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ORIGINAL ARTICLE

Pinocembrin Relieves *Mycoplasma pneumoniae* Infection-Induced Pneumonia in Mice Through the Inhibition of Oxidative Stress and Inflammatory Response

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Abstract

Pneumonia is a serious infectious disease with increased morbidity and mortality worldwide. The *M. pneumoniae* is a major airway pathogen that mainly affects respiratory tract and ultimately leads to the development of pneumonia. The current exploration was aimed to uncover the beneficial properties of pinocembrin against the *M. pneumoniae*-triggered pneumonia in mice via its anti-inflammatory property. The pneumonia was stimulated to the BALB/c mice via infecting them with *M. pneumoniae* (100 µl) for 2 days through nasal drops and concomitantly treated with pinocembrin (10 mg/kg) for 3 days. The azithromycin (100 mg/kg) was used as a standard drug. Then the lung weight, nitric oxide, and myeloperoxidase (MPO) activity was assessed. The content of MDA, GSH, and SOD activity was scrutinized using kits. The total cells and DNA amount present in the bronchoalveolar lavage fluid (BALF) was assessed by standard methods. The IL-1, IL-6, IL-8, TNF-α, and TGF contents in the BALF samples and NF-κB level in the lung tissues were assessed using kits. The lung histopathology was assessed microscopically to detect the histological alterations. The 10 mg/kg of pinocembrin treatment substantially decreased the lung weight, nitric oxide (NO) level, and MPO activity. The MDA level was decreased, and GSH content and SOD activity were improved by the pinocembrin treatment. The pinocembrin administered pneumonia animals also demonstrated the decreased total cells, DNA amount, IL-1, IL-6, IL-8, TNF-α, and TGF in the BALF and NF-κB level. The findings of histological studies also witnessed the beneficial role of pinocembrin against *M. pneumoniae*-infected pneumonia. In conclusion, our findings confirmed that the pinocembrin effectively ameliorated the *M. pneumoniae*-provoked inflammation and oxidative stress in the pneumonia mice model. Hence, it could be a hopeful therapeutic agent to treat the pneumonia in the future.

Keywords Pneumonia · Airway inflammation · Pinocembrin · *M. pneumoniae* · Myeloperoxidase

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Introduction

Mycoplasma pneumoniae is the most crucial pathogen that mainly affects human respiratory tract and causes pneumonia, asthma, pharyngitis, and tracheobronchitis [1]. *M. pneumoniae* primarily causes lower and upper airway diseases and responsible for 4 to 8% of community-acquired pneumonia incidences [2]. The *M. pneumoniae* can be spread and transmitted by airborne droplets among people by sneezing and coughing [3]. In the majority of *M. pneumoniae* infected pneumonia incidences are benign, and some incidences may progress into series and refractory pneumonia with bronchiectasis, pleural effusion, and dysfunction of multi-organs [4]. Additionally, the clear underlying mechanisms of the development of *M. pneumoniae* provoked pneumonia leftovers undiscovered. Several earlier literatures highlighted that several immune cells like neutrophils and lymphocytes are major players of the pneumonia development [5]. Moreover, the increased generation of host-immune responses and over-accumulation of IL-6, IL-8, IL-1β, and TNF-α is believed to be a vital contributor of the progression of pneumonia [6].

The increased immune responses are thought to cause several inflammatory ailments. Several previous studies mentioned that immune cells like neutrophils and macrophages recognize lipoproteins from *M. pneumoniae* by pattern recognition receptors and stimulated downstream signaling cascades to activate the immune responses via triggering of several cytokines production like IL-6 [7–9]. Neutrophils are the critical players of inflammatory responses. The infiltration of neutrophil is generally identified as a clinical feature of pneumonia. It was already reported that levels of neutrophils were found elevated in the BALF and blood samples of pneumonia patients [10, 11]. Additionally, the NF-ƙB is an essential player of immune reactions developed during *M. pneumoniae* infection [12]. The infection of *M. pneumoniae* activates the macrophages and NF-ƙB signaling through toll-like receptors to discharge the immuno-regulatory factors that can further aggravate the immune responses and leads to the development of pneumonia [13]. The several inflammatory mediators play a crucial role during *M. pneumoniae*-triggered inflammatory reactions. Several previous literatures mentioned that the IL-1β and TNF-α contents were found elevated in the lung tissues of *M. pneumoniae*-infected animal models. Among them, the IL-8 is believed to be a crucial communicator between the neutrophils and *M. pneumoniae* [14].

Oxidative stress is a vital player of host's immune response to invading pathogens and augments the generation of inflammatory regulators in the airway system [15]. Oxidative stress is tightly associated with the inflammatory responses. The elevated generation of inflammatory regulators attracts the inflammatory cells and augments the accumulation of oxidants [16]. The lung cells discharge several inflammatory factors due to augmented oxidative stress. TNF-α is a critical player of mitochondria-mediated generation of ROS that further activates the signaling NF-κB [17]. Hence, the amelioration of oxidative stress and inflammatory responses would be benefitted to reduce the pulmonary injury during pneumonia.

In most cases, the pneumonia patients successfully recovered from the *M. pneumoniae* pneumonia by the treatment with macrolides [18]. Nowadays, *M. pneumoniae* strains become drug resistant due to the increased usage of antibiotics, which leads to the higher incidences of multi-drug-resistant infection worldwide [19, 20]. Pinocembrin is a flavonoid extensively found in the propolis, euphorbia, eucalyptus, and *Sparattosperma leucanthum* with several pharmacological properties [21] like anti-apoptotic, antioxidant, and anti-inflammatory effects [22]. Pinocembrin also demonstrated the anti-fibrotic and antiarrhythmic property [23]. Pinocembrin ameliorated the CCl4-stimulated liver fibrosis [24] and

exhibited anti-oxidative [25], anti-inflammatory [26], and neuroprotective and pharmacological properties [27, 28]. The antimicrobial [29], anti-inflammatory [30], neuroprotective [31], and anti-atherosclerosis [32] and anticancer [33–35] properties of the pinocembrin were also reported. However, the beneficial properties of pinocembrin against the pneumonia were not systematically discussed yet. Thus, the current study was designed to disclose the beneficial properties of pinocembrin against the *M. pneumoniae*-triggered pneumonia in mice via its anti-inflammatory property.

Materials and Methods

Chemicals

Pinocembrin, azithromycin, and additional chemicals were acquired from Sigma-Aldrich, USA. The ELISA kits for respective markers were procured from the Thermo Fisher, USA.

Experimental Animals

The 4–6-week old BALB/c mice were selected for this study, and mice were purchased from the institutional animal house. The experimental mice were imprisoned in a clean polypropylene cabin. The mice were maintained at well-organized laboratory conditions throughout the study temperature 23 ± 5 °C, humidity 50–60%, and 12 h of light/dark sequence. The experimental mice were permitted freely to get the pellet food and drinking water throughout the study. The mice were subjected to acclimatization for a week in the laboratory before the commencement of experiments.

Experimental Groups

All the animals were randomly assigned into four clusters with six mice in each. Group I was set as a normal control without treatments. Group II mice were pneumonia-provoked animals via infecting with the 100 µl of *M. pneumoniae* for 2 days via nasal drops. The animals from group III were challenged with *M. pneumoniae* to provoke the pneumonia and concomitantly administered with the pinocembrin 10 mg/kg for 3 days. The mice from group IV were challenged with *M. pneumoniae* and concomitantly treated with the standard medication azithromycin (100 mg/kg) for 3 days. Followed by the achievement of treatments, all animals were anesthetized and sacrificed by cervical displacement, and then blood and lung tissues were gathered from animals in order to conduct the further biochemical experiments.

Determination of Nitric Oxide (NO) and Myeloperoxidase (MPO) Enzyme Activity in the Lung Tissues

The removed lung tissues from the experimental animals were homogenized with buffer and centrifuged at $2000 \times g$ for 10 min at 4 °C. The resultant supernatant was utilized to detect the level of NO (#EMSNO) and activity of MPO (#EMMPOX5) with the help of respective kits as per the procedures recommended by the manufacturer (Thermo Fisher, USA).

Determination of Oxidative and Antioxidant Biomarkers in the Lung Tissues

The excised lung tissues from the experimental mice were homogenized with buffer and centrifuged at 2000 × *g* at 4 °C for 10 min. the resultant supernatant were used for the determination of antioxidant biomarkers. The content of MDA (#MBS269473), GSH (#MBS3805433), and SOD (#MBS265351) activity in the lung tissue homogenate of both normal and experimental mice was scrutinized by using the marker-specific kits according to the protocols recommended by the manufacturer (MyBioSource, USA).

Collection of Bronchoalveolar Lavage Fluid (BALF) and Total Cell Count

The BALF from the control and experimental mice were collected by the instillation of 30 ml aliquots of saline into right middle lobe of the animals. Then the collected BALF was centrifuged immediately at 1500 × *g* for 5 min to separate the cell pellets. Then total cell counts in the samples were enumerated with the help of hemocytometer using light microscope.

Measurement of M. pneumoniae DNA Amount

The lung tissue homogenates from the control and experimental mice were added into the DNA extraction buffer and stand for 10 min at 37 °C. Then, suspension was centrifuged at 13,000 rpm for 5 min at 4 °C to discrete the DNA from the samples. Finally, the amount of *M. pneumoniae* DNA present in the samples were scrutinized by using PCR technique. The primers for 16rRNA which are upstream 5′- GAATCAAAGTTGAAAGGACCTGC-3′ and downstream 5′-CTCTAGCCATTACCTGCTAAAGTC-3′ were used [36].

Measurement of Pro-inflammatory Markers in the BALF Samples

The collected BALF samples from control and experimental mice were subjected to determine the contents of inflammatory biomarkers. The IL-1 (#BMS611TEN), IL-6 (#KMC0061), IL-8 (#ERA31RBX5), TNF-α (#BMS607-3TWO), and TGF (#BMS608-4TWO) contents in the BALF samples of animals were assessed with the aid of marker-specific ELISA kits using guidelines recommended by the manufacturer (Thermo Fisher Scientific, USA).

Determination of NF-κB Level in the Lung Tissues

The NF-κB (#MBS2023542) content in the lung tissue homogenate of control and experimental mice was assessed with the help of ELISA kit using protocols recommended by the manufacturer (MyBioSource, USA).

Histopathological Analysis

The changes in the lung histopathology of experimental animals were assessed microscopically. For this, the collected lung tissues from control and experimental animals were processed with neutral formalin (10%) and fixed in paraffin. Then paraffinized lung tissues were cut into slices at 5-mm size and then stained with hematoxylin and eosin. The tissues were microscopically scrutinized to detect the histological alterations using light microscope at 40 × magnification.

Statistical Analysis

The results of the experiments were assessed statistically by using SPSS software, and outcomes were presented as mean ± SD of three separate assessments. The results were studied by one-way ANOVA and Tukey's post hoc assay to measure the variations between experimental groups, and $p < 0.05$ were set as significant.

Results

Effect of Pinocembrin on the Lung Weight, NO Level, and MPO Activity in the Experimental Mice

Figure 1 depicts the lung weight index, NO content, and MPO activity in the control and *M. pneumoniae*-triggered pneumonia mice. The *M. pneumoniae*-initiated pneumonia animals displayed the significant elevation ($p < 0.05$) on the lung weight index, NO, and MPO activity when related with control. These alterations were substantially ameliorated by the pinocembrin. The administration of 10 mg/kg of pinocembrin demonstrated the significant reduction ($p < 0.01$) in the lung weight index, NO level, and MPO activity in the pneumonia mice. The standard drug azithromycin-treated pneumonia animals also exhibited the notable diminution in the lung weight index, NO content, and MPO activity in the lung tissues.

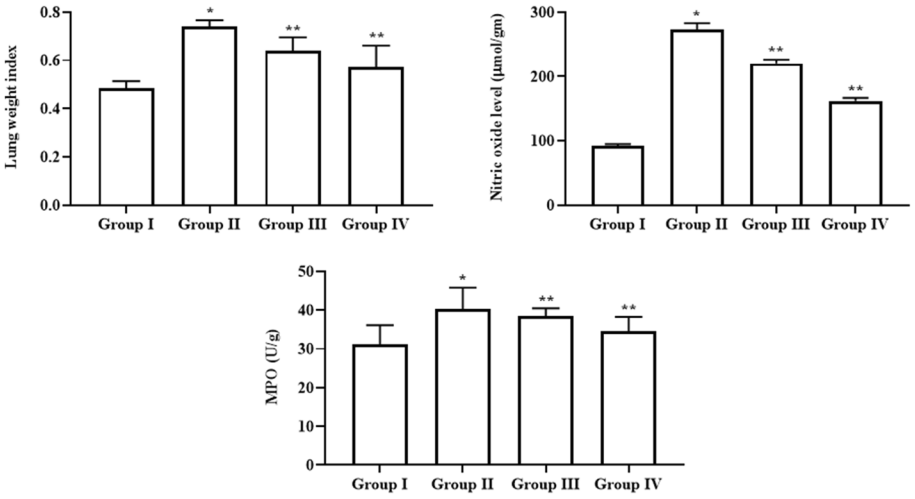

Fig. 1 Effect of pinocembrin on the lung weight, NO level, and MPO activity in the experimental mice. Outcomes were depicted as mean ± SD of three separate assays. Values were statistically inspected by one-way ANOVA and Tukey's post hoc assay. Note: "Single asterisk" indicates that outcomes significantly vary at $p < 0.05$ from control, and "double asterisks" indicate that outcomes significantly vary at $p < 0.01$ from pneumonia mice. Group I, normal control; group II, pneumonia-induced mice using 100 µl of *M. pneumoniae*; group III, pneumonia mice treated with 10 mg/kg of pinocembrin for 3 days; group IV, pneumonia mice treated with standard drug azithromycin (100 mg/kg) for 3 days

Effect of Pinocembrin Treatment on the Oxidative and Antioxidant Biomarkers in the Lung Tissues of Experimental Animals

Figure 2 demonstrates the effects of pinocembrin treatment on the GSH content, GSH level, and SOD activity in the pneumonia animals. The significant elevation ($p < 0.05$) in the MDA content, reduction in GSH, and SOD was found in the pneumonia animals when related to the control. However, the pinocembrin treatment appreciably regularized these changes on MDA and GSH contents and SOD activity. The administration of 10 mg/kg of pinocembrin significantly reduced ($p < 0.01$) the MDA and improved the GSH and SOD. In similar manner, the standard drug azithromycin also decreased the MDA and improved the GSH and SOD in the pneumonia animals.

Effect of Pinocembrin on the Total Cells and DNA Amount in the Lung Tissues of Experimental Animals

Figure 3 displays the levels of total cells and DNA amount in the control and pinocembrin-administered animals. The *M. pneumoniae*-triggered pneumonia mice significantly elevated ($p < 0.05$) total cells and DNA amount in the lung tissues. These elevations in the pneumonia mice were appreciably ameliorated by the pinocembrin treatment. The levels of total cells and *M. pneumoniae* DNA amount were significantly decreased ($p < 0.01$) by the 10 mg/kg of pinocembrin treatment. The administration of standard drug azithromycin also effectively depleted the total cells and *M. pneumoniae* DNA amount in the lung tissues, which is comparable to the pinocembrin treatment.

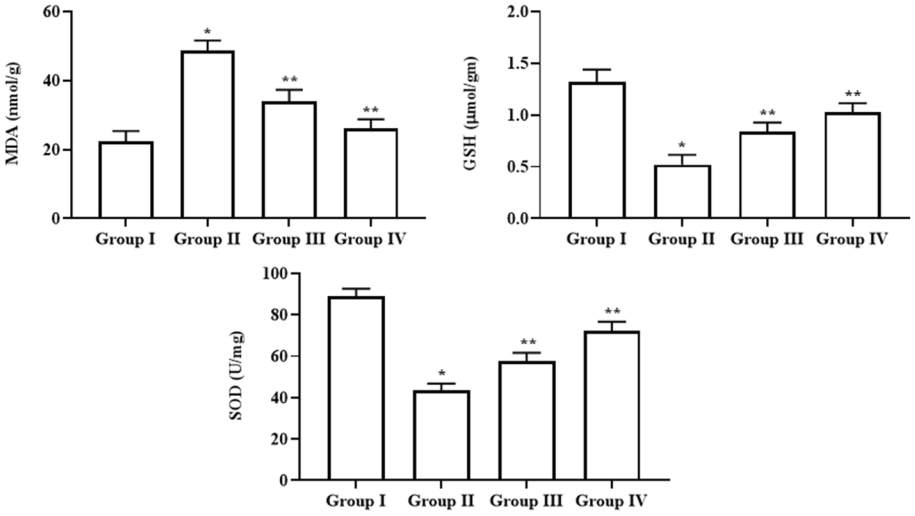

Fig. 2 Effect of pinocembrin treatment on the oxidative and antioxidant biomarkers in the lung tissues of experimental mice. Outcomes were depicted as mean ± SD of three separate assays. Values were statistically inspected by one-way ANOVA and Tukey's post hoc assay. Note: "Single asterisk" indicates that outcomes significantly vary at $p < 0.05$ from control, and "double asterisks" indicate that outcomes significantly vary at $p < 0.01$ from pneumonia mice

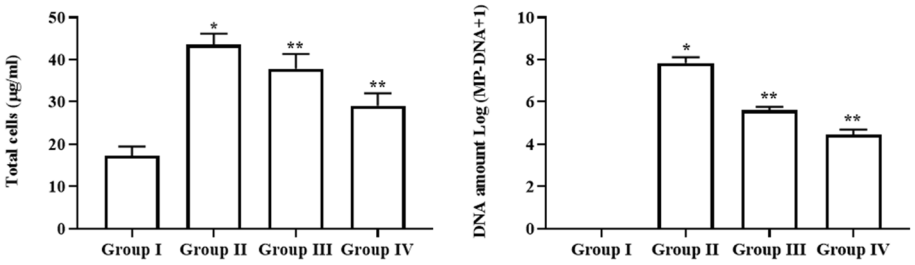

Fig. 3 Effect of pinocembrin on the total cells and DNA amount in the lung tissues of experimental mice. Outcomes were depicted as mean ± SD of three separate assays. Values were statistically inspected by one-way ANOVA and Tukey's post hoc assay. Note: "Single asterisk" indicates that outcomes significantly vary at $p < 0.05$ from control, and "double asterisks" indicate that outcomes significantly vary at $p < 0.01$ from pneumonia mice

Effect of Pinocembrin on the Levels of Inflammatory Markers in the BALF of Experimental Animals

Figure 4 demonstrates the influence of pinocembrin treatment on the contents of inflammatory biomarkers like IL-8, IL-6, IL-1, TNF-α, and TGF in the BALF of control and experimental animals. The pneumonia mice displayed the significant increase ($p < 0.05$) in the contents of IL-8, IL-6, IL-1, TNF-α, and TGF in the BALF when related to the control. Substantially, these elevations were notably attenuated by the pinocembrin. The significant reduction ($p < 0.01$) in the contents of IL-8, IL-6, IL-1, TNF-α, and TGF were found on the BALF of 10 mg/kg of pinocembrin-treated pneumonia mice. In similar manner, the azithromycin supplementation also appreciably depleted the contents of IL-8, IL-6, IL-1, TNF-α, and TGF in the BALF of *M. pneumoniae*-infected pneumonia mice.

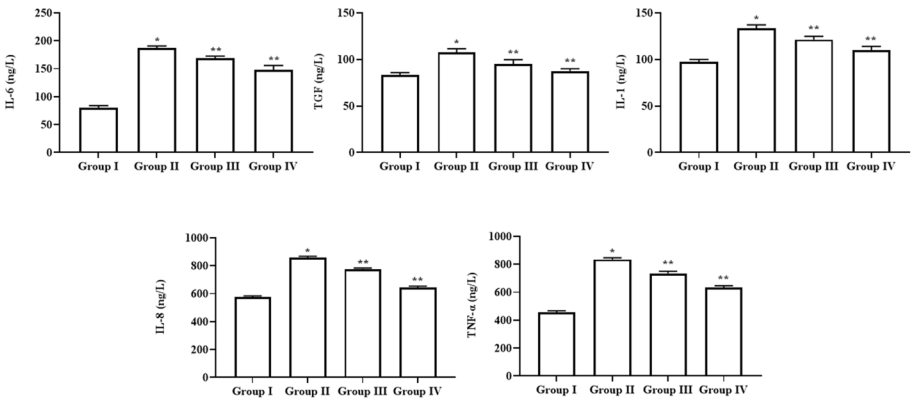

Fig. 4 Effect of pinocembrin on the inflammatory cytokines in the BALF of experimental animals. Outcomes were depicted as mean ± SD of three separate assays. Values were statistically inspected by one-way ANOVA and Tukey's post hoc assay. Note: "Single asterisk" indicates that outcomes significantly vary at $p < 0.05$ from control, and "double asterisks" indicate that outcomes significantly vary at $p < 0.01$ from pneumonia mice

Effect of Pinocembrin on the Level of NF-κB in the Lung Tissues of Experimental Animals

Figure 5 displays the level of NF-κB in the control and *M. pneumoniae*-triggered pneumonia animals. The pneumonia-triggered animals displayed the significant elevation ($p < 0.05$) in the NF-κB content in the lung tissues when related with control. Interestingly, the 10 mg/kg of pinocembrin administration significantly decreased ($p < 0.01$) the NF-κB level in the pneumonia animals. Similarly, the azithromycin supplementation also lessened the NF-κB level in the pneumonia animals.

Effect of Pinocembrin on the Lung Histopathology of Experimental Mice

Figure 6 displays the variations in lung histopathology of control and experimental animals. The normal alveolar arrangements without any lesion and inflammatory signs were appeared in the lung tissues of control mice. However, pneumonia-triggered animals displayed the increased inflammatory cell infiltrations, thickened alveolar walls, and narrowed bronchial tubes in the lung tissues. The 10 mg/kg of pinocembrin administration demonstrated appreciable reduction in the histological alterations in the lung tissues. The pinocembrin treatment appreciably decreased the infiltrations of inflammatory cells, narrowed bronchial tubes, and hyperemia signs (Fig. 6). The standard drug azithromycin administration also effectively ameliorated the histological alterations in the lung tissues of pneumonia animals.

Discussion

Pneumonia is a major infectious disease with increased morbidity and mortality rates worldwide. The *M. pneumoniae* is the major airway pathogen that causes infection at respiratory tract and ultimately leads to the development of pneumonia [37, 38]. The infection by *M. pneumoniae* generally affects the host-immune responses, thus triggering the inflammatory reactions through activating the intracellular signaling cascades [39]. Moreover, infection by *M. pneumoniae* can trigger several complications like asthma, pulmonary fibrosis, and pneumonia, which needs to be hospitalized with intensive care [40]. The underlying mechanisms of *M. pneumoniae* pneumonia development are highly complicated and include adhesion injury, membrane fusion destruction, depletion of nutrition, and immune and inflammatory

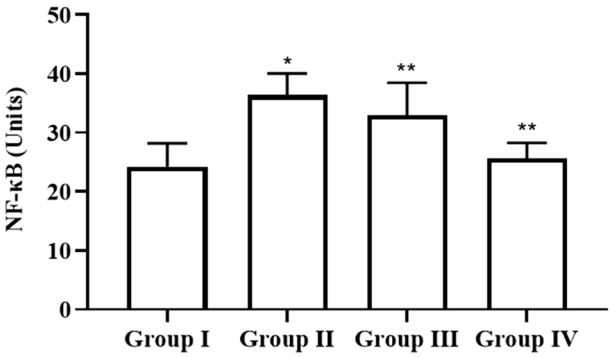

Fig. 5 Effect of pinocembrin on the level of NF-κB in the lung tissues of experimental animals. Outcomes were depicted as mean ± SD of three separate assays. Values were statistically inspected by one-way ANOVA and Tukey's post hoc assay. Note: "Single asterisk" indicates that outcomes significantly vary at $p < 0.05$ from control, and "double asterisks" indicate that outcomes significantly vary at $p < 0.01$ from pneumonia mice

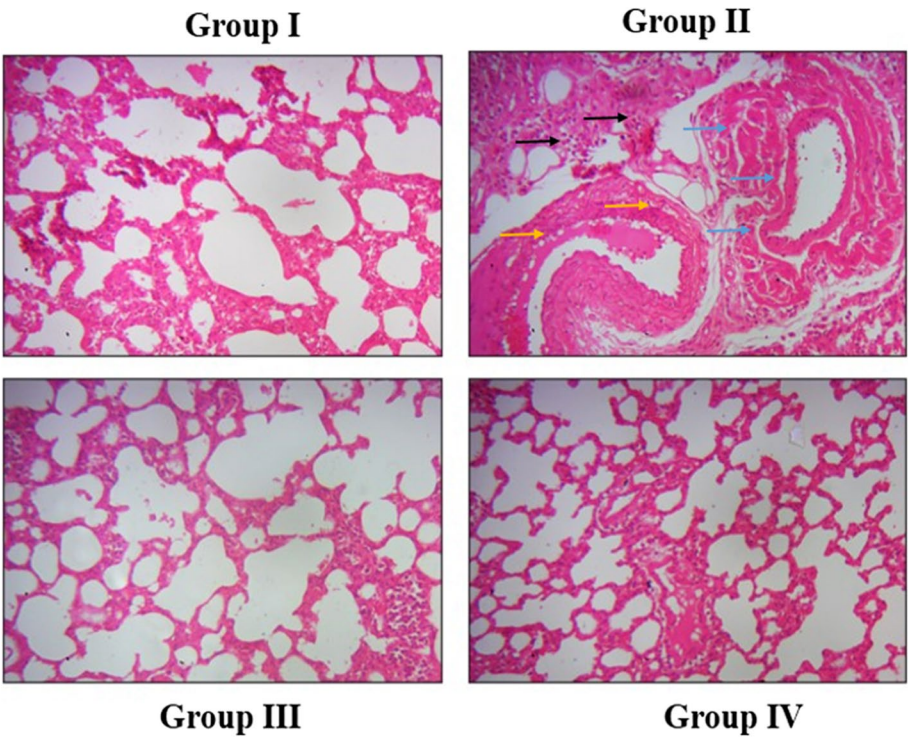

Fig. 6 Effect of pinocembrin on the lung histopathology of experimental animals. Control animals demonstrate the normal alveolar arrangements without any lesion and inflammatory signs (group I). *M. pneumoniae*-infected pneumonia animals exhibited the higher inflammatory cell infiltrations (black arrows), thickened alveolar walls (blue arrows), and narrowed bronchial tubes (yellow arrows) in the lung tissues (group II). The 10 mg/kg of pinocembrin treatment appreciably reduced the histological alterations in the lung tissues (group III). The azithromycin administration also effectively attenuated the histological alterations in the lung tissues (group IV)

injuries. The immunological reactions play a vital role during the progression and exacerbation of *M. pneumoniae* pneumonia. *M. pneumoniae* generates numerous infectious factors like invasive nucleases, polysaccharides, and lipoproteins that can cause several pathophysiological alterations [41]. In recent decades, *M. pneumoniae* has appeared as highly resistant to the existing antibiotics and leads to elevated global incidences of pneumonia and them very challenging to treat [42]. Hence, the present work is motivated to unveil the beneficial properties of pinocembrin against the pneumonia in mice. Our findings confirmed the beneficial roles of pinocembrin against the *M. pneumoniae*-provoked pneumonia in mice through its antioxidant and anti-inflammatory properties.

Oxidative stress arises due to the host immune response to the invading pathogens, and augments the generation of regulators of lung inflammation [43]. It was highlighted that the oxidative stress was found augmented in pneumonia patients than in normal peoples [44]. GSH is a well-established and most effective antioxidant in the lung, and its reduction signifies the increased oxidative stress [45]. Furthermore, the remarkably decreased contents of GSH, SOD, and GPx were found in the blood samples of pneumonia-affected patients than in the normal peoples, which suggest the role of oxidative stress on the pneumonia development

[46]. In similar manner, the current findings from this study suggested that the pinocembrin treatment appreciably depleted the MDA and improved the GSH content and SOD activity in the pneumonia animals. These outcomes suggest the antioxidant role of pinocembrin.

The inflammatory biomarkers like IL-6, IL-1β, and TNF-α play a major role during the progression of *M. pneumoniae*-triggered inflammatory responses. A previous literature highlighted that during *M.pneumoniae* infection in the experimental animals, the expressions of TNF-α, IL-1β, and IL-6 were found augmented in the lung tissues [47]. It was already stated that *M. pneumoniae* infection can stimulate the generation of IL-1β, which is a crucial player of several inflammatory damage [48]. Lee et al. [49] highlighted that during *M. pneumoniae* infection, IL-8 content augments rapidly in the blood, which further aggravates the inflammatory responses. He et al. [50] highlighted that the TNF-α content was amplified after the *M. pneumoniae* infection. Numerous previous literatures highlighted that the inflammatory reactions in the lung tissues speed up the pneumonia due to increased production of IL-6, TNF-α, and IL-1β [51, 52]. Several previous clinical studies demonstrated that the contents of inflammatory biomarkers like IL-6, IL-10, and TNF-α found augmented in the serum of the pneumonia patients than in normal volunteers [53, 54]. IL-6 is a vital pro-inflammatory mediator and a valuable marker to predict the mortality in hospitalized pneumonia patients [55].

In most cases, the contents of IL-1β and IL-8 were found elevated in the BALF of pneumonia patients that suggests their potential roles during the progression of *M. pneumoniae* pneumonia. IL-8 is an imperative chemokine generated by the macrophages and other inflammatory cells [49]. IL-1β is primarily generated by stimulated immune cells like monocytes and macrophages [56]. Gui et al. [57] highlighted that the contents of IL-1β and IL-8 were found augmented in the BALF of pneumonia-infected children, signifying that the elevated IL-8 and IL-1β contents in BALF participated in the progression of *M. pneumoniae* pneumonia. Similarly, our findings of this study suggested that the pinocembrin treatment substantially decreased the contents of IL-8, IL-6, IL-1, TNF-α, and TGF in the pneumonia animals. This finding suggested the strong anti-inflammatory roles of pinocembrin.

The elevated accumulation of pro-inflammatory biomarkers can further worsen the lung damage via exacerbating injury to the respiratory epithelial cells distinguished by cilia loss, exfoliation, and vacuolation. The production of inflammatory mediators is a complex process, in that NF-κB, an imperative inflammatory protein, performs a vital function [58]. As per the previous literature, the lipoprotein ingredients of *M. pneumoniae* pneumonia can identify the TLRs and then stimulate the NF-κB to control the generation of numerous inflammatory arbitrators, therefore accumulating airway inflammation [59]. Among the several pro-inflammatory mediators, the IL-8 and TNF-α was highlighted to be a prominent to evaluate the *M. pneumoniae* pneumonia [60]. Here, we witnessed that the pinocembrin treatment effectively diminished the NF-κB content in the lung tissues of pneumonia animals. Hence, it was clear that the pinocembrin can decrease the inflammatory responses during pneumonia development via its anti-inflammatory roles.

It has already been reported that the neutrophils perform a crucial functions in removing the invading pathogens via degranulation and autophagy. These mechanisms produce increased ROS and proteases that can not only clear bacterial invasion but also partly cause the oxidant–antioxidant disproportion [61]. Yan et al. [62] found the relatively augmented level of neutrophils in the blood of patients with drug-resistant refractory *M. pneumoniae* pneumonia. The neutrophils are not necessary to clear the *M. pneumoniae* from lung tissues; instead increased neutrophil generation could result in the hyper-inflammatory condition due to the MPO release [63].

MPO is the most abundant enzyme that actively participates in the immune responses via generation of microbicidal ROS [64]. Additionally, increased accumulation of MPO will result in the oxidative stress and cell injury [65]. In this exploration, our outcomes witnessed that the pinocembrin treatment effectively decreased the NO level and MPO activity. Additionally, the histopathological studies also confirmed that the pinocembrin treatment decreased the neutrophil penetrations into the lung tissues. These outcomes evidence the therapeutic properties of pinocembrin against the pneumonia.

Conclusion

In conclusion, our findings confirmed that the pinocembrin effectively ameliorated the *M. pneumoniae*-provoked inflammation and oxidative stress in the pneumonia mice model. Pinocembrin treatment appreciably improved the antioxidants, decreased the inflammatory biomarkers, and ameliorated the histological changes. Hence, it could be a promising salutary candidate for the management of pneumonia in the future, though further studies are still warranted in the future in order to understand the underlying mechanisms of therapeutic actions of pinocembrin against the pneumonia.

Author Contribution The authors contributed equally.

Data Availability Not applicable.

Declarations

Ethics Approval All work has been done under the guidelines of the Institutional Ethics Committee.

Consent to Participate All authors have their consent to participate.

Consent for Publication All authors have their consent to publish their work.

Competing Interests The authors declare no competing interests.

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