



Anti-inflammatory effects of *N*-Acetylcysteine and *Elaeagnus angustifolia* extract on acute lung injury induced by λ -carrageenan in rat

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Abstract

N-Acetylcysteine (NAC) is a chemical compound with anti-inflammatory and antioxidant activity and acts as a free radical scavenger. *Elaeagnus angustifolia* (EA) is a plant native to the western part of Iran, with antioxidant and anti-inflammatory properties. The present study been taken evaluated the protective effect afforded by EA and NAC extracts on carrageenan-induced acute lung injury in Wistar rats. In this study, 42 rats were randomly assigned into seven groups. NAC and EA extracts were orally administered once/day for 21 continuous days. Pulmonary damage was induced by intratracheal injection of 100 μ l of 2% λ -Carrageenan on day 21. Twenty-four hours post-surgery, the rats were euthanized and the samples were collected. Pretreatment with NAC and EA extracts reduced the total and differential cell accumulation as well as IL-6, and TNF- α cytokines. Antioxidant indicators demonstrate that in the groups receiving NAC and EA extract, MDA decreased while thiol and antioxidant capacity elevated. Treatment with NAC and EA significantly reduced Carrageenan-induced pathological pulmonary tissue injury. NAC and EA extract has protective effects on acute carrageenan-induced lung injury.

Keywords Acute lung injury · Anti-inflammatory · *N*-acetylcysteine · *Elaeagnus angustifolia* · λ -carrageenan

Introduction

Acute lung injury (ALI) and its more severe form, Acute respiratory distress syndrome (ARDS), are clinical complications with high mortality, increased permeability of the alveolar-capillary barrier impairs the function of pulmonary capillary endothelial cells, (Wu et al. 2020; Gurusamy et al. 2021) and contributes to high protein content and pulmonary edema (Laffey and Matthay 2017). ALI can result from infection, trauma, burns, or sepsis. Pro-inflammatory

mediators released following tissue damage can lead to tissue damage, respiratory failure, and subsequent infiltration of neutrophil and other inflammatory cells into the interstitial and alveolar spaces. Activation and transport of neutrophils are important in developing ALI (Lee et al. 2019). Neutrophils play an essential role in the innate immune system, as these cells are the first leukocytes to migrate to areas of acute inflammation (Bian et al. 2012). Neutrophils secrete large amounts of cytokines and chemokines that affect other immune cells and are, therefore, the primary regulators of inflammation (Hilda and Das 2016). About 5% of patients with coronavirus disease 2019 (COVID-19) suffer from exacerbations of ARDS, septic shock, or multiple organ failure, and require hospitalization in the intensive care unit (ICU) (De Flora et al. 2020). ARDS is the leading cause of death in COVID-19 patients, including cytokine storm and systemic inflammatory response caused by the release of many pro-inflammatory cytokines and chemokines that trigger the immune system to attack (Li et al. 2020). High levels of pro-inflammatory cytokines may cause extensive lung damage, leading to widespread infiltration of neutrophils and macrophages, impaired alveolar diffusion by forming hyaline membranes, and diffuse thickening of the alveolar wall

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(Cao 2020). Inflammation is treated with nonsteroidal anti-inflammatory drugs (NSAIDs) or steroidal anti-inflammatory-immunity drugs (SAIDs). NSAIDs cause serious gastrointestinal injury and hepatotoxicity. SAIDs may result in sodium retention, obesity, and osteoporosis; these result in serious health problems (Ou et al. 2019). Besides, drugs and methods currently used to treat acute lung injury have not responded to patients fully. Despite decades of efforts to treat acute lung injury, the mortality rate of patients with an acute injury is still higher than 50% (Ye and Liu 2020). N-acetylcysteine (NAC), is a chemical compound that controls and reduces inflammatory mediators (Bhatia and Moolhala 2004). NAC can directly scavenge oxygen free radicals and has immune regulatory effects such as inhibiting tumor necrosis factor- α (TNF- α) overproduction, increasing T cell activity, and improving the oxidation state, especially under severe oxidative stress (Qiao et al. 2019; Shen et al. 2016). *Elaeagnus Angustifolia* (EA) is one of the herbal medicines traditionally used to treat rheumatoid arthritis, osteoporosis, and asthma due in part to its therapeutic effects, such as antioxidant, anti-inflammatory, and antimicrobial, activities (Tehranzadeh et al. 2016). Phytochemical studies of EA fruit extract have identified various chemicals as flavonoids, phytosterols, polar glycosides, terpenoids, coumarins, phenols, carboxylic acids, saponins, and carotenoids (Okmen and Turkcan 2014). To the best of our knowledge, there is no study on its effect on pulmonary inflammation. Carrageenan is a potent inflammatory agent with neutrophil induction capacity and the production of reactive oxygen species (ROS). Induction of inflammation by carrageenan elevates the levels of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Alam et al. 2019, de Campos Facchin et al. 2020). Accordingly, the present study aimed to elucidate the anti-inflammatory effect of NAC and EA on Carrageenan-induced acute lung injury in mice.

Materials and methods

Materials

λ -Carrageenan (EC Number 232–953-5) was purchased from (Sigma Co. Steinheim, Germany.) N-acetylcysteine (NAC) tablets from (HEXAL Co. Holzkirchen, Germany) were used.

Preparation of aqueous extract of EA fruit

To prepare the aqueous extract of *Elaeagnus angustifolia* fruit (herbarium code E-1180 FUMH), 5 g of EA powder was mixed with 100 ml of distilled water and boiled at 95 °C for 15 min. The mixture was centrifuged at 2000 rpm for 3 min. Finally, the extract was filtered using Whatman filter

paper, stored at –30 °C for 24 h, and then dried using the freeze-drying technique.

Experimental animals

42 male Wistar rats (200–250 g) were enrolled in this research. All rats were kept under standard conditions, 22 \pm 2 °C, 12 h of illumination, and 12 h of darkness. During the experiment, rats had free access to water and food. All experiments with laboratory animals were performed according to the instructions of the ethics committee of Birjand University of Medical Sciences (Birjand, Iran). The experiments were reviewed and approved, with ethics code IR.BUMS.REC.1399.474.

Animal groups

Animals were randomly assigned into 7 groups of Control (saline), Carrageenan (intratracheal instillation of 0.1 ml, 2%), Carrageenan + EA (100 mg/kg, gavage), Carrageenan + EA (400 mg/kg, gavage), Carrageenan + NAC (50 mg/kg, gavage), Carrageenan + NAC (200 mg/kg, gavage) and Carrageenan + EA + NAC (400 mg/kg, gavage).

Carrageenan-induced acute lung injury

Rats were treated with EA extract and NAC for 21 days. On day 22, mice were anesthetized with ketamine [100 mg/kg, intramuscular (i.m)] and xylazine (10 mg/kg, i.m). ALI was induced by 0.1 ml intratracheal injection of 2% carrageenan in saline. Rats were anesthetized 24 h post-Carrageenan administration and bronchoalveolar lavage (BAL) fluid, blood, and lung tissues were harvested.

Bronchoalveolar lavage fluid (BALF) analysis

BAL was collected from anesthetized mice through a 20-gage angiocath as previously described (Gurusamy et al. 2021). Briefly, 4 ml of sterile PBS was instilled into the rat lung and lavaged three times. Cell numbers were standardized/ml BAL recovered. BAL recovery volume range was 3.4–3.7 ml. The cell-free supernatant was stored at -80 to determine the level of cytokines and total protein content. BAL cell counts were determined using a standard hemocytometer. Differential cell counts were subsequently performed on Giemsa-wright stained (Microscopy Hemacolor-Merck; Germany) cytospin preparations. Cell numbers were standardized/ml of BAL collected and results were expressed as number/ml \times total volume.

Measurement of wet to dry ratio of the lungs

The lower lobes of the right lung were harvested in all animals to determine the edema index (wet/dry ratio) as previously described (Gurusamy et al. 2021). In brief, The wet weight of the right lower lungs was measured at the time scarification. The dry weight was determined after lungs were incubated for 48 h at 80 °C in an oven. Finally, the W/D ratio was calculated.

Lung pathology

One part of the upper left lung was fixed in 10% formalin. Formalin-fixed paraffin-embedded tissue was sectioned (5 µm thick), hematoxylin- and eosin-stained, and analyzed under a light microscope. Three sections per lung tissue were assessed for each rat, with ten areas per section analyzed. A trained pathologist who was blinded to experimental groups/treatments scored the degree of lung injury using a 5-point scoring system measuring edema, neutrophil infiltration, hemorrhage, and disorganization of lung parenchyma as previously described (Honari et al. 2021). Higher scores indicate more severe lung abnormalities: 0 = normal, 1 = light, 2 = moderate, 3 = severe, and 4 = very severe (Nasseri et al. 2015). Scores for each of the four categories were combined to provide a total lung injury score.

Measurements of inflammatory cytokines in BALF

50 µl of BALF supernatant was used to measure the total concentration of inflammatory mediators. Enzyme immunoassay commercially available kits for rat IL-6 and TNF-α (R & D Systems Inc., Minneapolis, MN, USA) were used to determine BAL fluid concentrations of these mediators according to the manufacturer's instruction.

Measurements of protein concentration in BALF

BALF supernatant was used to measure protein concentration. The protein of BALF was measured by a Bradford detection kit (Kiazist, Iran).

Serum levels of lipid peroxidation, total antioxidant capacity, and thiol

Lipid peroxidation, total antioxidant capacity (TAC), and the level of thiol were measured according to the company's instruction [ZANTOX (K.A.A), Birjand, Iran]. Lipid peroxidation was measured according to the thiobarbituric acid reactive substance (TBARs) method. Total antioxidant capacity was measured by a ferric reducing antioxidant power (FRAP) assay. Elman method was used to measure thiol groups.

Statistical analysis

The results were reported in terms of mean ± standard error. Statistical tests used included one-way ANOVA and Tukey test. The Significance limit was defined as ($P < 0.05$). All statistical analyses were performed using SPSS software version 16.

Results

EA and NAC reduced immune cell influx into the lungs of carrageenan-challenged rats

Intratracheal carrageenan injection resulted in a marked increase in lung permeability as evidenced by excessive total inflammatory cell infiltration and neutrophils in the carrageenan group, compared with the control animals ($P < 0.001$). Treatment with EA extract (100 mg/kg) and NAC (50 mg/kg) however, significantly decreased BALF' total cell influx (Fig. 1A). Neutrophil counts were significantly reduced in all treated groups compared with carrageenan animals ($P < 0.05$) (Fig. 1B). The number of total cellular infiltrates, and neutrophils in the BAL fluid was reduced by 58% and 27%, respectively, in rats treated with EA extract (100 mg/kg), 45% and 19% reduction in animals treated with NAC (50 mg/kg) compared with carrageenan group.

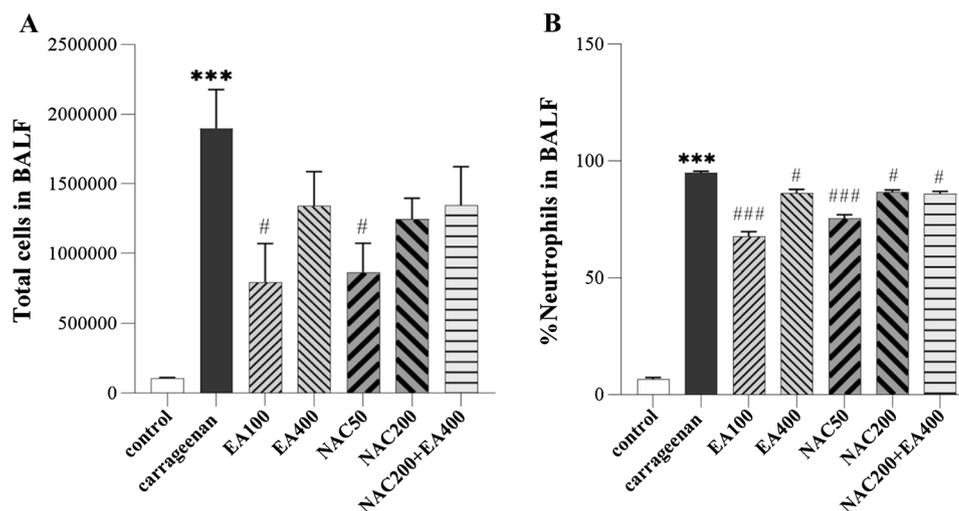
EA and NAC attenuated interstitial edema of carrageenan-challenged rats

Administration of Carrageenan into the trachea resulted in a marked increase in lung permeability as evidenced by an increase in lung's water content. The Carrageenan group indicated a significant increase in wet to dry ratio (W/D) compared to the control group. Meanwhile, EA extract (100 mg/kg) and NAC (50 mg/kg) treatments, profoundly reduced W/D ratio by 67% and 69% respectively ($P < 0.05$) (Fig. 2A).

EA and NAC attenuated lung's histological destruction induced by carrageenan

Intratracheal carrageenan injection-induced ALI characterized by destruction of lung architecture, increased cell infiltration, and hemorrhage compared with the control group (Fig. 2C). Destruction of lung architecture as evidenced by significantly increased lung histology scores (Fig. 2B). All carrageenan-induced pathological changes were attenuated in rats treated with EA extract (100 mg/

Fig. 1 The effects of NAC and *Elaeagnus angustifolia* on carrageenan-induced lung injury. Values are presented as mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to the control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared to the carrageenan-induced lung injury group. **A** Total cell count in BALF. **B** Neutrophils counts in BALF



kg) and NAC (50 mg/kg) by 47% and 57%, respectively ($P<0.05$).

EA and NAC ameliorated BAL's levels of inflammatory cytokines induced by carrageenan

To determine the impact of EA and NAC treatments on Carrageenan-induced ALI, the levels of TNF- α and IL-6 into the BAL fluid were measured. Post intratracheal carrageenan injection, Inflammatory cell influx into the airways was accompanied by elevated levels of both inflammatory mediators, TNF- α and IL-6, in comparison with the control rats ($P<0.001$) (Fig. 3A, B). EA extracts (100 and 400 mg/kg) and NAC (50 and 200 mg/kg) could significantly ameliorate TNF- α levels, while only with EA extract (100 mg/kg) and NAC (50 mg/kg) reduced IL-6 levels to a significant degree compared with carrageenan-treated animals.

EA and NAC reduced BALF total protein content induced by carrageenan

Total protein concentration in the carrageenan group significantly increased ($P<0.001$) compared to the control group. Treatment with EA extract (100 and 400 mg/kg) and NAC (50 and 200 mg/kg); however, profoundly attenuated the presence of total protein content in BAL compared to the carrageenan only rats. significant ($P<0.001$) showed a decrease in the protein concentration compared to the carrageenan group. (Fig. 3C).

EA and NAC reduced carrageenan-induced levels of serum lipid peroxidation, total antioxidant capacity, and thiol

Intratracheal administration of carrageenan significantly elevated the amount of lipid peroxidation and reduced

total antioxidant capacity and thiol contents in serum samples. The MDA test showed the rate of lipid peroxidation increased significantly in the carrageenan-treated group compared to the control animals ($P<0.05$) (Fig. 4A). EA (400 mg/kg), NAC (200 mg/kg) and EA (400 mg/kg)+NAC (200 mg/kg) administrations, however attenuated the amounts of lipid peroxidation. Furthermore, the FRAP levels (antioxidant capacity) in the carrageenan group were significantly reduced compared to the control rats (Fig. 4B). This situation was reversed via EA (400 mg/kg), NAC (200 mg/kg) and EA (400 mg/kg)+NAC (200 mg/kg) treatments ($P<0.05$). Finally, the thiol content of the carrageenan rats was profoundly decreased compared to the control group ($P<0.001$). Administration of NAC (200 mg/kg) and EA (400 mg/kg)+NAC (200 mg/kg) however, ameliorated thiol content of the serum ($P<0.05$) (Fig. 4C).

Discussion

Acute lung injury refers to activating the immune responses in the lungs. During lung inflammation, immune cells are activated, resulting in increased inflammatory cytokines such as IL-6 and TNF- α (Pedersen 2017; Ye et al. 2019). Our results in the present study demonstrated that carrageenan-mediated ALI induction can cause neutrophilic infiltration, hemorrhage, and increased lung tissue injury. In addition, carrageenan elevated the concentration of infiltrated total proteins, TNF- α and IL-6 in BALF. The underlying mechanisms for acute lung injury as a devastating disease and its more severe form, ARDS, are complex. Meanwhile, it is suggested that oxidative stress and systemic inflammatory responses may play an important role in the development of ALI/ARDS (Tasaka et al. 2008). Oxidative stress is induced by an imbalance of conditions between the production of oxygen free radicals and the body's

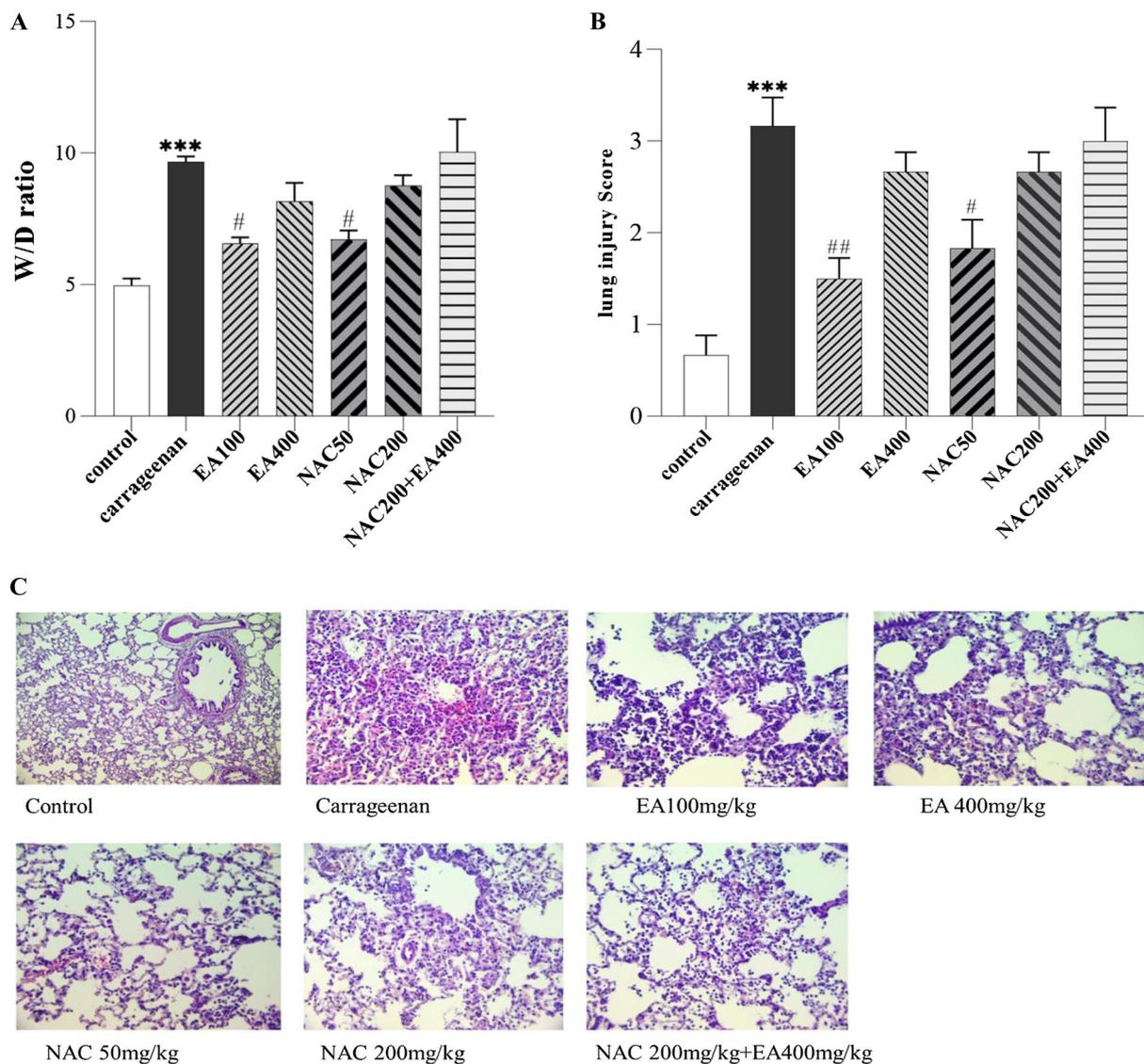


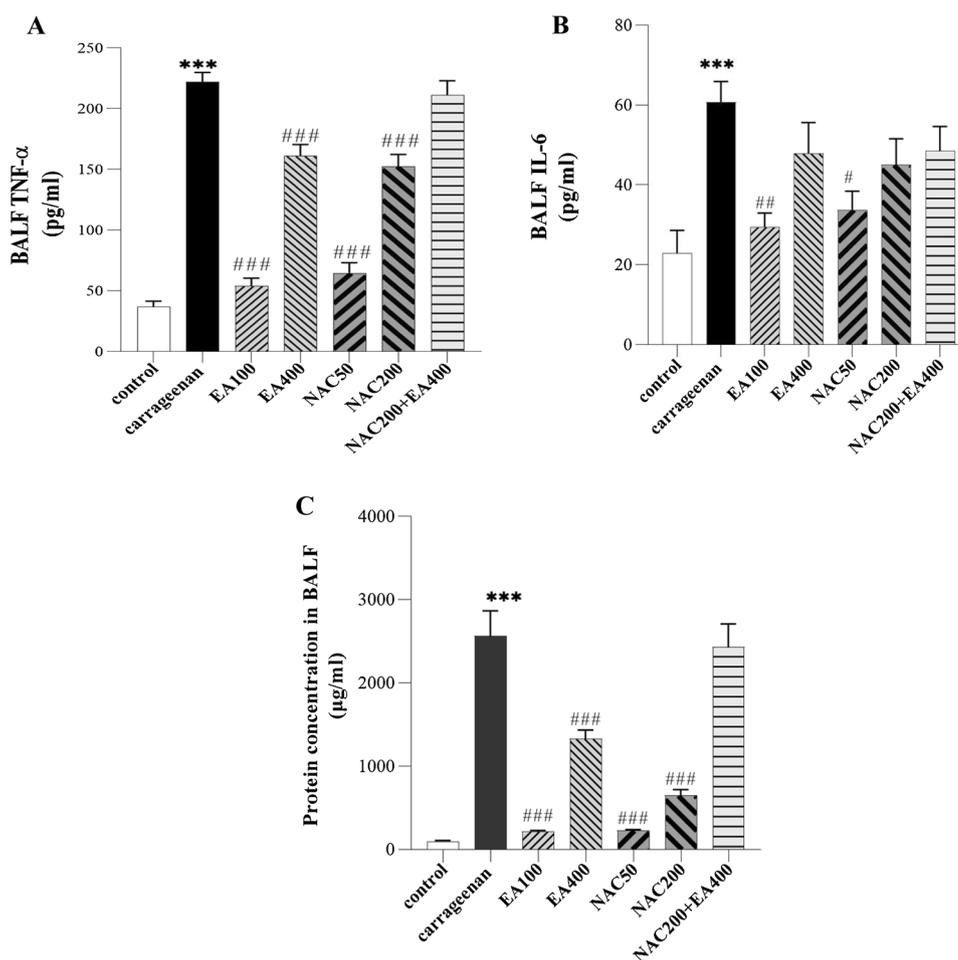
Fig. 2 The effects of NAC and *Elaeagnus angustifolia* on carrageenan-induced lung injury. Values are presented as mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to the control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared to the car-

rageenan-induced lung injury group. **A** lung wet/dry (W/D) weight ratio of rat in each group. **B** the comparison of lung injury scoring among the groups. **C** Pathological changes of lung tissues of rat detected by H&E staining

antioxidant capacity, which can subsequently activate inflammatory cells leading to serious disorder of ALI (Tasaka et al. 2008; Yang et al. 2019). Inflammation involves multiple cellular and molecular mechanisms in which various mediators participate effectively and appropriately (Medzhitov 2008; Adefegha et al. 2017). Inhibiting both inflammation and oxidative stress therefore considered as effective strategies in ameliorating the ALI/ARDS symptoms (Chen et al. 2017). Carrageenan-induced ALI is broadly used to analyze inflammatory responses (Ekuadzi et al. 2018). In this model, the development of oxidative stress and inflammation are jointly participate via neutrophil infiltration, overproduction of ROS, and generation of

inflammatory cytokines. (Yang et al. 2020). The present study confirmed that *Elaeagnus angustifolia* and *N*-Acetylcysteine decreased the levels of inflammatory mediators TNF- α and IL-6 in a significant manner. Assessment of the degree of lung damage also demonstrated that EA and NAC ameliorated detrimental effects of carrageenan in reducing lung damage, inflammatory cytokines, and preventing neutrophil infiltration. The pro-inflammatory cytokines TNF- α and IL-6 are mainly secreted by neutrophils, play a major role in initiating and progressing inflammatory responses initially via the overproduction of adhesion molecules (de Campos Facchin et al. 2020). Previous studies have shown that the migration of neutrophils from the blood into the

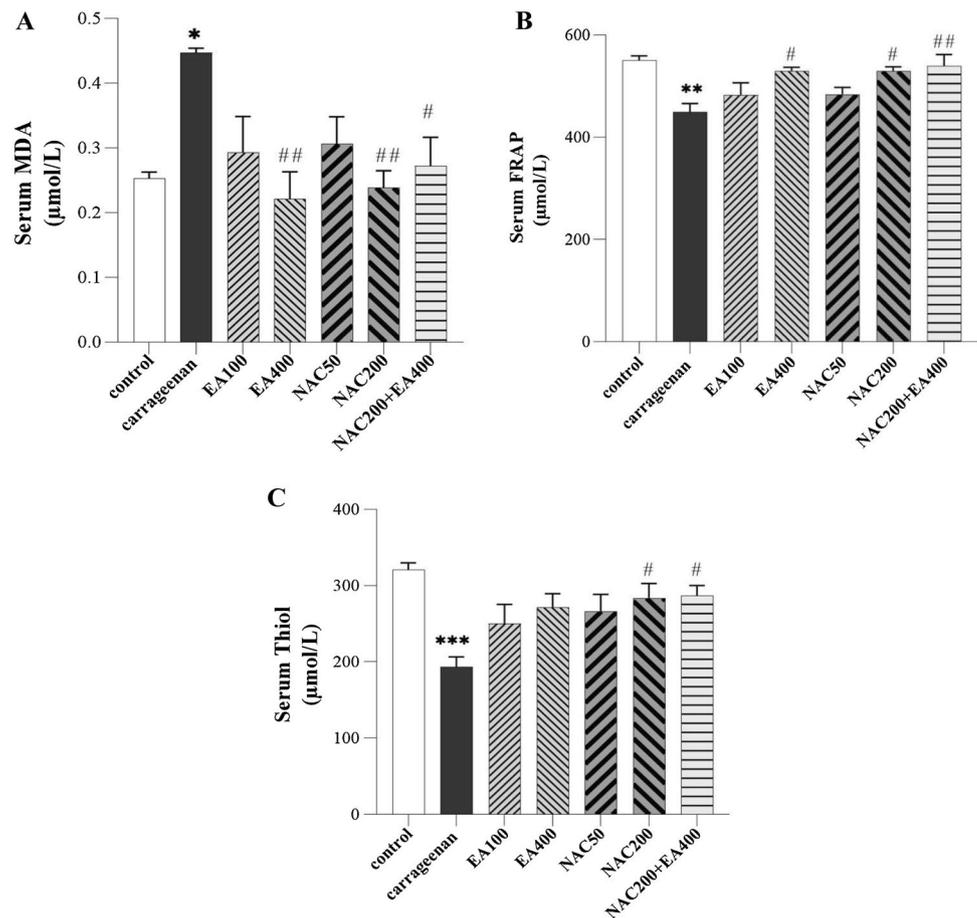
Fig. 3 The effects of NAC and *Elaeagnus angustifolia* on carrageenan-induced lung injury. Values are presented as mean \pm SEM ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the carrageenan-induced lung injury group. **A** Tumor necrosis factor alpha (TNF- α) in BALF. **B** Interleukin-6 (IL-6) levels in BALF. **C** Protein concentration in BALF



cavity and chest tissues plays an important role in producing pro-inflammatory adhesion molecules (ICAM-1 and VCAM-1) (Gao et al. 2020). The induction of pro-inflammatory cytokines such as TNF- α and IL-6 in ALI model is mainly mediated by the action of carrageenan and other high affinity ligands on toll-like receptor 4 (TLR4). As a pattern recognition receptor (PRR), TLR4 is responsible for the activation of various intracellular pathways, such as the NF- κ B and p38 MAPK (Bhattacharyya et al. 2008). Carrageenan have demonstrated to mediated NF- κ B activation via subsequent phosphorylation of the inhibitor of NF- κ B (I κ B) in the cytosol and rapidly degrades I κ B through the ubiquitin–proteasome pathway. The p65 phosphorylated subunit of NF- κ B in turn causes the overproduction of TNF- α , IL-1 β , IL-6, and other pro-inflammatory mediators (Cho et al. 2009; Pereira dos Santos Nascimento et al. 2016). It's reported that SARS-CoV-2 and other high affinity ligands for TLR4 might induce NF- κ B activation in a similar manner (Kircheis et al. 2020). Consistent with the present report, carrageenan-induced pleurisy represented similar inflammatory responses as increased levels of cytokines, induction of neutrophil migration, and increase in total protein content (Dos Santos

et al. 2021; Bezerra Rodrigues Dantas et al. 2020). In line with our study, NAC has been shown to inhibit the lipopolysaccharide-induced TNF- α and IL-6 levels in the inflammatory response in bone marrow mesenchymal stem cells (Wang et al. 2020). *N*-acetylcysteine can also prevent lung inflammation and neutrophil infiltration after short-term inhalation exposure to concentrated ambient particles (Rhoden et al. 2004). Besides the main anti-inflammatory therapeutic effects of NAC, this compound is well known as a mucolytic drug, anti-pulmonary fibrosis, and a hepatic protective agent (Schwalfenberg, 2021). Recently, NAC has been shown to be effective against neutrophilic airway inflammation in patients with cystic fibrosis, suppresses ubiquitination and degradation of I- κ B (NF- κ B inhibitor) and thereby blocking NF- κ B nuclear translocation and activation (Ryu et al. 2019) (Pajonk et al. 2002; Pei et al. 2018). Surprisingly, studies have demonstrated the anti-COVID-9 effects of NAC (Poe and Corn 2020; Assimakopoulos and Marangos 2020). It's shown that NAC may interfere with the binding of SARS-COV-2 to angiotensin-converting enzyme-2 (ACE2) receptor (Poe and Corn 2020; Andreou et al. 2020), suggestive as a promising candidate against

Fig. 4 The effects of NAC and *Elaeagnus angustifolia* on carrageenan-induced lung injury. Values are presented as mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to the control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ compared to the carrageenan-induced lung injury group. **A** lipid peroxidation (MDA) in serum **B** Total antioxidant capacity (TAC) in serum. **C** Thiol content in serum



COVID-19 to prevent the development of ARDS (Assimakopoulos and Marangos 2020, Cadejani 2020). Phytochemical studies have shown that EA fruit extract contains flavonoids, terpenoids, and other chemicals (Motevalian et al. 2017; Ahmadiani et al. 2000). Flavonoids and terpenoids have been reported for their anti-inflammatory and inhibitory effects on arachidonic acid metabolism and the activities of pro-inflammatory cytokines and adhesion molecules in vitro (del Carmen Recio et al. 1995) (Ali et al. 2011; Serafini et al. 2010). Besides, the anti-inflammatory activity of *Elaeagnus angustifolia* fruit extract has been proven on rat paw edema (Motevalian et al. 2017). In our study, serum antioxidant evaluations indicate that carrageenan increased MDA levels and decreased thiol groups and antioxidant capacity (FRAP) in acute lung injury while EA extracts and NAC could reversed aforementioned conditions. The carrageenan-induced inflammatory response is closely associated with decreased antioxidant enzyme activity, free radical production, and lipid peroxidation. Lipid peroxidation acts as a marker of cell damage and induces inflammatory processes (Lu et al. 2007). Similar to our data, a study conducted on carrageenan-induced pleurisy in mice, it was demonstrated that carrageenan increased MDA and decreased

GSH (Yang et al. 2020). GSH is the body's main antioxidant with diverse cellular functions. GSH is present in high concentrations in BALF fluid and protects the lungs against oxidative damage caused by internal or external stimuli. Reduction in the lung's environment is associated with an increased risk of lung injury and disease. One strategy to limit oxidative lung damage is to increase the intracellular glutathione content using its precursors, such as NAC, a compound containing thiol and glutathione groups (Galvão et al. 2011). Studies have shown that thiol-containing antioxidants can prevent the release of inflammatory mediators from epithelial cells, thus increasing intracellular GSH and reducing free radicals and NF- κ B activation (Caglikulekci et al. 2006). The NAC eradicates free radicals and reacts directly with its free thiol side chain with electrophilic groups such as hydroxyl radical (OH \cdot), Nitrogen dioxide (NO $_2$), and carbon dioxide ion (CO $_3$ $^{2-}$) with the eventual consequence of detoxification of ROS produced by leukocytes (Akca et al. 2005). The indirect antioxidant function of NAC relies on intracellular GSH replenishment by inserting cysteine into the GSH biosynthesis pathway (Aldini et al. 2018). A recent study conducted on acute kidney injury in septic rats has depicted, that NAC can reduce the level of

MDA and increase antioxidant enzymes followed by an increase in antioxidant capacity (Fan et al. 2020). Plant extracts generally exert their protective effect against oxidative stress damage through increased antioxidant, free radical scavenging and anti-lipid peroxidation capacities due in part to their active compounds of flavonoids, tannins, triterpenoids, and alkaloids (Amini et al. 2019). We have illustrated in the present report the therapeutic efficacy of NAC and EA substances against carrageenan-induced ALI phenotype in Wistar rats in reducing inflammation and oxidative stress from various cellular, immunological and biochemical aspects. It is important to highlight the notion that the generation of different species of ROS is strongly involved in ALI progression and deterioration and herbal extracts as well as NAC are capable of diminishing their detrimental effects (Lu et al. 2019). However, our study suffered from some limitations: Our lack of knowledge regarding precise molecular signaling of EA extract and NAC and their effectiveness in carrageenan-induced ALI was the most important one. On that account, we didn't evaluate a wide spectrum of involved molecular mechanisms and focused on the main preliminary objectives of the project. Further evaluations are necessary to clarify the efficacy of these two therapeutic substances in ALI model.

Conclusions

The results of this project clearly demonstrated a significant reduction in infiltration of inflammatory cells within the lung, reduction of pulmonary edema, reversibility of structural changes in lung tissue, effective reduction of inflammatory cytokines and finally a significant reduction of oxidative stress. Accordingly, EA extract and NAC can be considered promising treatment modalities against ALI/ARDS phenotype.

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Author contributions MM: investigation, methodology, data curation, formal analysis, software, writing—original draft. SN: conceptualization, investigation, methodology, formal analysis, writing—review and editing. YM: investigation, methodology, data curation, formal analysis, software. SA: formal analysis, histopathology. AZ: conceptualization, methodology, formal analysis, software, writing—review and editing, funding acquisition.

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Data availability statements The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest and are fully responsible for data collection, originality, interpretation, and writing of this manuscript.

Informed consent Not applicable.

Ethical approval All procedures involving animals were in accordance with the national guides in care and use of Laboratory Animals in Scientific Affairs provided by the Iranian Ministry of Health and Medical Education (2019). The guideline is following 1964 Helsinki declaration and its later amendments or comparable ethical standards. Moreover, the animal experiments were approved by the Birjand University of Medical Sciences Ethics Committee (permit code: IR.BUMS.REC.1399.474).

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