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ORIGINAL ARTICLE

Oleuropein Reduces *Mycoplasma pneumonia*-Induced Pneumonia in Mice via Down-Regulating the Inflammatory Response

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Abstract

Pneumonia is a common airway inflammatory disease, which is caused by several stimuli. *Mycoplasma pneumoniae* is the foremost cause of pneumonia, with the clinicopathological features ranging from coughing, fever, and breathing problems. The therapeutic effects of oleuropein were analyzed against *M. pneumoniae*-induced pneumonia in mice via regulating the inflammatory and oxidative stress responses. Swiss albino mice were challenged with *M. pneumoniae* (100 µl) through nasal drips for 2 days to initiate pneumonia. The mice were subsequently treated with 20 mg/kg of oleuropein for 3 days. The lung weight, nitric oxide, and myeloperoxidase levels in the mice were estimated. The oxidative stress markers malondialdehyde, superoxide dismutase, and glutathione, as well as inflammatory cytokines, were analyzed. The total cell count and DNA quantity were determined in the bronchoalveolar lavage fluid of the experimental mice. The histopathological analysis was done on lung tissues. The 20 mg/kg of oleuropein effectively reduced the lung weight, nitric oxide, and myeloperoxidase levels in the pneumonia mice. The malondialdehyde was decreased, and superoxide dismutase and glutathione levels were boosted by oleuropein treatment. The levels of inflammatory cytokines, including IL-1, IL-6, IL-8, TNF-α, and NF-κB, were effectively reduced by oleuropein treatment in the pneumonia model mice. Oleuropein also decreased the total cell count and DNA amount in the pneumonia infected mice. The findings of the histopathological analysis revealed a substantial decrease in inflammatory cell infiltration and alveolar damage in the pneumonia model mice. The present results validate the beneficial effects of oleuropein against *M. pneumoniae*-induced pneumonia in mice. Therefore, oleuropein can be a candidate for the management of pneumonia.

Keywords Cytokines · Lung inflammation · Malondialdehyde · Myeloperoxidase · Pneumonia · Seco-iridoide

Introduction

Mycoplasma pneumoniae is the most common and well-studied bacteria of the pathogenic *Mycoplasma* genus, which is a common human pathogen that causes pneumonia and other respiratory illnesses, especially in young children and teenagers. In most cases, *M. pneumoniae* infections are short-lived and uncomplicated (Kutty et al 2019). However, for some people, this condition might worsen to the point where it threatens their lives. Lower respiratory tract infections were found to be attributed with up to 40% of community-acquired pneumonia in children over the age of 5. It has been hypothesized that infections caused by *M. pneumoniae* contribute to the development of chronic lung illness and bronchial asthma (Waites et al. 2017). Several factors have been linked to the pathophysiology of this infections, including inflammation, oxidative stress, and cytokine fluctuations. Coughing, fever, hypoxemia, respiratory, and breathing problems are some of the clinicopathological symptoms of pneumonia. If these signs persist for an extended period, respiratory distress syndrome may develop, which can ultimately result in heart failure (Lee et al. 2015). Pneumonia is a particularly dangerous infectious immunological disease for youngsters, but it can be fatal for people of any age. Nearly 450 million people of all ages are affected by pneumonia each year, according to a population-based analysis, with *Streptococcus pneumoniae* accounting for 4.1 cases per 10,000 people and producing 2.4 cases per 10,000 people (Bjarnason et al. 2018).

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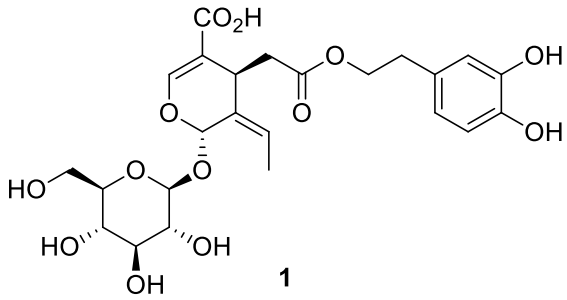
Mycoplasma infections are especially hazardous because they are transferred from person to person via respiratory droplets and cause local tissue damage, cytotoxicity, and an immunological response from the host. Epithelial cells are damaged when *M. pneumoniae* binds to them and begins to increase oxidative stress (Segovia et al. 2017). It is becoming clear that *M. pneumoniae*-induced symptoms are linked to the inflammatory cytokine release and the formation of pulmonary fibrosis (Maselli et al. 2018). Clinically, *M. pneumoniae* infection in children can cause serious problems, including bronchiectasis, potentially deadly pneumonia, and impairment of multisystem activity (Wood et al. 2017).

The pathophysiology of *M. pneumoniae*-induced pneumonia is characterized by a strong correlation between direct damage and immunological damage. The apical structure of *M. pneumoniae* allows it to attach to the membrane of the host cell. In addition to preventing respiratory clearance, this mechanism aids in nutritional uptake (Saraya et al. 2014). Excessive immunological response is thought to play a key function in the pathogenesis of *M. pneumoniae* infection, and cytokine content correlates strongly with its severity (Xu et al. 2016). Pneumonia etiology is profoundly influenced by cytokines, which modify both intercellular signal transmission and inflammation. It is well established that inflammatory cytokines are essential for controlling immune responses (Jiang et al. 2015).

The NF-κB pathway is an important transcription factor that is present in all cells in a latent state and can be activated by a variety of causes. Therefore, it plays a primary role in responding to a several stimuli, including microbial infections. Also, NF-kB is crucial in controlling the immune response to infections (Meffert et al. 2003). Infection with *M. pneumoniae* causes a rapid immunological response by stimulating the production of IL-8, IL-18, TNF-α, and IL-1 in macrophages through the activation of toll-like receptors (Oishi et al. 2011). In *M. pneumoniae*-induced inflammatory reactions, pro-inflammatory regulators play a crucial role.

Currently, macrolide antibiotics are the first line of treatment for pneumonia caused by *M. pneumoniae*. Conventional macrolide antibiotics often come with several side effects and have limited efficacy in the treatment of pneumonia due to the rise of mycoplasma resistance to these drugs (Qiu et al. 2020). Therefore, effective alternative agents are urgently needed for the successful management of pneumonia with fewer side effects. Oleuropein (**1**) is a glycosylated seco-iridoid compound and is extensively found in the olive leaves (*Olea europaea* L., Oleaceae). Oleuropein exhibits antidiabetic (Castejón et al. 2017), anti-inflammatory (Ahamad et al. 2019), antioxidant (Imran et al. 2018), and antitumor (Bulotta et al. 2014) and also demonstrates protective effects against cardiovascular (Zhang et al. 2018), neurological (Fki et al. 2020), and hepatic (Williamson et al. 1992) diseases. However, the beneficial properties of oleuropein were not studied against mycoplasma pneumonia. Therefore, the therapeutic effects of oleuropein were analyzed in the present study against *M. pneumoniae*-induced pneumonia model in mice via regulation of inflammatory and oxidative stress responses.



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Materials and Methods

Chemicals

The following chemicals and drugs were attained from Sigma-Chemical, USA: oleuropein (purity: ≥ 98%; Lot No.32619–42-4), azithromycin, and other chemicals. To estimate the biochemical markers, the assay kits were used which were obtained from Thermo Fisher Scientific, USA.

Experimental Mice

Swiss albino mice weighing above 27 ± 5 g of bodyweight was used in this study. Mice were housed in sterile polypropylene cages and kept in a stable environment at 23 ± 2 °C, with air humidity 50–60% and a 12-h dark/light cycle. All mice in the study had unrestricted access to the pellet food and sterile water throughout the experiment. All mice were acclimated to laboratory conditions for 7 days before the experiments began.

Experimental Groups

The experimental mice were distributed into four groups of six mice each ($n = 6$). Group I was normal control mice. Group II was mice were injected with *M. pneumoniae* (100 µl) culture for 2 days via nasal drips to initiate the pneumonia. Group III mice were injected with *M. pneumoniae* and subsequently treated with 20 mg/kg of oleuropein for 3 days. Group IV was pneumonia-induced mice administered with 100 mg/kg of the standard drug azithromycin for 3 days. After treatments, mice from each group were sacrificed by cervical dislocation, and then blood and lung tissue samples were taken for additional experiments. The lung wet and dry weights were assessed precisely.

Nitric Oxide and Myeloperoxidase Levels

The lung tissues excised from the experimental mice were collected and homogenized using an ice-cold buffer solution and then centrifuged at 12,000 × *g* for 15 min at 4 °C. The resultant was obtained and utilized for the assessment of nitric oxide (NO) and myeloperoxidase (MPO) levels using the corresponding assay kits by strictly following the guidelines described by the kit's manufacturer (Thermo Fisher, USA). The absorbance of the reaction solution was taken at 550 nm using a microplate reader. Each assay was done in triplicate.

Antioxidants and Malondialdehyde Levels

The levels of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) in the lung tissue homogenates of controls and treated mice were assessed using the corresponding assay kits. Each experiment was conducted in triplicates using the procedures given by the kits's manufacturer (MyBioSource, USA).

Bronchoalveolar Lavage Fluid and Total Cell Count

The right middle lobe of the experimental mice was injected with 30 ml aliquots of saline to collect the BALF samples. The BALF were then centrifuged 3000 × *g* for 5 min. The resultant BALF sample was then transferred to fresh vials for further analysis. Under an optical microscope, a hemocytometer was employed to count the cell numbers in the collected BALF sample.

Mycoplasma pneumoniae DNA Content

The removed lung tissues from both control and treated mice were homogenized and subjected to treatment with a DNA extraction solution for 10 min. Later, the solution was centrifuged for 5 min at 11,000 × *g* to separate the DNA samples. The total quantity of *M. pneumoniae* DNA samples in the solution was assessed by the PCR method as previously described (Akashi et al. 2018).

Inflammatory Cytokine Levels

The obtained BALF sample from both control and treated mice was utilized for the assessment of inflammatory cytokines such as IL-6, IL-1, IL-8, TNF-α, and TGF using the corresponding assay kits as per the guidelines given by the kit's manufacturer (Thermofisher, USA).

NF-κB Level

The assay kits purchased from Thermo Fisher, USA, were utilized to assess the total NF-κB level in the lung tissues of both control and treated mice, as per the instructions described by the kit's manufacturer.

Histopathological Analysis

The histopathological analysis was done in the lung tissues to detect the histological changes. Briefly, the tissues were processed with 10% neutral formalin and then fixed in paraffin. The paraffinized tissues were then cut into sections with a 5-µm diameter. The hematoxylin and eosin stains were utilized to stain the tissues, which were then subjected to microscopic examination to observe the changes in the histopathology of the lung tissues.

Statistical Analysis

The outcomes were analyzed statistically using SPSS software and data are displayed as mean ± SD of triplicate measurements. The data were analyzed by one-way ANOVA, and Tukey's post hoc test to find the significant changes between treatment groups with $p < 0.05$ was significant.

Results and Discussion

Effects on Lung Weight, MPO, and NO Levels

The lung weight, MPO activity, and NO level in the control and treated mice were examined, and the outcomes are revealed in Fig. 1. The mice with *Mycoplasma pneumoniae*-induced pneumonia revealed a substantial increase in lung weight, MPO activity, and NO levels. However, the treatment with 20 mg/kg of oleuropein effectively decreased these elevations in the pneumonia-induced mice. The treatment with the azithromycin also decreased the lung weight, MPO, and NO levels in the pneumonia-induced mice, which validated the activity of oleuropein treatment.

Effects in MDA SOD and GSH Levels

As illustrated in Fig. 2, a considerable elevation in the MDA and reduction in the SOD and GSH levels were observed in pneumonia-induced mice when compared to the control. Interestingly, treatment with 20 mg/kg of oleuropein effectively reduced MDA and remarkably boosted the SOD and GSH levels in mice with pneumonia. Similarly, a reduction in MDA level and an elevation in SOD and GSH status were

also noted in the azithromycin-treated mice, which supports the antioxidant properties of oleuropein.

Total Cell Counts and DNA Content

The changes in the total cell counts and DNA amounts were determined, and the outcomes are shown in Fig. 3. An increase in the total cell counts and DNA amounts was observed in the pneumonia-induced mice when compared to the control. Whereas the oleuropein (20 mg/kg) treatment caused a considerable reduction in the DNA amount and total cell counts. The activity of oleuropein treatment was supported by the findings of azithromycin treatment, which also decreased the total cell count and DNA amount in the pneumonia mice.

Inflammatory Cytokine Levels

The levels of inflammatory cytokines in the BALF of both control and treated mice are shown in Fig. 4. A substantial increase in the IL-8, IL-6, IL-1, TNF-α, and TGF levels was observed in the BALF of pneumonia-induced mice when compared to control. Interestingly, the 20 mg/kg of oleuropein treatment remarkably decreased the IL-8, IL-6, IL-1, TNF-α, and TGF levels in the pneumonia mice. The azithromycin treatment also effectively decreased the status of these inflammatory cytokines, which supports the anti-inflammatory properties of oleuropein.

NF-κB Level

The level of NF-κB in the lung tissues of both control and pneumonia-induced mice were examined using assay kits, and the outcomes are shown in Fig. 5. A considerable increment in NF-κB levels were noted in the lung tissues of pneumonia-induced mice when compared with controls, whereas the treatment with 20 mg/kg of oleuropein substantially decreased the NF-κB in the pneumonia-induced mice. Furthermore, the azithromycin treatment also reduced the NF-κB level, which supports the anti-inflammatory activity of oleuropein.

Lung Histopathology

The findings of the lung histopathological analysis of both control and treated mice are revealed in Fig. 6. The control mice revealed regular arrangements of alveolar cells without signs of inflammation or damage to the lung tissues, whereas pneumonia-induced mice revealed drastic histological changes such as alveolar wall thickening, narrowing of bronchial tubes, and elevated infiltration of inflammatory cells in the lung tissues. Interestingly, the oleuropein (20 mg/kg) caused a considerable decrease in the lung histopathological changes, including inflammatory cell infiltrations. The results of azithromycin treatment also revealed a considerable decrease in lung histopathological changes, which supports the therapeutic activity of oleuropein.

It has been shown that *M. pneumoniae* is a major pathogen in community-acquired pneumonia in both young adults and children. *Mycoplasma pneumoniae* is a contributing factor in up to 40% of all cases of acquired pneumonia in children, which raises mortality rates, hospitalizations, and overall healthcare costs. Pneumonia has been recorded in a small number of patients, despite the fact that pneumonia is typically a benign and self-limiting condition (Sauteur et al. 2016). In children with refractory pneumonia, pulmonary

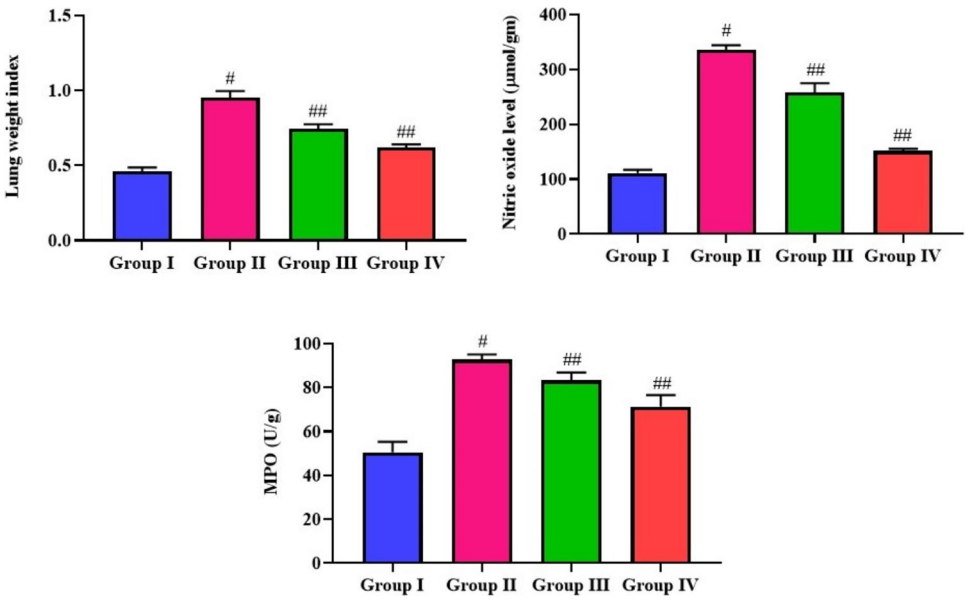

Fig. 1 Effect of oleuropein (**1**) on the lung weight, myeloperoxidase activity, and nitric oxide level in the experimental mice. Each bar reveals the value mean ± SD of three independent experiments. Outcomes are measured statistically using one-way ANOVA and Tukey's post hoc test. "#" signifies the significance at $p < 0.01$ from control, and "##" signifies the significance at $p < 0.05$ from pneumonia-induced mice

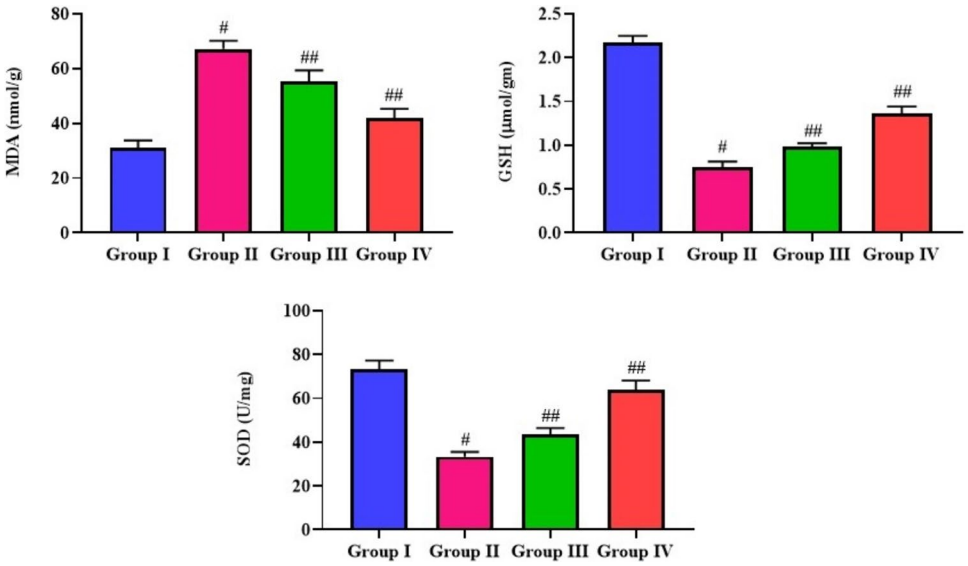

Fig. 2 Effect of oleuropein (**1**) on the malondialdehyde and antioxidant levels in the experimental mice. Each bar reveals the value mean ± SD of three independent experiments. Outcomes are measured statistically using one-way ANOVA and Tukey's post hoc test. "#" signifies the significance at $p < 0.01$ from control, and "##" signifies the significance at $p < 0.05$ from pneumonia-induced mice

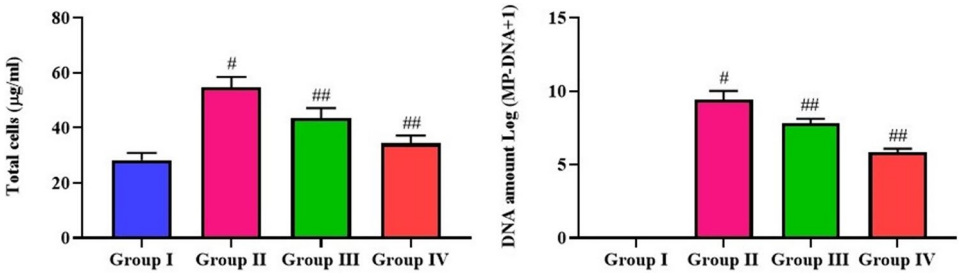

Fig. 3 Effect of oleuropein (**1**) on the total cell counts and DNA content in the lung tissues of experimental mice. Each bar reveals the value mean ± SD of three independent experiments. Outcomes are measured statistically using one-way ANOVA and Tukey's post hoc test. "#" signifies the significance at $p < 0.01$ from control, and "##" signifies the significance at $p < 0.05$ from pneumonia-induced mice

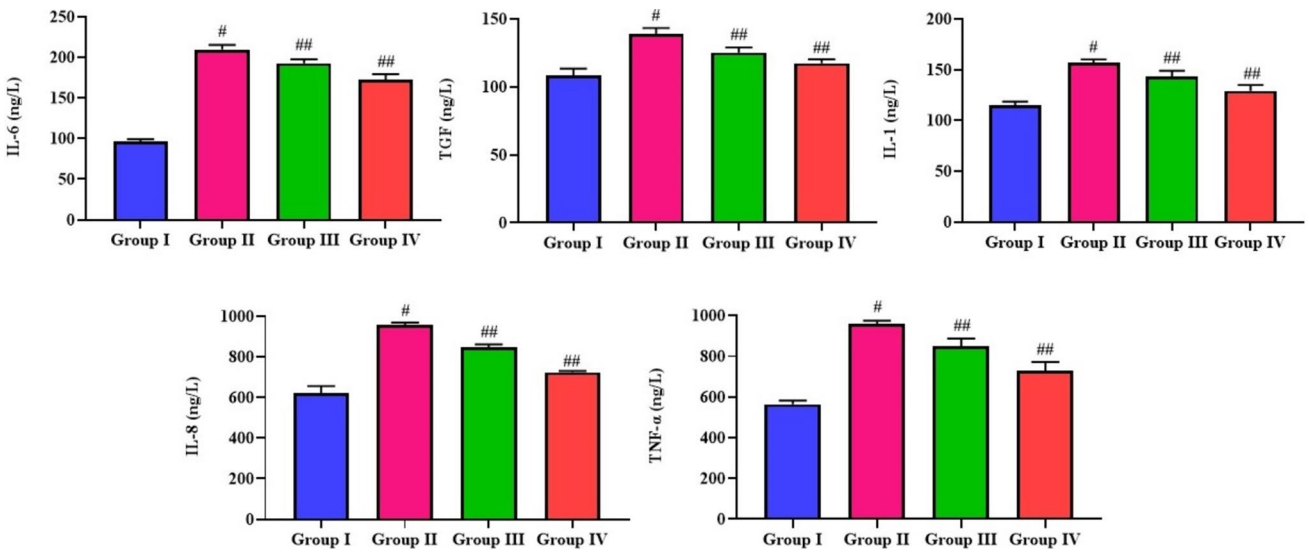

Fig. 4 Effect of oleuropein (**1**) on the inflammatory cytokine levels in the BALF of experimental mice. Each bar reveals the value mean ± SD of three independent experiments. Outcomes are measured statistically using one-way ANOVA and Tukey's post hoc test. "#" signifies the significance at $p < 0.01$ from control, and "##" signifies the significance at $p < 0.05$ from pneumonia-induced mice

sequelae such as bronchiectasis and atelectasis persisted long after the initial symptoms had subsided, sometimes resulting in plastic bronchitis or necessitating critical care (Moynihan et al. 2018). Multiple extrapulmonary consequences have been linked to *M. pneumoniae* infection. In addition, an immunological response caused by *M. pneumoniae* may be closely linked to the development of extrapulmonary problems. It's generally agreed that an *M. pneumoniae* epidemic would be a major public health crisis. *Mycoplasma pneumoniae* is generally found in the mucosal layer of the human respiratory, urogenital, mammary, and gastrointestinal tracts due to its tissue and organ specificity. Although *M. pneumoniae* is generally thought of as a membrane-bound parasite, it can occasionally invade cells and live there permanently (Santos et al. 2012).

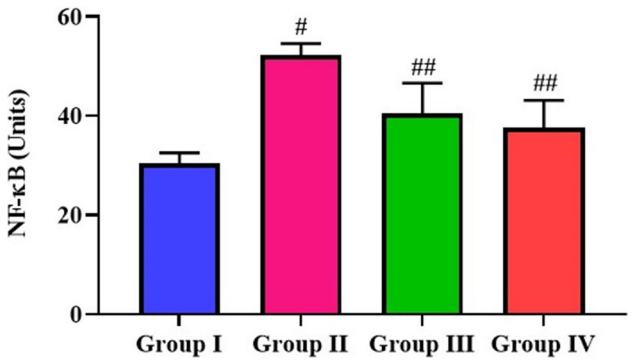

Fig. 5 Effect of oleuropein (**1**) on the NF-κB level in the lung tissues of experimental mice. Each bar reveals the value mean ± SD of three independent experiments. Outcomes are measured statistically using one-way ANOVA and Tukey's post hoc test. "#" signifies the significance at $p < 0.01$ from control, and "##" signifies the significance at $p < 0.05$ from pneumonia-induced mice

Myeloperoxidase is one of the most common protein enzymes, and it plays a role in immune protection by producing microbicidal reactive oxidants. Nevertheless, when MPO levels rise above their normal threshold, it may cause an increase in oxidative stress and tissue damage (Aratani 2018.). It has been hypothesized that the enzyme MPO, which is mostly found in neutrophil granules, possesses strong antibacterial properties. Therefore, MPO is a significant marker for neutrophil infiltration. In experimental animals, researchers found that MPO levels increased after LPS injection, implicating inflammatory cells such as neutrophils in the development of pneumonia (Kim et al. 2014). In addition, a study in LPS-exposed MPO (-/-) mice corroborated the role of MPO in the generation of cytokines (Alblihed 2021). The results of the current study showed that the *M. pneumoniae*-induced pneumonia mice had increased MPO activity, which demonstrates increased neutrophil infiltration and an aggravated inflammatory response. However, the oleuropein treatment effectively decreased the MPO activity, which proves its anti-inflammatory properties. An increase in the lung wet/dry weight ratio, another characteristic of inflammation, was observed following both systemic and local inflammatory infiltration (Kratzer et al. 2012). Similarly, the pneumonia-induced mice also showed an increase

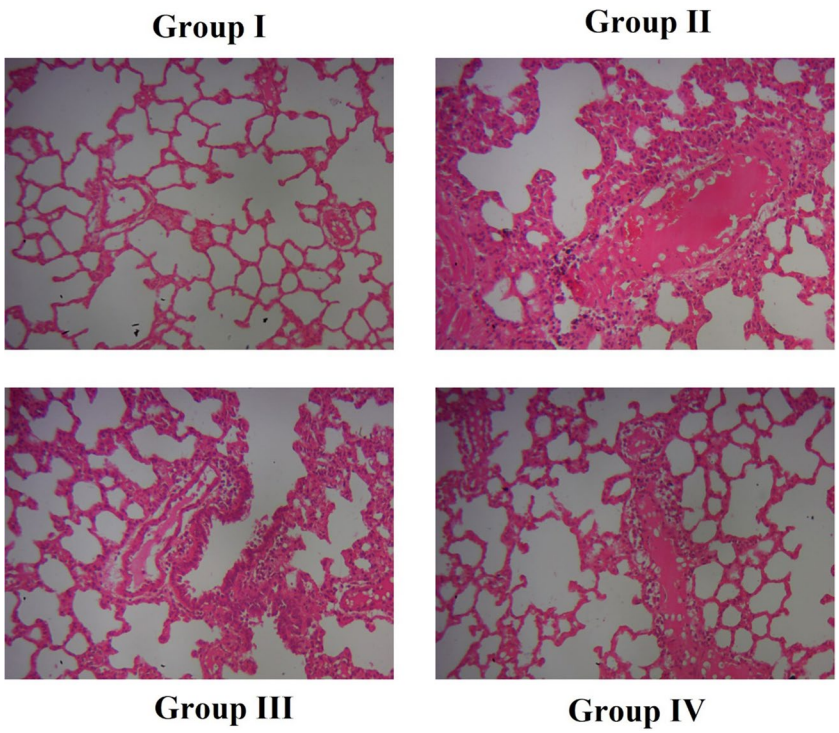

Fig. 6 Effect of oleuropein (**1**) on the lung histopathology of experimental mice. Group I: the control mice revealed a regular arrangement of alveolar cells without signs of inflammation and damages. Group II: pneumonia-induced mice revealed an alveolar wall thickening, narrowing of bronchial tubes, and increased infiltration of the inflammatory cells. Group III and VI: oleuropein (20 mg/kg) and azithromycin (100 mg/kg) treatment revealed a considerable decrease in the lung histopathological changes, including inflammatory cell infiltrations

in the lung weight ratio, which was also effectively reduced by the oleuropein.

Oxidative stress plays a major role in the pathophysiology of pneumonia. Patients with pneumonia have been shown to experience elevated levels of oxidative stress. The oxidative stress level in the lungs is up-regulated due to *M. pneumoniae* infection, which plays a foremost function in the innate immunological response of the host against foreign pathogens (Szczepanek et al. 2014). After adhering to host epithelial cells, *M. pneumoniae* cells release enormous amounts of H_2O_2 as a byproduct of their metabolism, which is known to cause oxidative damage, pathological alterations, and necrosis in the host cells. Normally, host cells have antioxidant defense systems that allow them to maintain an oxygen-free radical metabolism balance (Ni et al. 2015). Cell injury may result from mitochondrial dysfunction and a decrease in the antioxidants like SOD and GSH levels under pathological conditions due to oxidative stress caused by excess oxygen free radicals (Rao et al. 2011). Oxidative stress is typically accompanied by an increase in the formation of ROS and MDA. Thus, the fundamental underlying mechanism of cell injury is the disruption of multiple signal transduction pathways due to an increase in oxidative stress (Navarro-Yepes et al. 2014). It was already known that patients with community-acquired pneumonia had higher levels of oxidative stress than healthy controls (Bhargava et al. 2013). Similarly, the current findings also proved that the pneumonia-induced mice showed an elevated MDA status, while a reduced SOD and GSH levels was noted. Interestingly, the oleuropein treatment effectively increased the SOD and GSH levels while reducing the MDA levels, which proves the antioxidant properties of oleuropein.

The inflammatory cytokines play a pivotal role as mediators in bacterial pneumonia and pulmonary inflammation (Kumar 2020). Metabolites and toxins secreted by *M. pneumoniae* cells may serve as inflammatory agents and provoke an immune response. In response to invading pathogens, the host's innate immune system produces inflammatory mediators in the lungs (Moldoveanu et al. 2008). The infection of *M. pneumoniae* causes an increased release of cytokines like TNF-α, IL-1, IL-6, and IL-8, which may be strongly linked to the exacerbation of pneumonia and subsequent lung injury. One possible pathogenic mechanism of *M. pneumoniae* infection involves alterations in cytokine profiles (Luo et al. 2021). It was already reported that serum IL-8 and TNF-α levels rise in tandem with the progression of *M. pneumoniae* infection (Liu et al. 2019). A specific innate immune reaction in the lung can be triggered by the secretion of several inflammatory regulators. The cytokine profiles in the serum are known to fluctuate in response to invading pathogens. It is challenging to study host immune response patterns since many cytokines were shown to associate with the pneumonia severity (Zhang et al. 2023). Zhou and Ye (2017) highlighted that the IL-1, IL-6, IL-8, and TNF-α levels are up-regulated in the BALF of pneumonia patients. Similarly, the current findings also exhibited an increase in IL-1, IL-6, IL-8, and TNF-α levels in the pneumonia mice, which evidences the exacerbation of the inflammation. However, the oleuropein treatment substantially reduced these cytokine levels, which proved its anti-inflammatory activity.

Menendez et al. (2012) found that a rise in the secretion of inflammatory cytokines resulted in an increased inflammatory cells, which in turn activates inflammation-associated transcription factors like NF-κB. The inflammatory genes are highly expressed when NF-κB signaling is activated (Yu et al. 2018). It has previously been established that NF-κB signal transduction plays a pivotal function in controlling inflammatory responses. The NF-κB signaling cascade can be stimulated by a wide variety of cytokines (Son et al. 2021). Inflammatory responses in lung tissues are a common target of the NF-κB signaling cascade. The secretion of inflammatory mediators is highly dependent on the NF-κB cascade. Therefore, blocking NF-κB signal transduction may be a promising strategy for reducing *M. pneumoniae*-induced lung inflammation (Cyphert et al. 2015). The current results show that the pneumonia mice showed an increased NF-κB level, which indicates an exacerbated inflammatory response. However, treatment with oleuropein remarkably suppressed the NF-κB level, which may facilitate the reduction of the inflammatory response. These findings proved the anti-inflammatory properties of oleuropein.

Lung inflammation caused by inflammatory cell infiltration is a hallmark of *M. pneumoniae*-induced pneumonia. Since the degree of *M. pneumoniae* infection corresponds with the concentration of inflammatory cell infiltration in the lung (Zhang et al. 2021), the higher degree of *M. pneumoniae* may explain the larger neutrophil proportion. Lung inflammation mostly affects the tiny air sacs called alveoli. *Mycoplasma pneumoniae* can cause damage to the respiratory tract as well as various extrapulmonary problems. In addition, the production of inflammatory mediators by inflammatory cells substantially increased the permeability of the alveolar-capillary barrier (Zhou et al. 2019). Neutrophils are very important in innate immunity, which is attributable for the initiation inflammatory reactions (Rungelrath et al. 2020). The recruitment of neutrophils is thought to be a crucial phase of the innate immune reaction to an invading pathogen in lung tissues (Peniche et al. 2018). The findings of histopathological analysis showed increased inflammatory cell infiltrations and alveolar cell damage in the pneumonia mice. Interestingly, the oleuropein remarkably attenuated the infiltration of inflammatory cells and alveolar damages, which evidences its therapeutic effects against pneumonia.

Conclusion

The current findings validate the beneficial properties of oleuropein against *M. pneumoniae*-induced pneumonia in mice. The treatment with oleuropein resulted in a decrease in lung weight, reduced inflammatory cytokines and MDA levels, boosted antioxidants, and decreased NF-κB expression in the pneumonia-induced mice. These findings reveal the therapeutic properties of oleuropein against pneumonia. Therefore, oleuropein can be a helpful agent for the pneumonia treatment.

Author Contribution YD: draft preparation, conceptualization, and methodology. DC: data curation and validation.

Declarations

Ethics Approval All work has been done under the guidelines of Institutional Ethics Committee, Xi'an Zhongkai Animal Experiments Medical Research (IEC no: 4896).

Competing Interests The authors declare no competing interests.

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