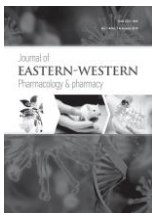

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THE PROTECTIVE EFFECTS OF SEABUCKTHORN (*HIPPOPHAE RHAMNOIDES* L.) LEAVES EXTRACT ON PM2.5-INDUCED ACUTE LUNG INJURY

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KEYWORDS

Acute lung injury; Particulate matter; Inflammation; Oxidative stress; *Hippophae rhamnoides* L.

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ABSTRACT

Background: Hippophae rhamnoides L. (also known as Seabuckthorn SLE), a unique and valuable plant, has recently received worldwide attention. Due to its nutritional and medicinal properties, it has now been domesticated in several regions of the world from the Atlantic coast of Europe to Central Asia, Siberia, northwestern China, and northwestern Mongolia.16 The plant has been extensively used in Indian, Chinese, Tibetan, and Mongolian folk remedies for gastric ulcers, cough, circulatory disorders, skin diseases, asthma, cardiovascular diseases, lung disorders, and liver damage.[17,18]

Materials and methods: Then, the mice model of ALI was induced by PM via intra-tracheally instilled with 50 mg/kg body weight of Standard Reference Material1648a (SRM1648a), and SLE (12.5, 25, 50 mg/kg) were administered orally 1 h prior to PM. The efficacy and molecular mechanisms in the presence or absence of SLE were elucidated.

Results: Eleven main ingredients were detected in SLE and the contents of homoorientin and berberine were quantified. Additionally, the results demonstrated that SLE profoundly inhibited weight loss in mice and ameliorated

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lung pathological injury induced by PM. Furthermore, we also found that SLE. significantly decreased the lung wet-to-dry weight (W/D) ratios, reduced total protein in bronchoalveolar lavage fluid (BALF), and effectively attenuated PM-induced increased leukocyte and macrophages in BALF. Meanwhile, SLE. could pronouncedly inhibit myeloperoxidase (MPO) activity in lung tissues, decreased the PM-induced inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), reduced nitric oxide (NO) and increased superoxide dismutase (SOD) in BALF.

Conclusion: These findings indicated that SLE attenuated PM-induced ALI by suppressing the release of inflammatory cytokines.

1. INTRODUCTION

In recent years, air pollution has become an increasingly common problem around the globe due to human malpractice and various natural disasters, leading to many diseases, such as bronchial asthma, chronic obstructive pulmonary disease (COPD), and pneumonia.[1] The severity of air pollution is evaluated by measuring particulate matter (PM) in the air.[2] PM is composed of fine particles and coarse particles: the aerodynamic diameter of fine particles ≤ 2.5 μm is called PM2.5, the aerodynamic diameter of coarse particles ≤ 10 μm is called PM10.[3]

PM2.5 contains many toxic chemicals including polycyclic aromatic hydrocarbons, transition metals, and endotoxins.[4] Previously, it has been established that inhalation of PM2.5 increases the risk of several respiratory diseases, including allergic respiratory infections, asthma, COPD, and acute lung injury (ALI).[5] ALI or its more severe form, acute respiratory distress syndrome (ARDS), is a severe clinical disease that is associated with high morbidity and mortality. It is usually caused by direct or indirect destruction of alveolar epithelial cells and capillary endothelial cells and is characterized by apoptosis, autophagy, oxidative stress, inflammatory reactions, pulmonary edema, and excessive production of inflammatory cytokines.[6]

NF-κB, an important transcription factor, plays a major role in the regulation of inflammation. The activation of NF-κB is essential for several gene expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). [7]

These pro-inflammatory cytokines are key mediators of lung tissue injury. The secretion of such cytokines leads to the stimulation of peripheral polymorphonuclear neutrophils (PMNs), whose excessive activation further exacerbates the inflammatory response and induces oxidative stress.[8] Oxidative stress causes excessive production of reactive oxygen species (ROS), myeloperoxidase (MPO) and malondialdehyde (MDA) formation, and reduces expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH), which are playing an important role in the protection against oxidative stress-induced lung damage.[9]

In the literature, it has been reported that NLRP3, a multiprotein complex composed of the Nod-like receptor (NLR) family, plays a key part in ALI. The NLRP3 inflammasomes are composed of three sections NLRP3, ASC, and caspase-1, and its activation results in the maturation and secretion of IL-1β, which is an important factor in the ALI development.[10] Cellular stress caused by the imbalance between antioxidants and oxidants and the imbalance of anti-inflammatory and pro-inflammatory cytokines play a key role in the progression and exacerbation of ALI.[11]

Hippophae rhamnoides L. (also known as Seabuckthorn), a unique and valuable plant, has recently received worldwide attention. Due to its nutritional and medicinal properties, it has now been domesticated in several regions of the world from the Atlantic coast of Europe to Central Asia, Siberia, northwestern China, and northwestern Mongolia.[12] The plant has been extensively used in Indian, Chinese, Tibetan, and Mongolian folk remedies for gastric ulcer, cough, circulatory disorders, skin diseases, asthma, cardiovascular diseases, lung disorders, and liver damage.[13,14] In recent years, research has been started

to understand and support the traditional use of SLE. A wide range of pharmacological properties of SLE such as antioxidant, mmunomodulatory, anti-inflammatory, hepatoprotective, anti-stress, radioprotective, tissue repair, and anti-atherogenic have been recently described in various literature.[15,16]

However, less is known about the signaling pathways that mediate the protective activities of SLE. Therefore, the objective of the present study was to investigate the antioxidant, anti-inflammatory, and mechanistic pathways that regulate the cytoprotective effects of SLE.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

SRM 1648a (PM), an urban PM, that was collected in St. Louis. Dexamethasone (DEX) (D1756) was purchased from Sigma-Aldrich St. (Louis, MO, USA). The SOD, MDA, and MPO kits were obtained from the Jianceng Bioengineering Institute (Nanjing, Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits for NO, TNF-α, IL-6, and IL-1β were purchased from ExCell Biotech (Taicang) Co., Ltd (Nanjing, China).

2.2. Plant material and preparation of extract

The fresh leaves of Seabuckthorn (*Hippophae rhamnoides* L.) were collected in August 2019 from Us Erdene Company Co Ltd., located in Uvs aimag in northwestern Mongolia. The leaves were washed thoroughly, sliced into smaller pieces and dried at room temperature under shade. Next, the leaves were powdered finely and subjected to extraction. The extraction of Seabuckthorn leaves was carried out by Maceration method, as described in previous report with some modification.[17] The powdered leaves were soaked with 30% ethanol (1:5 w/v) at room temperature (25±1°C) for 24 h and filtered through a muslin cloth. The supernatants were collected and the residues were re-immersed in the solvent. Those steps were repeated three times. After repetitions, all collected supernatants were evaporated using an evaporator at 40 °C until reaching the certain volume. The concentrated SLE were stored in airtight dark bottles at -20 °C.

2.3. Animals and experimental design

C57BL/6 male mice (18-22 g) were purchased from the Experimental Animal Center of Yangzhou University (Yangzhou, Jiangsu, China, certificate NO SCXK 2017-0007). After 3 days of adjustable feeding and acclimatization, the C57BL/6 mice were randomly allocated into six groups (n = 6, each). Mice were intratracheally administrated with PM (50 mg/kg, per mouse) and then orally administrated normal saline, SLE (12.5, 25 and 50 mg/kg) and DEX (10 mg/kg) 4 h later. After 72 h, all animals were sacrificed. Then lung tissue samples, serum and BALF were collected and used for hematoxylin and eosin (H&E) staining, western blot assay, immunofluorescence, ELISA test, SOD, MDA, NO and MPO quantification. All studies were performed according to the International Guidelines for Biomedical Research involving animals, published by the Council of International Organizations of Medical Sciences and Use of Laboratory Animals, and the protocols used were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.4. Histopathological analysis

Lung tissues fixed in 4% paraformaldehyde were embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin (H&E). Pathological images were examined using an optical microscope (Olympus, Japan).

2.5. Cells counting and protein concentration assay in BALF

After 72 h of PM-induced, mice were sacrificed and BALF was obtained by washing three times with 0.5 mL of cold sterile phosphate-buffered saline (PBS) through a tracheal cannula. BALF samples were then centrifuged at 3,000 rpm for 10 min at 4°C, the supernatants were used for the assessment of total protein concentration by BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Nanjing, Jiangsu), China. While the sedimented cells were resuspended in 50 μL PBS to obtain the total counts of cells and lymphocytes with a Hematology Analyzer (Siemens, Germany).

2.6. Analysis of vascular permeability in premetastatic lungs through Evans blue assay

Seventy-two hours after modeling, Evans blue dye (EBD) (50 mg/kg in 100 μL of normal saline) was injected into the mice tail vein. The mice were sacrificed after 2 h of administration, and the lungs were removed. The EBD was extracted from the lungs with 1 mL of formamide overnight at 55℃ and measured spectrophotometrically at 620 nm. The amount of EBD in each sample was calculated according to a standard curve generated from known amounts of EBD and expressed as μg of dye/g of lung tissue.

2.7. Measurement of MPO, NO, MDA, and SOD levels

The collected BALF and serum were used to measure the levels of MDA, SOD, MPO, and NO contents by using commercially available assay kits following the respective manufacturer's instructions. The enzymatic activity was detected at 460 nm using a microplate spectrophotometer (Tecan, Switzerland).

2.8. Enzyme-linked immunosorbent assay

To quantify the expression levels of TNF-α, IL-6, and IL-1β in BALF, ELISA kits were used according to the manufacturer's instructions respectively. All measurements were performed in duplicate and the optical density of each well was measured at 450 nm using a microplate spectrophotometer (Tecan, Switzerland).

2.9. Statistical analysis

GraphPad Prism (version 5.0) was used for statistical analysis. Data was expressed as the mean±standard deviation (SD). Comparisons among different groups were performed by the One-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. SLE treatment reduces inflammatory responses and alleviates PM-induced ALI in mice

To assess the protective effects of SLE on PM-induced ALI in mice, lung histopathological changes were measured. As shown in Fig. 1A, the lung tissues of PM-induced mice showed significant lung damage including lung edema, thickening of alveolar wall, and inflammatory cell infiltration compared to the control group. Conversely, the treatment of mice with incremental doses of SLE or DEX prevented ALI induced by the PM pretreatments in a dose-dependent manner compared to the PM model group. In addition, different pathological scores indicated that mice treated with PM had a high lung inflammatory score compared to the control group (Fig. 1B). However, less lung inflammatory score was found in the SLE 50 mg/kg group among other two groups (12.5 and 25 mg/kg) or DEX (10 mg/kg). These results were in accordance with the H&E staining. Next, we measured the PM-induced inflammatory cell infiltration and total protein levels in mice BALF. As presented in Fig. 1C, 1D and 1E, there was a significant increase in the total number of cells, macrophages, and the total protein content in mice BALF treated with PM only as compared to the control group. However, the administration of SLE or DEX (10 mg/kg) dramatically decreased the total number of cells in BALF. Figure 1F showed that the vascular permeability of mice in the SLE groups with different doses and the positive drug group (DEX) decreased in a dose-dependent manner. Importantly, SLE treatment significantly reduced total cells, macrophages count, and the total protein amount in BALF in a dose-dependent manner. These findings suggest that the SLE treatment effectively diminishes inflammatory responses and alleviates PM-induced ALI in mice.

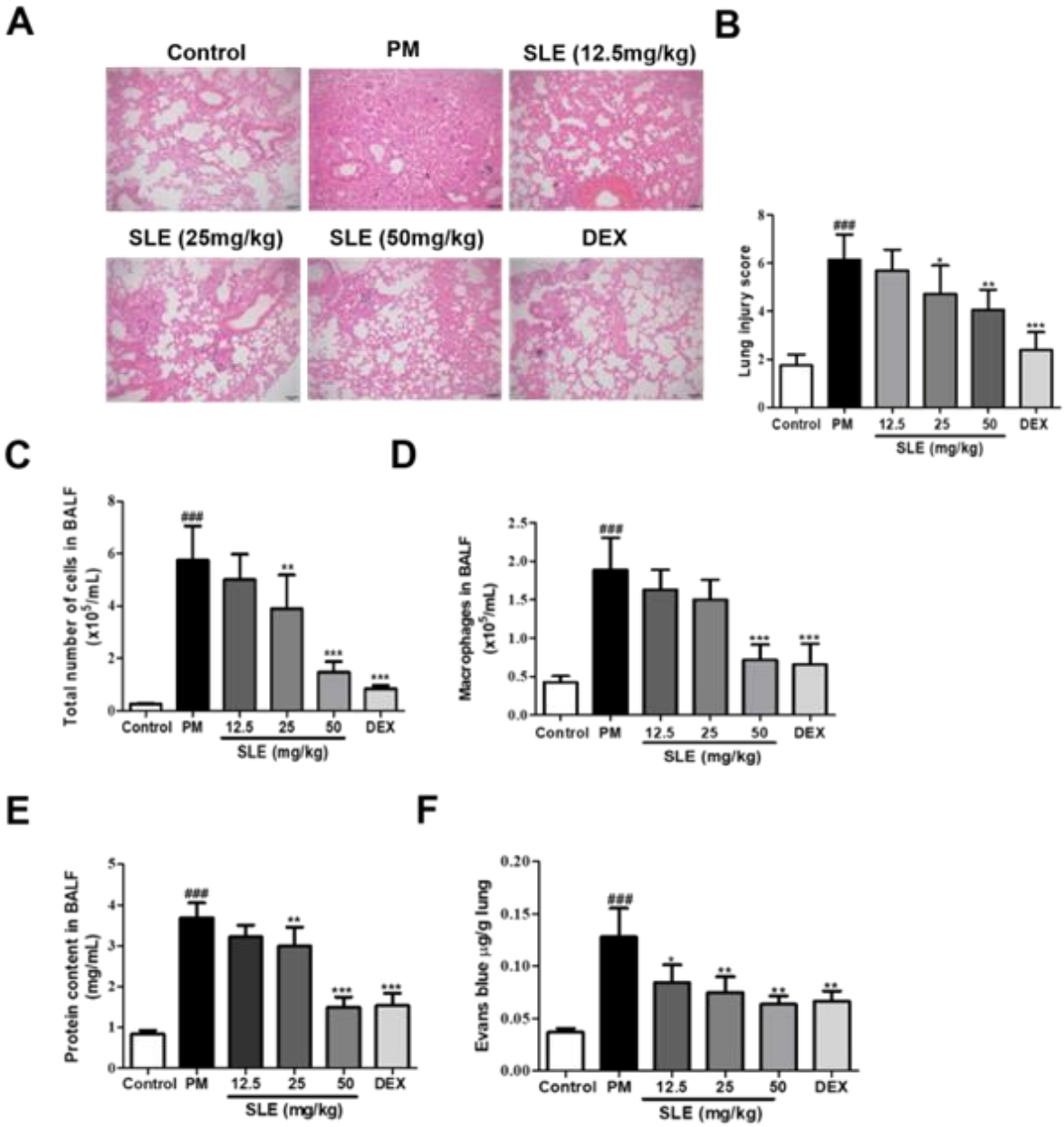

Figure 1. Effects of the SLE treatment on inflammation in PM-induced ALI in mice. (A) H&E staining of lung tissue in the different groups (H&E staining, magnificationЧ200, Scale bar = 50 μm). (B) Pathological scores of ALI. (C) Total number of cells in BALF. (D) Macrophages in BALF. (E) Total protein content in BALF. (F) Quantification of EBD levels in lungs. Data was presented as the means ± SD (n=4). ##P < 0.01 compared with the control group; **P < 0.01 compared with the PM model group; *P < 0.05 compared with the PM model group.

3.2. SLE treatment impedes PM-induced oxidative stress in mice

It has been shown that overproduction of oxidants within the lung leads to ALI [14], therefore, we proposed that SLE may perform its protective role in PM-induced ALI by inhibiting ROS generation. For this purpose, we measured the levels of oxidative stress marker, MDA, and the antioxidant enzyme, SOD, in the different groups of animals. As shown in Fig. 2A and 2B, increased levels of MDA while decreased levels of SOD were observed in the BALF of the PM model group in comparison to the control group. However, SLE administration or DEX (10 mg/kg) treatment significantly upregulated SOD activity, while downregulated MDA levels in a dose-dependent fashion, indicating that SLE might provide a significant protection against PM-induced oxidative damage.

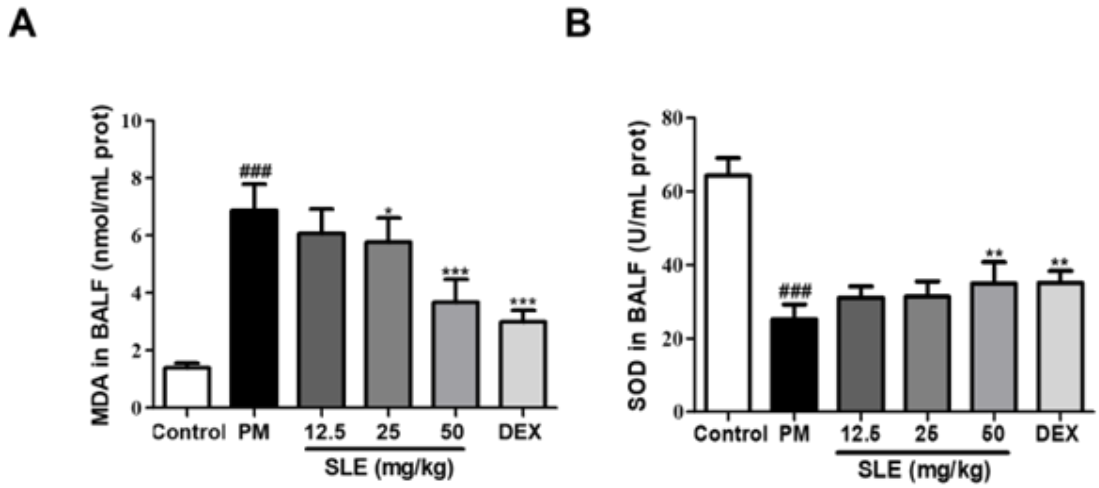

Figure 2. Effects of the SLE treatment inhibits oxidative stress in PM-induced ALI in mice. Mice were intratracheally administrated with PM (50 mg/kg, per mice) and then orally administrated normal saline, SLE (12.5, 25, and 50 mg/ kg) and DEX (10 mg/kg) 4 h later. After 72 h, all animals were sacrificed. (A) Levels of MDA in BALF. (B) Levels of SOD in BALF. All data were presented as the means ± SD (n=6). ##P < 0.01 vs. control group; **P < 0.01 vs. PM model group; *P < 0.05 vs. PM model group. EBD levels in lungs. Data was presented as the means ± SD (n=4).
**P < 0.01 compared with the control group; **P < 0.01 compared with the PM model group; *P < 0.05 compared with the PM model group.

3.3. SLE treatment reduces PM-induced lung inflammation in mice

NO, and pro-inflammatory cytokines production, such as TNF-α, IL-1β, IL-6, and COX-2 are important indicators that reflect inflammatory processes, and increased MPO activity in the lung indicates neutrophil tissue infiltration. As shown in Fig. 3A, the MPO activity in BALF in mice exposed to PM increased in comparison with the control group. Similarly, the administration of PM also resulted in the increased concentrations of NO, TNF-α, IL-1β, IL-6, and COX-2 in the plasma, lung tissue and BALF (Fig. 3B-F). In contrast, co-administration of SLE or treatment of mice with DEX caused a reduction in the levels of MPO, NO, TNF-α, IL-1β, IL-6, and COX-2, especially at the high dose 50 mg/kg of SLE compared to the PM model group. These data suggest that the treatment of mice with SLE can effectively ameliorate PM-induced lung inflammation in a dose trend manner.

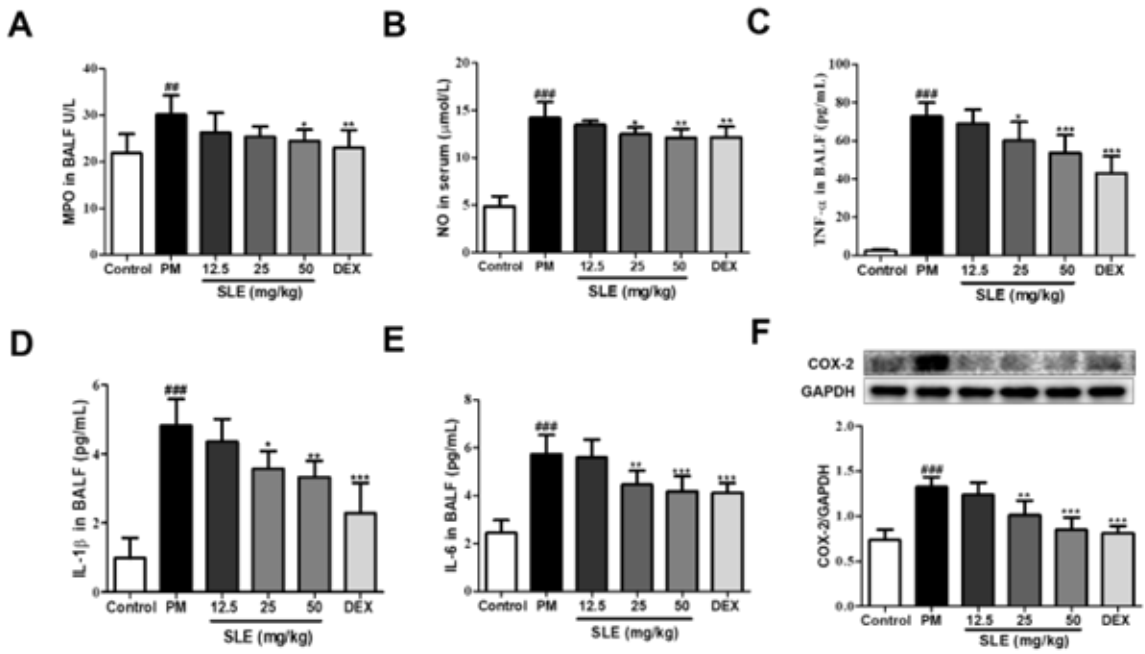

Figure 3. Effects of the SLE treatment on PM-induced lung inflammation in mice. Mice were intratracheally administrated with PM (50 mg/kg, per mice) and then orally administrated normal saline, SLE (12.5, 25 and 50 mg/ kg) and DEX (10 mg/kg) 4 h later. After 72 h, all animals were sacrificed. (A) Concentration of MPO in BALF. (B) Concentration of NO in plasma. (C) Concentration of TNF-α in BALF. (D) Concentration of IL-1β in BALF. (E) Concentration of IL-6 in BALF. (F) Western blot analysis for COX-2 inflammatory protein. All data were presented as the means ± SD (n=6). ##P < 0.01 against the control group; **P < 0.01 against the PM model group; *P < 0.05 against the PM model group.

4. DISCUSSION

In the past few years, due to various natural disasters and human activities, the amount of PM and other air pollutants has increased dramatically. PM exposures are always associated with excessive inflammatory responses and overt oxidative stress.[23,24] Accumulating evidence shows that PM is capable of absorbing many cytotoxic pollutants such as heavy metals, volatile organic compounds (VOCs), biological components, polycyclic aromatic hydrocarbons (PAHs).[18,19] These pollutants may deposit in the alveolar and activate cells in the airway (epithelium cells, macrophages, neutrophils, etc.) causing overt neutrophil infiltration, edema, increase capillary permeability, and overt production of inflammatory cytokines leading to ALI.[20,21]

Seabuckthorn (*Hippophae rhamnoides* L.) belonging to family of Eleagnaceae is a wonderful plant that contains many antioxidants and nourishment.[22] All parts of this plant (seeds, leaves, pulp, bark and fruits) are considered to be a good source of a large number of biologically active substances, such as carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, and minerals that more contributes to its widespread use as a natural antioxidant.[23] Seabuckthorn is widely used in traditional medicine, especially India, China and Mongolia. Its medicinal and nutritional value has been proven in pharmacology, phytochemicals, and various scientific studies. Previous studies have demonstrated that SLE has protective effects against various cancers, cardiovascular, skin, liver, and gastrointestinal disorders, anti-inflammatory activity, as well as antioxidant properties against ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and DPPH (2,2-diphenyl-1-picryl-

hydrazyl) free radicals, and HepG2 human liver cancer cells in vitro. [21-25]

The increase in total protein and pulmonary albumin levels can explain the increased pulmonary edema and pulmonary permeability of PM-perfused lungs.[26] In our study, the treatment of mice induced by PM with incremental doses of SLE significantly reduced PM-induced pulmonary edema, inflammatory cell infiltration, reduced macrophage count, total protein amount and pulmonary albumin level. These findings demonstrated that SLE protected against the ALI induced by PM in mice.

PM has the ability to induce oxidative stress by excessive production of intracellular ROS that in turn causes excessive release of inflammatory cytokines and DNA damage, leading to lung injury.[27] PM exposures also cause lipid peroxidation and inflammation of the cell membrane.[28] Lipid peroxidation is one of the main mechanisms of ROS-induced cell damage. As one of the lipid peroxidation products, MDA may reflect the degree of lipid peroxidation and the intensity of cells attacked by free radicals.[29] Besides, exposure to PM can also promote oxidative stress by decreasing the expression of antioxidant enzymes such as SOD.[30] In the present study, PM administrations significantly increased MDA levels while considerably decreased SOD levels indicative of oxidative stress-induced ALI. In contrast, co-administration of SLE evidently reduced PM-induced oxidative stress and the reduction in MDA level, increase of SOD content levels suggested that SLE may act as a kind of antioxidant in mice lung.

It is well documented that PM-induced macrophages activation and neutrophils increase in the lung, is associated with excessive inflammatory mediator's secretion, chemokines release and ROS generation.[31,32] Long-term inflammatory mediators, including MPO, NO, TNF-α, IL-6, IL-1β, and COX-2, are closely related to the development of acute and chronic inflammatory diseases.[33] An increased amount of these inflammatory cytokines were also detected in patients with lung injury.[34] The production of these inflammatory mediators at the injury site is one of the characteristics of ALI. In our experiments, PM administrations significantly elevated TNF-α, IL-1β, and IL-6 secretion as well as MPO, NO and COX-2 expression indicative of PM-induced ALI damage *in vivo*. However, the treatment of ALI mice with incremental doses of SLE effectively reduced secretion of TNF-α, IL-1β, and IL-6 and suppressed MPO and NO level.

5. CONCLUSION

In summary, the indicators of PM2.5-induced ALI mice given SLE showed that SLE significantly improved the lung tissue damage, inflammatory injury, and oxidative stress imbalance in PM2.5-induced ALI mice, and had a significant effect on lung wet-dry weight ratio, total protein content in BALF, inhibition of a total number of inflammatory cell. Further studies on the regulation of apoptosis, autophagy, and signaling pathway needs to be done.

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