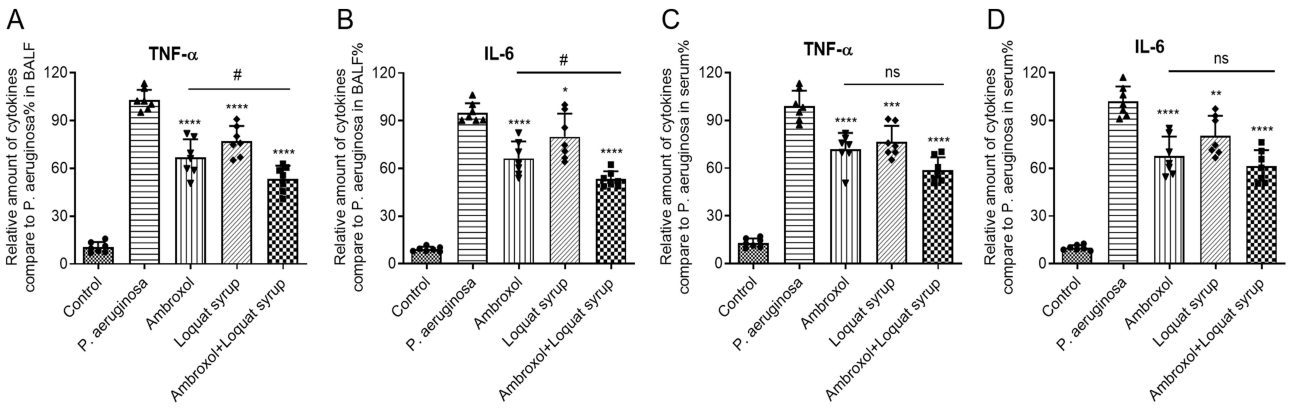

Figure 4 Ambroxol combined with loquat syrup administration inhibited TNF-α and IL-6 mRNA transcription in P. aeruginosa-induced bacterial pneumonia mice. (**A** and **B**), Levels of TNF-α and IL-6 mRNA transcription in bronchoalveolar lavage fluid (BALF) samples from mice infected with P. aeruginosa. (**C** and **D**), Levels of TNF-α and IL-6 mRNA transcription in serum samples from mice infected with P. aeruginosa. Mice randomly divided into five groups (n=7 per group), except control group, all mice were infected with 50 μL of P. aeruginosa bacterial suspension (1×10^6 CFU/mL, suspended in 50 μL of 1× PBS). After 2h of P. aeruginosa infection, mice were then treated with PBS, ambroxol (6mg/kg) alone, loquat syrup (3mL/kg) alone, or ambroxol (6mg/kg) + loquat syrup (3mL/kg) for another 22 h, respectively. Data are represented as mean ± SD. ns means not significant with $p> 0.05$, *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$ vs P. aeruginosa group; $^{\#}p< 0.05$.

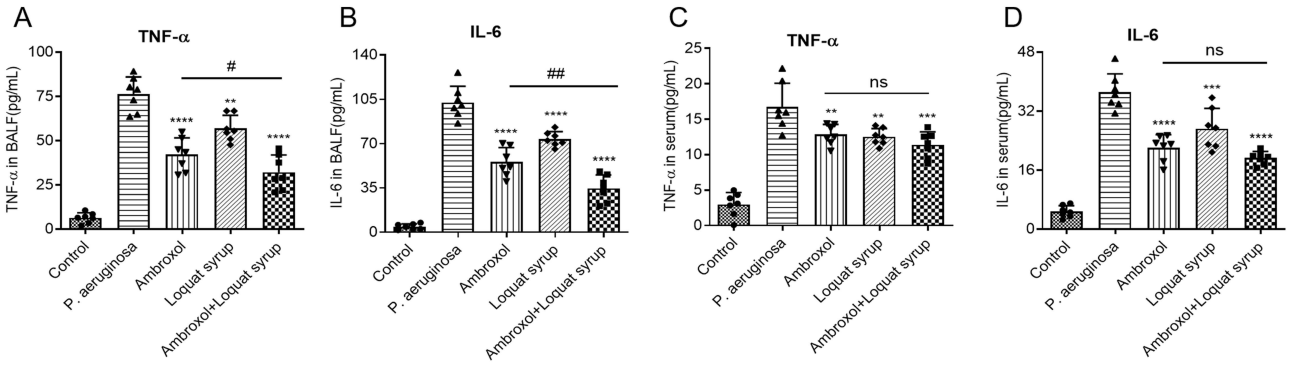

Figure 5 Ambroxol combined with loquat syrup administration inhibited TNF-α and IL-6 secretion in P. aeruginosa-induced bacterial pneumonia mice. (**A** and **B**), Levels of TNF-α and IL-6 secretion in bronchoalveolar lavage fluid (BALF) samples from mice infected with P. aeruginosa. (**C** and **D**), Levels of TNF-α and IL-6 secretion in serum samples from mice infected with P. aeruginosa. Data are represented as mean ± SD. n = 7 per groups. ns means not significant, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$ vs P. aeruginosa group; $^{\#}p< 0.05$, $^{\#\#}p< 0.01$.

Consistent with the results of the qPCR and ELISA assays, immunohistochemical (IHC) staining using antibodies against TNF-α and IL-6 revealed a significant increase in the number of TNF-α and/or IL-6-positive foci in the lung interstitial regions of mice with P. aeruginosa-induced bacterial pneumonia (Figure 6A). However, this increase was significantly reduced in all treatment groups, with the most pronounced effect observed in the group receiving a combination of ambroxol and loquat syrup (Figure 6A). Quantitative analysis results indicated that there was a statistically significant difference in the level of inflammation between the ambroxol administration and the control group ($p< 0.05$), or the combination administration group and the single administration group, further corroborated these experimental findings ($p< 0.05$) (Figure 6B and C). According to these findings, the combination of ambroxol and loquat syrup demonstrates superior effectiveness in alleviating inflammation induced by P. aeruginosa in mice.

Discussion

The present study aimed to assess the effectiveness of combined therapy using ambroxol and loquat syrup for treating bacterial pneumonia. The results demonstrated that combined therapy with ambroxol and loquat syrup markedly increased P. aeruginosa excretion, decreased P. aeruginosa load in lung tissues, and efficiently mitigated the inflammatory response induced by P. aeruginosa, thereby attenuating lung damage subsequent to P. aeruginosa infection. A comprehensive analysis of the study results indicates that the combined therapy with ambroxol and loquat syrup is

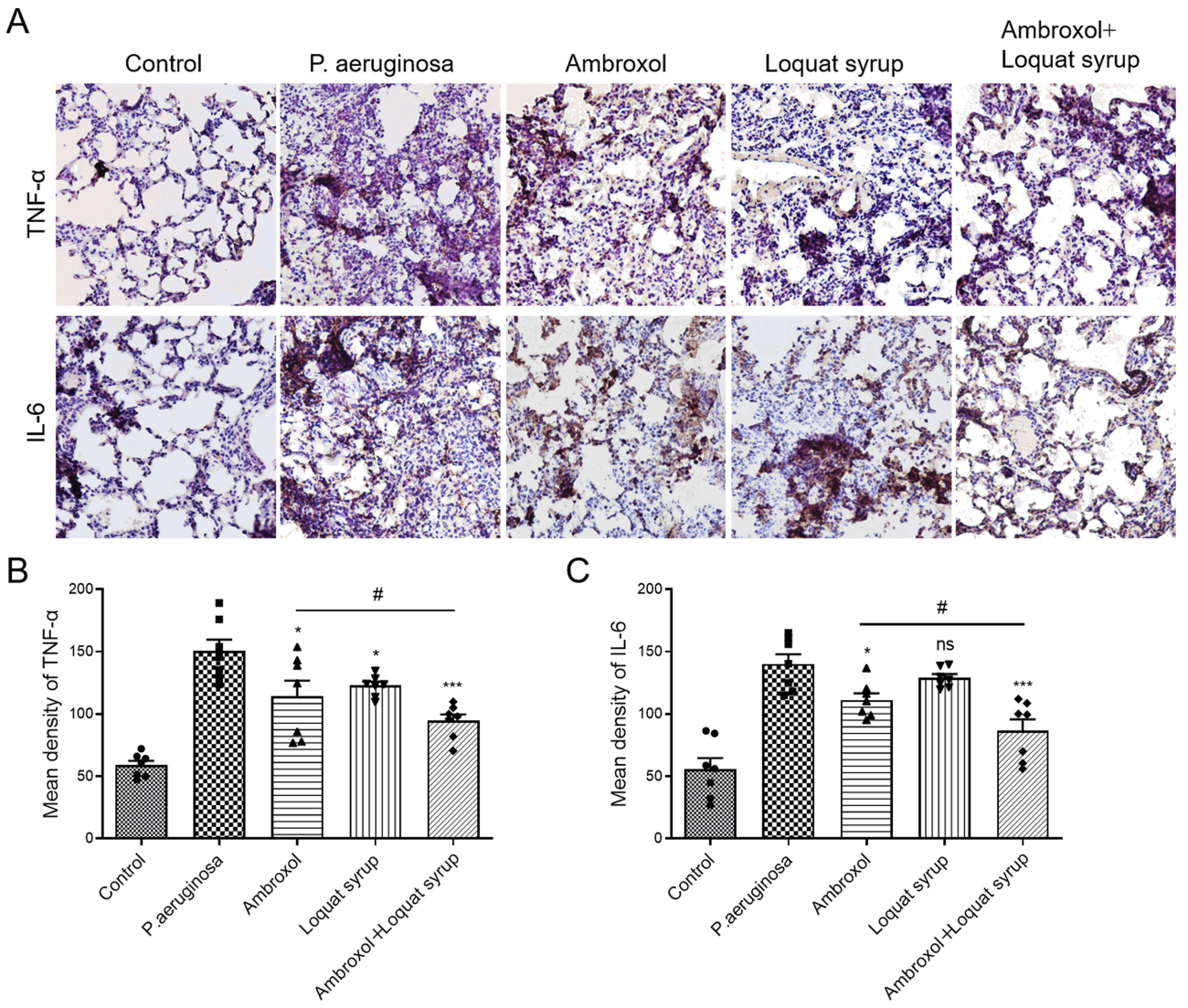

Figure 6 Ambroxol combined with loquat syrup administration inhibited lung inflammatory cytokine levels in P. aeruginosa-induced bacterial pneumonia mice. (**A**), Representative immunostaining of mouse lung tissues for TNF-α and IL-6. Slides were counterstained with haematoxylin (blue). (**B**), Quantification of TNF-α immunoreactivity showed in A. (**C**), Quantification of IL-6 immunoreactivity showed in A. Data are represented as mean ± SD. n = 7 per groups. ns means not significant with $p > 0.05$, *$p < 0.05$, ***$p < 0.001$, vs P. aeruginosa group; #$p < 0.05$.

superior to the individual use of ambroxol or loquat syrup in treating P. aeruginosa-induced bacterial pneumonia. This implies that the combined therapy may exert a synergistic effect, resulting in enhanced treatment outcomes.

Ambroxol, known both as an expectorant and a cough medicine *in clinical* settings, offers a range of clinical benefits.[17,19,31] Evidence indicates that ambroxol reduces mucus viscosity and adhesion by promoting the biosynthesis of pulmonary surfactant, resulting in rapid ciliary clearance.[32] Previous study has demonstrated that ambroxol can alleviate cough symptoms caused by mucus irritation through the reduction of airway mucus secretion and promotion of its expulsion.[33] Loquat syrup, formulated with loquat leaf as its primary ingredient, is commonly employed in Chinese medicine for treating respiratory diseases and associated symptoms. Previous study has verified that, owing to the pharmacological activity of loquat leaf, loquat syrup exhibits expectorant, cough-relieving, and soothing effects,[34] thereby aiding in the accelerated expulsion of mucus. In summary, compared to treatment with ambroxol or loquat syrup alone, the combined administration of ambroxol and loquat syrup demonstrated a superior therapeutic effect against P. aeruginosa-induced bacterial pneumonia for the following reasons: (i) augmented stimulation of the respiratory mucosa by the combination of ambroxol and loquat syrup; (ii) promotion of secretion clearance in the pulmonary lesion

area by the combined therapy; (iii) potentiation of bacterial efflux by the combination of ambroxol and loquat syrup, facilitating pathogen clearance in the lungs and reducing bacterial replication and infection severity. Thus, although ambroxol and strong loquat lack intrinsic antibacterial or bacteriostatic properties, their combination can still mitigate the reduction of bacterial load in the lungs.

Close correlation exists among bacterial infection, inflammation, and lung injury, which collectively influence the onset and progression of bacterial pneumonia.[35–37] Bacterial infection initiates lung inflammation,[38] which, if excessive, can result in lung injury.[39] Additionally, lung injury exacerbates inflammation, perpetuating a harmful cycle.[40,41] Consequently, prioritizing anti-inflammatory therapy is crucial in bacterial pneumonia management. This study observed that the combination of ambroxol and loquat syrup exerted a more robust protective effect against P. aeruginosa-induced lung injury by mitigating lung inflammation. This therapeutic effect of combining ambroxol with loquat syrup may be directly associated with their ability to promote bacterial efflux and decrease bacterial burden in the lungs, thereby ameliorating lung symptoms, inflammation, and facilitating the repair and regeneration of lung tissue in mice with P. aeruginosa-induced bacterial pneumonia.

In conclusion, the combination of ambroxol and loquat syrup offers significant advantages over treatment with ambroxol or loquat syrup alone in the management of P. aeruginosa-induced bacterial pneumonia in mice, providing a novel therapeutic approach for bacterial pneumonia. Furthermore, this study can serve as a valuable reference for future clinical practice.

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Disclosure

The authors declare that they have no conflicts of interest.

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