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The effect of *Holothuria atra* body wall extract on pulmonary fibrosis and oxidative stress caused by bleomycin in Wistar rat

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ABSTRACT

Background & Aim: Idiopathic pulmonary fibrosis (IPF) leads to the misplaced deposition of the extracellular matrix and the stiffness parenchyma and the creation of scarring (fibrosis) in the lung. This study was conducted with the aim of investigating the effectiveness of different doses of *H.atra* body wall extract on bleomycin-induced pulmonary fibrosis.

Experimental: The animals were randomly divided into six groups (n = 6). Group 1 (Control) was administered normal saline intratracheally (single dose) on day 1, Group 2 (bleomycin) received single dose of bleomycin (BLM, 7.5 IU/kg) intratracheally and daily doses of normal saline by gavage, Group 3 (pirfenidone) received single dose of BLM (7.5 IU/kg) intratracheally and daily doses of pirfenidone by gavage, Groups (4, 5 and 6) received single dose of BLM (7.5 IU/kg) intratracheally and received daily doses of *H. atra* extract (50, 100 and 200 mg/kg, respectively). Finally, the rats were euthanized, and the lung tissues were taken out for histological and biochemical assessments.

Results: Histological tests showed that bleomycin could induce marked pulmonary fibrosis within two weeks. Administration of hydroalcoholic extract of *H. atra* body wall reduced these damages in lung tissue in a dose-dependent manner. The best results were obtained for 200 mg/kg per day of *H. atra* body wall extract.

Recommended applications/industries: Based on the present findings, *H. atra* body wall extract can reduce the toxic effects of bleomycin on lung tissues. Such effects can be attributed to the compounds with anti-inflammatory and antioxidant properties in this plant.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a spontaneous interstitial lung disease of unknown etiology that often results in progressive loss of lung function through thickening and scarring of the lung tissue. The precise etiology of IPF is still unclear despite considerable research, hence the term 'idiopathic'. The typical survival time following diagnosis for this illness is 2 to 5 years, and it is linked to severe morbidity and death (Desai, 2024). This disease begins with a progressive damage to the alveolar epithelial cells, which causes the release of inflammatory mediators and free radicals, and ultimately leads to lung tissue damage and respiratory failure (Kim, 2015). Among the risk factors of this disease are environmental factors: (smoking, dust, metals), genetic factors: Mutation of SP-C, ELMOD2, ABCA3 genes, apoptosis egulatory genes

such as BCL-2 and BAX may participate in epithelial cell apoptosis in pulmonary fibrosis (Safaeian, 2009) and aging as well as pathogenic mechanisms such as oxidative stress were mentioned (Glass, 2022). Pulmonary fibrosis treatment aims to reduce inflammation and the process of fibrosis. The use of corticosteroids and immunosuppressants are common treatments. Prednisolone, pirfenidone, nintedanib, and azathioprine can be mentioned among the drugs used in the treatment of pulmonary fibrosis. The guidelines suggest a regimen of two drugs consisting of prednisolone and azathiopurine or cyclophosphamide for IPF patients (Fernandez, 2012). Side effects such as infection, diabetes, osteoporosis, myopathy, bone marrow suppression, hepatitis, nephrotoxicity, and drug-induced pneumonia can occur, and patients with a history of tuberculosis, hepatitis, kidney failure, and other active infections will need early treatment (Tolle, 2018). Pirfenidone is an oral biological derivative of pyridone that exhibits both anti-inflammatory and anti-fibrotic properties. It has been shown to ameliorate bleomycin-induced lung injury in rats. Pirfenidone suppresses TGF-B-induced myofibroblast differentiation and fibrogenic activity of human lung fibroblasts. But its side effects, such as digestive problems, increase in liver enzymes, as well as high treatment costs, may limit its use (Peikert, 2008; Yang, 2009). Considering the high cost of current chemical treatments and the many side effects and low effectiveness, researchers are looking for alternative and more effective treatments. Nowadays, many therapeutic approaches have been directed towards drugs of plant and animal origin, among the reasons for which are the reduction of their side effects compared to chemical treatments and the greater cooperation of patients in their consumption (Meyer, 2017). It has been proven that inflammation and inflammatory factors play a major role in the development of pulmonary fibrosis. First, inflammatory processes occur in the lung and over time it becomes fibrosis (Ghelani, 2022). *Holothuria atra*, commonly known as black sea cucumber or loll fish, a species of marine invertebrates that was placed under the genus *Holodeima* by Pearson in 1914, and hence its full scientific name was *Holothuria atra*. *H.atra* is in the *Echinodermata* branch and the *Holothuroidea* family (Woo, 2018). To evaluate the cell protection potential against oxidative stress, we found that *H. atra* lysophospholipids inhibit H2O2-induced apoptosis in macrophage (Khotimchenko, 2018). Hexadecanoicacid, methyl ester, 9-12-octadecanoic acid in *H. atra* extract have anti-inflammatory and antioxidant properties (Nishikawa, 2015). Flavonoids are polar compounds capable of protecting against free radicals. Flavonoids in *H. atra* can be used as anti-inflammatory, anti-tumor, anti-viral and antibiotic. The presence of terpenoids in *H. atra* in the methanolic extract in the presence of Lieberman-Bourchad reagent causes the formation of a brown ring at the boundary of the solution. Terpenoidsare used as diabetes, malaria, anti-inflammatory, leukemia and allergy drugs (Chandra, 2022). Due to the limited number of drugs available in the treatment of this disease and their various side effects, Finding new drugs, especially natural compounds alone or together with chemical drugs with better efficacy and less side effects, is of interest to researchers. This study aims to investigate the antioxidant and anti-inflammatory effects of different doses of *H. atra* body wall extract on fibrosis caused by bleomycin.

2. Materials and Methods

2.1. Preparation of extract

H. atra are collected and identified from the Persian Gulf. The soaking method was used to prepare the extract. First, *H. atra* were cut into small pieces and then soaked in 75% ethanol solvent (25% distilled water - 75% ethanol) for 24 hours. After one day, the resulting extract was filtered. The ethanol extraction process was repeated 5 times. Then the obtained extract was passed through a filter paper and concentrated by a rotary evaporator (40ºC). Finally, the dry extract was obtained by freeze-drying.

2.2. Animal study

In this study, 36 adult male Wistar rats were purchased from the animal shelter of the Faculty of Pharmacy and kept for 7 days in the laboratory in polypropylene cages at ambient temperature (25 ± 4 ºC) to get used to the environment. In all experiments, conditions of 12 hours of light and 12 hours of darkness as well as suitable humidity with free access to food and water are provided for them. On average, 3 rat will be kept in each cage. Then, they were randomly divided.

The animals were randomly divided into 6 groups (n=6) which were induced by a single dose of

bleomycin (7.5 IU/Kg) intratracheally, the groups are treated according to the following schedule:

Control group, which received daily normal saline orally for 28 days.

Bleomycin group, which received a single dose of bleomycin (7.5 IU/Kg) intratracheally and daily normal saline orally for 28 days.

Pirfenidone group, which received a single dose of bleomycin (7.5 IU/Kg) intratracheally and daily pirfenidone (100 mg/kg) orally for 28 days.

Sea cucumber groups (*H. atra*) included 3 groups and received 50, 100, 200 mg/kg of *H. atra* body wall extract intraperitoneally for 28 days. After finishing the administration of the extracts, the animals were sacrificed.

The lungs were carefully removed and the weight of the lungs were measured. After washing the lungs with cold normal saline, the left lung was separated for histological studies and a part of it was placed in 10% formalin solution as a fixative. In order to evaluate histopathology, six slides were prepared from each group, one slide for each animal (and five fields are examined in each slide). The rest of the lung tissue was stored at -80 ºC for biochemical analysis.

2.3. Hydroxyproline assay

Kiazist kit (Kiazist Company, Hamadan, Iran) was used to measure hydroxyproline content. Accordingly, 20 to 40 mg of tissue was transferred to a clean microtube and homogenized in 100 µL of deionized water. Then, 100 µL of 12 M HCl was added and incubated at 120°C for 3-4 hours. At the end of the preparation stage, the microtube was opened and allowed to dry completely at 90°C. After that, 50 µL of Assay Buffer was added to each microtube and mixed well. Activated Charcoal (5 mg) was added to each sample and stirred completely. The resulting mixture was centrifuged at 12000 rpm for 15 min and the absorbance of the supernatant was read at 540 to 560 nm.

2.4. Total antioxidant capacity assay

Total antioxidant capacity was measured by spectrophotometric method using a commercial kit (Kiazist, Iran). Approximately 10 to 20 mg of tissue was lysed by sonication or homogenization in PBS buffer (or alternative buffer) and centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was freezed at -80 ºC. Add 100 microliters of TAC DMSO to the Trolox vial and mixed well to dissolve Trolox powder. 25 µL of dissolved Trolox was mixed with 975 µL of PBS to make a 1 mM standard. Finally, read the absorbance of the plate at a wavelength of 450 nm (Mahlooji, 2022).

2.5. Malondialdehyde assay

Kiazist kit was used for measurement. Accordingly, remove 10 to 20 mg of tissue and wash with PBS buffer. Then mix 300 µL of MDA Lysis Buffer with 3 µL of BHD 100X and homogenize the sample with it on ice. Centrifuge the resulting homogenate at 6000 rpm for 10 minutes and separate the supernatant to perform the test. Add 200 µL of sample or blank and standard to each microtube, then add 600 µL of TBA Solution to each. Close them tightly with parafilm and incubate for 60 minutes at 95C. Then cool for 10 minutes at room temperature. Take 200 µL of the sample or standard supernatant and fry it in a 96-well plate. For calorimetric reading, the plate should be read at a wavelength of 532 to 560 (Esfahani *et al.*, 2023).

2.6. Histopathology assessment

Hematoxylin-eosin staining was used to identify inflammatory cells and Masson trichrome staining was used to identify collagen deposition. Description and scoring of histopathological lesions and evaluation of fibrosis changes were carried out as previously described by Greco *et al.* (1997) with some modifications. Briefly, 10 fields for each lung section were systematically examined using a ×10 objective and each field was scored using the following grading scheme:

grade 0 for normal tissue and grades 1-4 for the presence of pulmonary inflammation and fibrosis. The severity of lesions was graded as 1 (mild), 2 (moderate), 3 (severe) and 4 (severe inflammation accompanied by total distortion of the structure). The extent of lesions was graded as 1(>10% of the slide), 2 (10%-40%), 3 (40%-70%), and 4 (<70%) of tissue affected. Fields predominantly occupiedby portions of large bronchi or vessels were not counted (Hosseini-Sharifabad, 2021).

2.7. Statistical analysis

The results were reported as mean ± SEM. Data analysis was done by Graphpad Prism software using one-way analysis of variance (ANOVA) followed by

Tukey's post hoc test. The difference with P-value less than 0.05 was considered significant.

3. Results and discussion

Pulmonary fibrosis is a dangerous disease that changes the normal structure of the lungs and can cause threatening consequences in patients. Since pulmonary fibrosis is considered as an inflammatory response, it can affect endothelial cells, fibroblasts and other lung cells and cause advanced tissue damage through persistent inflammatory reactions. The most important manifestation is the increase of fibroblasts as well as the production of collagen and its accumulation in the alveolar spaces (Hemmati *et al.*, 2018). Currently, FDA approved drugs for idiopathic pulmonary fibrosis including nintedanib and pirfenidone, which have poor efficacy in most patients. Therefore, we need to discover new drugs with more efficacies and less side effects for the treatment of idiopathic pulmonary fibrosis. Recently, several agents, especially those derived from plant and animal sources, with strong antioxidant and anti-inflammatory activities, have shown protective effects against bleomycin-induced lung injury (Dhaouafi *et al.*, 2023).

3.1. Effect of H. atra extract on rat body weight

The change in body weight of rats in different groups was determined between days 0 and 28, and the results were presented in Table 1. Bleomycin caused significant (P<0.05) weight loss in exposed rats compared to the control group. While, the control and *H. atra* groups showed a gradual and significant (P<0.05) increase in body weight from the 7th day (Table 1). Treatment with *H. atra* extract with different doses (50, 100 and 200 mg/kg) tended to prevent body weight loss two weeks after drug administration (P<0.05).

Table 1. Effect of bleomycin instillation and *H. atra* extract treatment on rat body weight variation.

Treatments	Day 0	Day 7	Day14	Day 21	Day 28
CON	193.48	195.83	198.56	201.33	202.94
PFD	204.28	187.84	194.68	197.58	201.64
BLM	192.38	183.24[a]	187.65[a]	190.42[a]	193.36[a]
HA-50	191.66	172.25[a,b]	183.75[a,b]	192.58[a]	194.68[a]
HA-100	195.36	176.87[a,b]	188.75[a]	193.52[a]	197.32[a,b]
HA-200	202.41[a,b]	186.71[a]	192.41[a,b]	196.57[a,b]	201.82[b]

Number of rat: n= 6/group. CON: Control: Negative control group, BLM: Bleomycin: Intratracheal instilled bleomycin group, PFD: Pirfenidone, HA: *H. atra* extract at different dose (50, 100 and 200 mg/kg) after bleomycin instillation. [a] p<0.05 compared to control group. [b] p<0.05 compared to BLM group.

3.2 The effect of H. atra extract on hydroxyproline level

An essential pathological feature of pulmonary fibrosis is the severe deposition of collagen and hydroxyproline, a specific component of collagen hydrolysis. Therefore, the hydroxyproline content of lung tissue is considered as a sign of pulmonary fibrosis (Zaafan *et al.*, 2016). The results of the present study showed that bleomycin increased the content of hydroxyproline in the lung tissue compared to the control group. After treatment with *H. atra* extract (200 mg/kg), the level of hydroxyproline significantly decreased compared to the group treated with bleomycin. Pirfenidone (100 mg/kg) also significantly reduced the hydroxyproline content in treated rats compared to the bleomycin-treated group (Fig. 1). These findings suggest that the protective activities of *H. atra* extract on bleomycin-induced pulmonary fibrosis are closely related to the reduction of lung hydroxyproline levels.

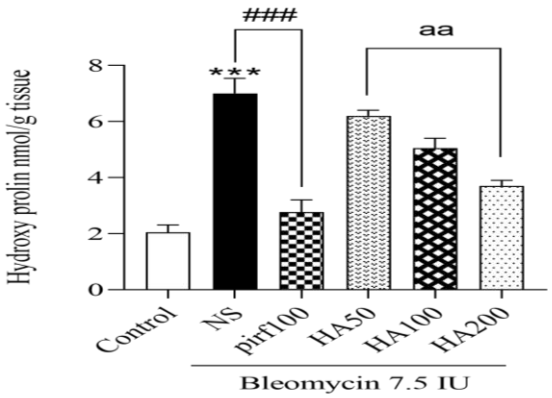

Fig. 1. Effect of *H. atra* extract on HYP content of BLM-induced pulmonary fibrosis in rats. Values are presented as mean ± SEM of six independent observation in each group. ***P<0.001 significant differences compared with the control group ###P<0.001, aaP<0.01. NS: Bleomycin; HA: *Holothuria atra*; Pirf: Pirfenidone.

3.2 The effect of H. atra extract on malondialdehyde level

The lungs are exposed to a highly oxidizing environment, triggering a variety of mechanisms. Oxidative stress can be linked to the biology of aging, which includes DNA damage responses, loss of proteostasis, and mitochondrial dysfunction. The role of reactive oxygen species (ROS) in certain fibrotic processes such as macrophage polarization and immunity, apoptosis and aging of alveolar epithelial cells, differentiation and aging of myofibroblast and changes in the extracellular matrix are significant (Esmat *et al.*, 2013). Inflammation is a key factor in the development of pulmonary fibrosis. Acute lung injury (ALI) and its more severe manifestation, respiratory distress syndrome (ARDS), are specific forms of lung inflammation. In response to lung injury, macrophages convert to proinflammatory M1 phenotypes and start to secrete pro-inflammatory cytokines (IL-6, TNF-a, IL-1, IL-8) and chemokines (CCL2, CCL7), which leads to increased chemotaxis and gradual enrichment of alveolar spaces by monocytes and neutrophils (Puspitasari *et al.*, 2023). Oxidative stress plays an important role in liver damage and fibrosis. Recent interest in food phenolics has increased due to their role as antioxidants and scavengers of reactive oxygen species and free radicals. Bioactive phenolic compounds in *H. atra* body wall extract have been investigated to evaluate the hepatoprotective potential against thioacetamide (TAA)-induced liver fibrosis in rats (Otoupalova *et al.*, 2011). Intracellular ROS attack the cell membrane through the breakdown of polyunsaturated fatty acids, resulting in increased malondialdehyde, elucidating the next challenge of bleomycin. Malondialdehyde is a reactive carbon agent that is used as a marker of lipid peroxidation (Del Rio *et al.*, 2005). The present study showed that intratracheal injection of bleomycin in rats causes a significant increase in malondialdehyde levels compared to the control group. Treatment with *H. atra* extract (200 mg/kg) significantly prevented the increase in malondialdehyde level compared to the group treated with bleomycin. Pirfenidone (100 mg/kg) also significantly reduced malondialdehyde levels in treated rats compared to the bleomycin group (Fig. 2). These findings indicate that *H. atra* extract can reduce lung fibrosis by inhibiting oxidative stress pathways.

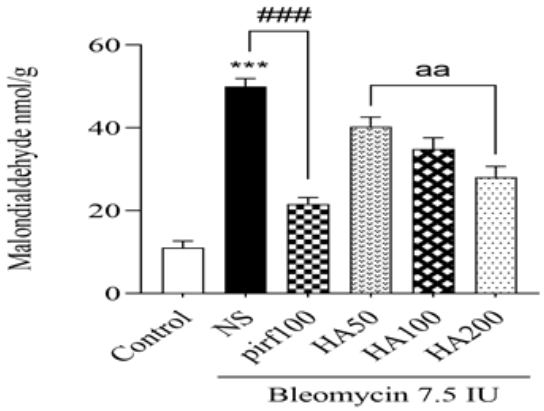

Fig. 2 . Effect of *H. atra* extract on MDA content of BLM-induced pulmonary fibrosis in rats. Values are presented as mean ± SEM of six independent observation in each group.***P<0.001 indicate significant differences compared with the control group؛ ###P<0.001, aaP<0.01 bleomycin-treated rats. NS: Bleomycin; HA: *Holothuria atra;* Pirf: Pirfenidone.

3.3. The effect of H. atra extract on total antioxidant capacity level

Total antioxidant capacity (TAC) refers to the total amount of antioxidant substances in a biological sample, and its changes contribute to the development of diseases related to free radicals.

This study showed that the TAC level in bleomycin-treated rats was significantly lower than the control group. In the group treated with *H. atra* extract (200mg/kg), the level of total antioxidant capacity increased compared to the group receiving bleomycin. The increase in the level of total antioxidant capacity in rats treated with *H. atra* extract was in line with the dose of extract (50, 100, 200mg/kg), respectively. Pirfenidone (100 mg/kg) also significantly increased the level of total antioxidant capacity in treated rats compared to the bleomycin group (Fig. 3).

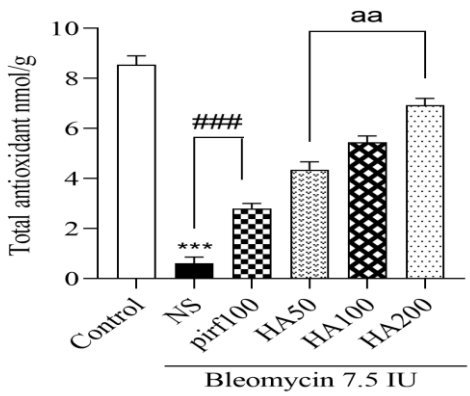

Fig. 3 . Effect of *H. atra* extract on TAC content of BLM-induced pulmonary fibrosis in rats. Values are presented as mean ± SEM of six independent observation in each group.***P<0.001 indicate significant differences compared with the control group؛ ###P<0.001, aaP<0.01 bleomycin-treated rats. NS: Bleomycin; HA: *Holothuria atra;* Pirf: Pirfenidone.

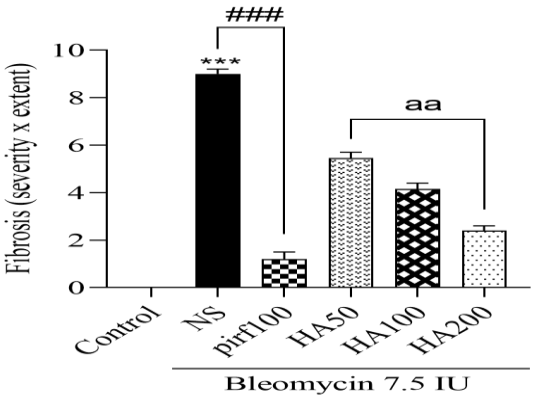

Fig. 4 . Numerical scoring of the severity and extent of bleomycin induced fibrosis in rat lungs. NS: Bleomycin; HA: *Holothuria atra;* Pirf: Pirfenidone. Data are expressed as multiplication of severity and extent of fibrosis and presented as mean±SEM (n=6). Stars show statistically significant difference with the vehicle treated group (****P*<0.001).

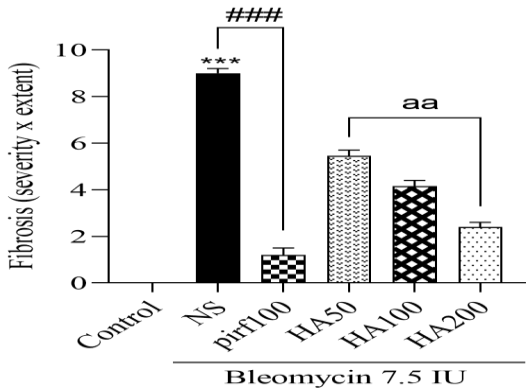

Fig. 5. Numerical scoring of the severity and extent of bleomycin induced inflammation in rat lungs. NS: Bleomycin; HA: *Holothuria atra;* Pirf: Pirfenidone. Ddata are expressed as multiplication of severity and extent of inflammation and presented as mean ± SEM (n=6). Stars show statistically significant difference with the vehicle treated group (****P*<0.001).

Previous research by Kobayashi et al. (1991) and Van Dyck et al. (2010) showed that *H. atra* contains triterpene sulfated glycosides, such as echinoside A, echinoside B, and holothurins B1, B2, B3, B4. In addition, dehydroquinoside A-24 was also detected in the body wall of *H. atra* . Other saponins from *H. atra* such as calcigroside B, holothurin A, holothurin D, holothurinogenin B, holothurinoside K1, sulfated echinoside B and A5 holothurin were also identified. Saponins have been shown to have many physiological functions, including antihyperglycemic, anti-inflammatory and antioxidant activity (Savin *et al.*, 2022). Phytochemical investigation of *H. atra* showed the presence of alkaloids, glycosides, phenol hydroquinone, saponins, steroids, triterpenoids, flavonoids and phenolic compounds, which could explain that *H. atra* is a common medicine for the treatment of various diseases. In addition, the crude extract showed moderate antioxidant activity related to neutralization of diphenyl picrylhydrazyl (DPPH) radicals (Awad *et al.*, 2023).

3.4. Histopathological assessment

The extent and severity of the lesions, especially the development of necrosis, inflammation and fibrosis in the groups treated with extract were significantly less compared to the bleomycin-treated group and most therapeutic effect was observed for the concentration of 200 mg/kg (Figs 5 and 6). In general, a high dose of the extract (200 mg/kg) showed a stronger preventive effect against lungs damaged than lower doses.

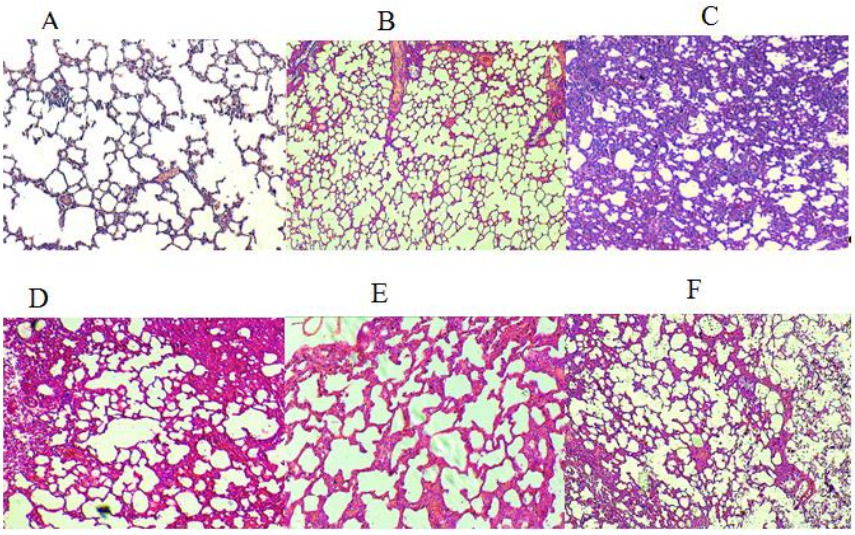

Fig. 6. Histopathological findings (H&E) in lung tissue. (A) Control: Normal lung tissue; (B) Pirfenidone: Decrease in inflammatory cells and fibrosis; (C) Bleomycin: Severe infiltration of inflammatory cells and fibrosis; (D) *Holothuria atra* extract-50: Reduces inflammation and fibrosis; (E) *Holothuria atra* extract-100: Decrease in fibrosis in the interstitial tissue; (F) *Holothuria atra* extract-200: Reduction in the thickening of the intra-alveolar space.

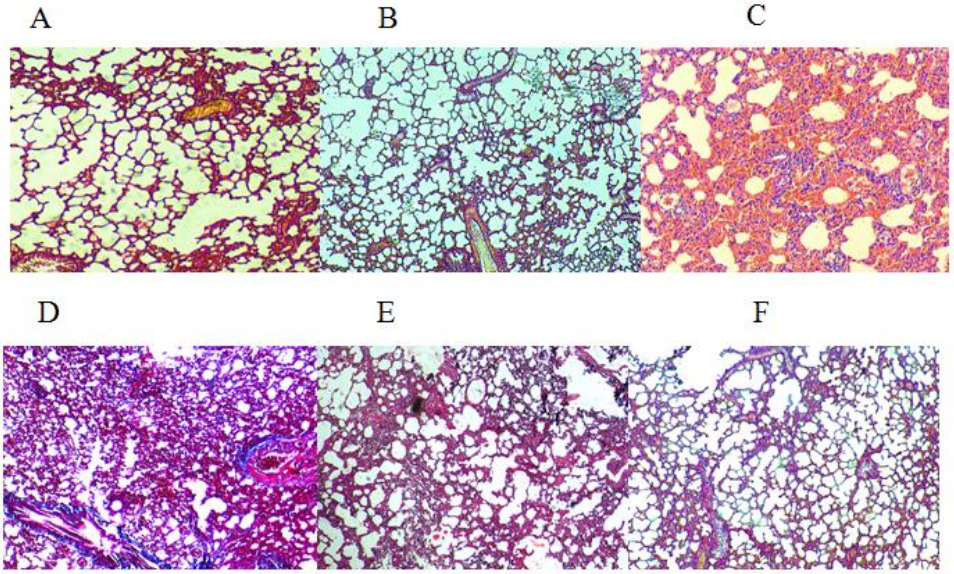

Fig. 7. Histopathological findings (Masson's trichrome) in lung tissue. (A) Control: Normal lung tissue; (B) Pirfenidone: Greater decrease in collagen deposition, (C) Bleomycin: Excessive deposition of collagen in the lung tissue.(D) *Holothuria atra* extract-50, (E) *Holothuria atra* extract-100, (F) *Holothuria atra* extract-200: decrease in collagen interstitial deposition in the lung tissue.

Figure 6 shows the histopathological findings in the lung tissue stained with hematoxylin-eosin. The control group showed normal appearance in the lung parenchyma (Fig. 6A). The pirfenidone group (100 mg/kg) compared to the bleomycin group caused a significant decrease in inflammatory cells and pulmonary fibrosis (Fig. 6B). Bleomycin caused destruction of the alveolar structure, emphysema, severe necrosis, bleeding in the lungs, thickening of the inner wall of the alveoli, and severe infiltration of inflammatory cells in the interstitial space and the space around the bronchi (Fig. 6C). In rats treated with hydroalcoholic extract of *H. atra* (50 mg/kg) the progress of necrosis, inflammation and fibrosis decreased compared to the bleomycin group (Fig. 6D). In rats treated with the hydroalcoholic extract of *H. atra* (100 mg/kg) a moderate penetration of inflammatory cells and a decrease in fibrosis in interstitial tissue was observed (Fig. 6E). In the group treated with 200 mg/kg of extract, a mild infiltration of inflammatory cells and a reduction in the thickening of the intra-alveolar space was evident (Fig. 6F).

Figure7 shows the histopathological findings in the lung tissue stained with Masson's trichrome. The control group showed normal lung tissue (Fig. 7A). In the lungs of rats treated with hydroalcoholic extract of *H. atra* with an increase in the dose (50, 100, 200mg/kg) a decrease in collagen interstitial deposition was observed in the lung tissue (Fig. 7D-F), especially in the group with a high dose of *H. atra* extract (200 mg/kg) and also in the pirfenidone group (100 mg/kg) (Fig. 7B). In the bleomycin group, it showed excessive deposition of collagen in the interstitial space of the lung tissue. Dense bundles of collagen were significantly evident in the bleomycin group (Fig. 7C).

4. Conclusion

This study showed the anti-fibrotic effect of *H. atra* extract against bleomycin-induced pulmonary fibrosis in rats. It seems that *H. atra* extract has a protective effect by improving antioxidant and anti-inflammatory status and by reducing collagen accumulation in lung tissue. These results suggest the therapeutic properties of *Holothuria atra* for the prevention or treatment of pulmonary fibrosis. However, the exact mechanisms of *H. atra* extract against bleomycin-induced pulmonary fibrosis need to be further investigated.

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