Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Antifibrotic Effects of Lilii Bulbus Extract in a Mouse Model of Bleomycin-induced Pulmonary Fibrosis

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Submitted: 17-Mar-2021 Revised: 09-Jun-2021 Accepted: 26-Jul-2021 Published: 24-Jan-2022

ABSTRACT

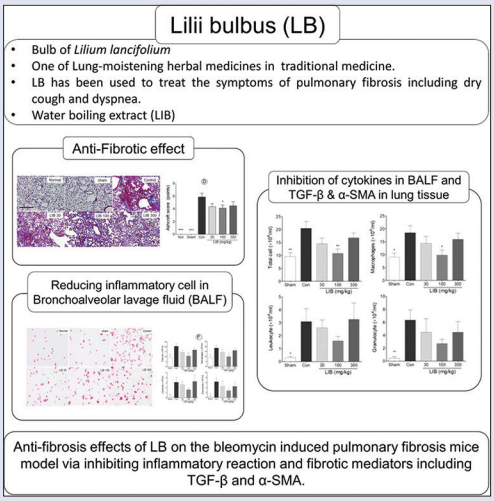
Objectives: Lilii Bulbus (LB), a lung-moistening herbal medicine, has been used to treat the symptoms of pulmonary fibrosis, including dry cough and dyspnea. In this study, we investigated whether LB water extract (LIB) has antifibrotic effects in a mouse model of bleomycin-induced pulmonary fibrosis. **Materials and Methods:** The effects of LIB and its mechanisms of action were investigated in a mouse model of bleomycin-induced (2 mg/kg, intratracheal) pulmonary fibrosis. LIB (30, 100, and 300 mg/kg) was administered orally twice daily for 10 days after induction. Changes in body weight, lung histology, inflammatory cells in bronchoalveolar lavage fluid (BALF), and the levels of transforming growth factor (TGF)-β, α-smooth muscle actin (α-SMA), and proinflammatory cytokines were evaluated. **Results:** LIB treatment decreased histological fibrotic change according to Ashcroft score, inflammatory cells, including total cells, macrophages, inflammatory cytokines (tumor necrosis factor-α and interleukin-6) in BALF, and TGF-β and α-SMA production in lung tissue. **Conclusion:** LIB exerted antifibrotic effects in a mouse model of bleomycin-induced pulmonary fibrosis by inhibiting inflammatory reactions and fibrotic mediators including TGF-β and α-SMA.

Key words: Bleomycin, herbal medicine, Lilii Bulbus, pulmonary fibrosis, transforming growth factor-β

SUMMARY

- Lilii Bulbus water extract showed antifibrotic effects in a bleomycin-induced pulmonary fibrosis mouse model through inhibiting inflammatory reactions and fibrotic mediators including transforming growth factor-β and α-smooth muscle actin.

Abbreviations used: α-SMA: α-smooth muscle actin; BALF: Bronchoalveolar lavage fluid; H and E: Hematoxylin and eosin; IL: Interleukin; IPF: Idiopathic pulmonary fibrosis; LB: Lilii Bulbus; LIB: Lilii Bulbus water extract; LPS: Lipopolysaccharide; TGF: Transforming growth factor; TNF: Tumor necrosis factor.



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DOI: 10.4103/pm.pm_117_21

Access this article online
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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common cause of chronic progressive interstitial lung disease. The primary symptoms of IPF include dry cough, dyspnea, general malaise, and weight loss. The pathological mechanisms of IPF are not fully understood; however, several contributing factors, including growth factors, such as transforming growth factor (TGF), myofibroblast differentiation, and collagen accumulation, have been reported.[1,2]

Steroids, interferon-γ, N-acetylcysteine, immunosuppressants, and anticoagulants have been recognized as therapeutic agents for IPF; however, they have been found to have little to no benefit in the treatment of patients with the disease.[3] Recently, two approved therapies, pirfenidone and nintedanib, were shown to slow the progression of IPF. However, treatment was discontinued in approximately 20% of patients due to side effects including skin rash, photosensitivity, hepatotoxicity, and gastrointestinal tract symptoms.[1,3] Thus, alternative therapeutics need to be developed in various fields, including traditional medicine and natural products.

Lilii Bulbus (LB), the bulb of *Lilium lancifolium* Thunb., is a kind of lily that originated from Asia, including Korea, China, and Japan. LB is well

Cite this article as: Chiang SC, Park J, Bae J, Joo H, Lee D, Kim I, *et al*. Antifibrotic effects of lilii bulbus extract in a mouse model of bleomycin-induced pulmonary fibrosis. Phcog Mag 2021;17:679-83.

known for the effect of moistening the lung to suppress cough in traditional medicine[4] and has been used to treat various pulmonary diseases including pneumonia, bronchitis, asthma, and so on.[5,6] It has been reported to have pharmacological effects such as antifungal, antibacterial, and antioxidant activities.[5,7-9] In addition, an anti-inflammatory effect was also reported in LPS-stimulated Raw264.7 cells[8] and a cigarette smoke-exposed pulmonary inflammation mouse model.[10]

Considering the typical symptoms of IPF, including dry cough and dyspnea, it could be regarded as a chronic pulmonary disease correlated with lung Yin deficiency syndrome in traditional medicine.[11,12] Thus, lung-moistening herbal medicines may be a potential therapeutic option for IPF. We previously screened the antifibrotic effects of lung-moistening herbal medicines and found that LB was a candidate for preventing the progression of IPF.[4] The current study was designed to assess the antifibrotic effects of LB according to dose and to investigate its mechanisms of action.

MATERIALS AND METHODS

Sample preparation

A dried bulb of LB, which was collected in Hunan province of China in October, was obtained from Kyung Hee University Korean Medicine hospital (Seoul, Korea). Sample, HLL-003 (sample number), was morphologically identified and stored in the Department of Herbal Pharmacology, College of Korean Medicine Kyung Hee University.[4] Two hundred grams of LB with 1500 ml of water was boiled using a Reflux extractor for 2 h to obtain 440 ml of LB water extract. Then, the extract was filtered with filter paper and concentrated using a rotary vacuum evaporator and dried using a freeze dryer (FD-5508, IlShin Lab Co., Yangjugun, Korea) to obtain 18.98 g of dried extract (yield 9.49%, LIB).

Animals

Male ICR mice (7 w of age; 31 ± 2 g, Daehan Biolink, Korea) were housed for 7 d on a 12 h light/dark cycle at a temperature of 22°C ± 1°C and humidity of 55% ± 10%, with *ad libitum* access to food and water. The study protocol was approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHMC-IACUC 18-177).

Induction of pulmonary fibrosis in a mouse model and sample administration

A mouse model of pulmonary fibrosis was established according to a method described in previous studies.[4,13] Briefly, mice were anesthetized with 2% isoflurane (O_2:N_2O [30:70]) and bleomycin was instilled into the trachea (2 mg/kg). The mice were divided into groups as follows: normal, sham, control, and those treated with 30, 100, and 300 mg/kg of LIB, respectively. The control and LIB groups received bleomycin, while the sham group received saline, and the normal group received nothing. LIB was orally administered twice per day for 10 days after induction, while the control and sham groups were treated with the same volume of water (3.3 mL/kg). Bronchoalveolar lavage fluid (BALF) collection and lung isolation for histology and protein assay procedure were conducted at the 11th day after induction [Figure 1].

Bronchoalveolar lavage fluid analysis and cytokine measurement

BALF collection and inflammatory cell analysis were performed using a method described in a previous study.[4] Briefly, mice were anesthetized with urethane (1.2 g/kg, i. p.) on the 11th day, and a midline neck incision was made followed by exposure of the trachea. BALF was collected through the bronchus three times using 0.5 mL of phosphate-buffered saline. Total cells were counted using a hemocytometer, and the mean number of macrophages, lymphocytes, and neutrophils was calculated after hematoxylin and eosin (H and E) staining by two blinded researchers.

Measurement of cytokines in bronchoalveolar lavage fluid

Tumor necrosis factor (TNF)-α and interleukin (IL)-6 in BALF were analyzed using the TNF-α and IL-6 ELISA Kit (BD Biosciences, USA) according to the manufacturer's protocol. In brief, BALF samples or standard were added to each ELISA diluted well and incubated for 2 h at room temperature, respectively. After washing, a detection antibody for mouse TNF-α or IL-6 was added to each well and then an enzyme-linked polyclonal antibody was added. Then, substrate reagent and stop solution were added in sequence. The absorbance of TNF-α and IL-6 was measured at 450 nm using a microplate reader (SpectraMax® Plus, Molecular Devices Corp, USA).

Histological analysis

Mice were perfused transcardially with 4% paraformaldehyde solution on the 11th day after induction. The lung was then isolated and postfixed in the same solution for making a paraffin block. The tissue was sliced (5 μm thick) and subsequently stained with Masson's trichrome. Two researchers blindly evaluated the degree of histological changes following lung fibrosis based on the Ashcroft scale.[14]

Measurement of transforming growth factor-β and α-smooth muscle actin in lung tissue

Lungs were isolated after anesthesia on the 11th day of induction, homogenized using a homogenizer (Bullet Blender Tissue Homogenizer, USA) and then centrifuged (13,000 rpm, 4 min, 4°C) to obtain a supernatant. TGF-β and α-smooth muscle actin (SMA) in lung tissue were measured using a TGF-β ELISA kit (RandD Systems, USA) and an α-SMA ELISA kit (Novus Biologicals, USA) according to the manufacturer's protocol. In brief, diluted samples or standards were added to 96 well microplates prepared with the diluted capture antibody. The mouse TGF-β or α-SMA detection antibody, Streptavidin-HRP conjugate working solution, and substrate reagent solution were sequentially treated for reaction, and the reaction was terminated by the stop solution. The absorbance was measured at 450 nm using a microplate reader (SpectraMax® Plus, Molecular Devices Corp, USA).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance was followed by Dunnett's test using GraphPad Prism version 5.0 (GraphPad Inc., San Diego, CA, USA) to compare each group with the control group.

RESULTS

Fibrotic change in lung tissue and body weight

Various fibrotic changes in the lung tissue were apparent in the control group, including alveolar wall thickening, alveolar collapse, inflammatory cell deposition, and collagen accumulation. The normal and sham groups did not exhibit any histological changes in lung tissue stained with Masson's trichrome compared with the control group. Mice in the LIB-treated groups exhibited decreases in fibrotic changes [Figure 2a and b]. Ashcroft scale scores of the control group increased up to 6 points, while those of normal and sham groups showed 0 points. The LIB group had a reduced scale compared to the control group. In particular, mice treated with 100 mg/kg LIB exhibited a significant decrease [Figure 2d]. In addition, the LIB-treated groups did not exhibit recovery of body weight [Figure 2c].

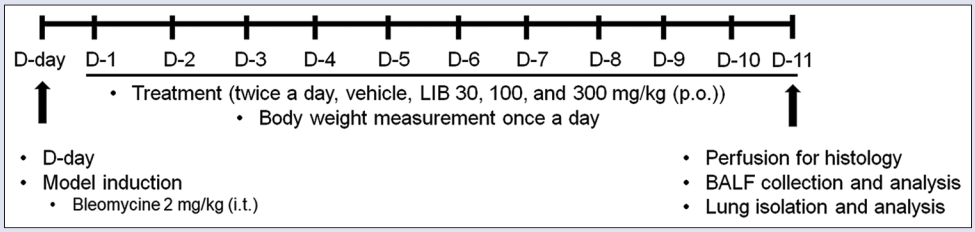

Figure 1: The scheme of the experiment

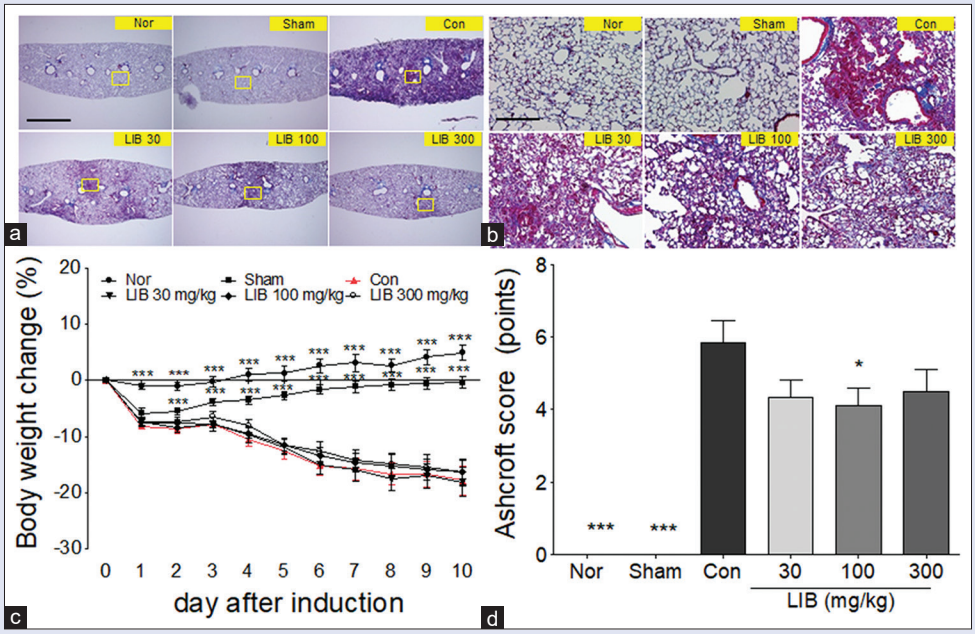

Figure 2: The effects of LIB on pulmonary fibrotic change and body weight in a mouse model of pulmonary fibrosis. Masson's trichrome-stained lung sections (a and b); changes in body weight (c); Ashcroft score (d). Each photograph in B is the magnification of the yellow box of each photograph in A. The scale bar in A and B is 400 and 40 μm, respectively. Nor: normal group; Sham: sham group; Con: control group; LIB: water extract of Lilii Bulbus; *indicates a statistically significant difference from the control group (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Inflammatory cells and cytokines in bronchoalveolar lavage fluid

There was an increase in the total number of cells, macrophages, lymphocytes, and granulocytes in the BALF of the control group compared with the sham group. All LIB-treated groups exhibited a decrease in total cells, macrophages, lymphocytes, and granulocytes in BALF compared with the control group [Figure 3]. In particular, the LIB 100 mg/kg group exhibited a significant decrease in the total number of cells and macrophages compared with the control group [Figure 3b].

BALF levels of TNF-α and IL-6 in the control group were significantly increased by 41.3% and 348.5%, respectively, compared with the sham group. The LIB 100 mg/kg group exhibited a significant decrease of 15.8% and 31.7%, respectively [Figure 4].

Transforming growth factor-β and α-smooth muscle actin levels in lung tissue

Mice in the control group exhibited significantly increased levels of TGF-β and α-SMA (41.0% and 55.2%, respectively), compared with the sham group. However, the LIB 100 mg/kg group exhibited significant reductions in TGF-β and α-SMA levels (16.5% and 11.8%, respectively) compared to the control group [Figure 5].

DISCUSSION

In the current study, LIB treatment reduced Ashcroft score, TGF-β and α-SMA levels in lung tissue, and total cells, macrophages, TNF-α, and IL-6 in BALF without a change in body weight.

LIB treatment tended to reduce Ashcroft scale scores. In particular, the LIB 100 mg/kg group exhibited a significant and maximal effect. The bleomycin-induced pulmonary fibrosis model is most clinically similar to IPF and has the advantage of inducing short-term pulmonary fibrosis in animal studies.[15] Intratracheal instillation of bleomycin leads to an inflammatory reaction at the first (approximately 7 days) due to damage of alveolar epithelial cells. Fibrotic changes occur in the interstitial tissues of the lungs, including collagen accumulation, followed by myofibroblast differentiation (7–14 days).[15,16] The Ashcroft scale is one of the most commonly used tools for evaluating alveolar structural changes, inflammatory cell deposition, and collagen levels in the lung interstitium.[14] Thus, the results of the current study suggest that LIB could inhibit pulmonary fibrosis and that 100 mg/kg may be an optimal dose in mice. Although LIB 300 mg/kg in the current study did not show statistical significance compared with previous studies,[4] it showed similar data to LIB 100 mg/kg, which showed the maximal efficacy, in terms of the Ashcroft score, the inflammatory cytokines in BALF, and the fibrosis factors in lung tissue. A LIB dose of 100 mg/kg

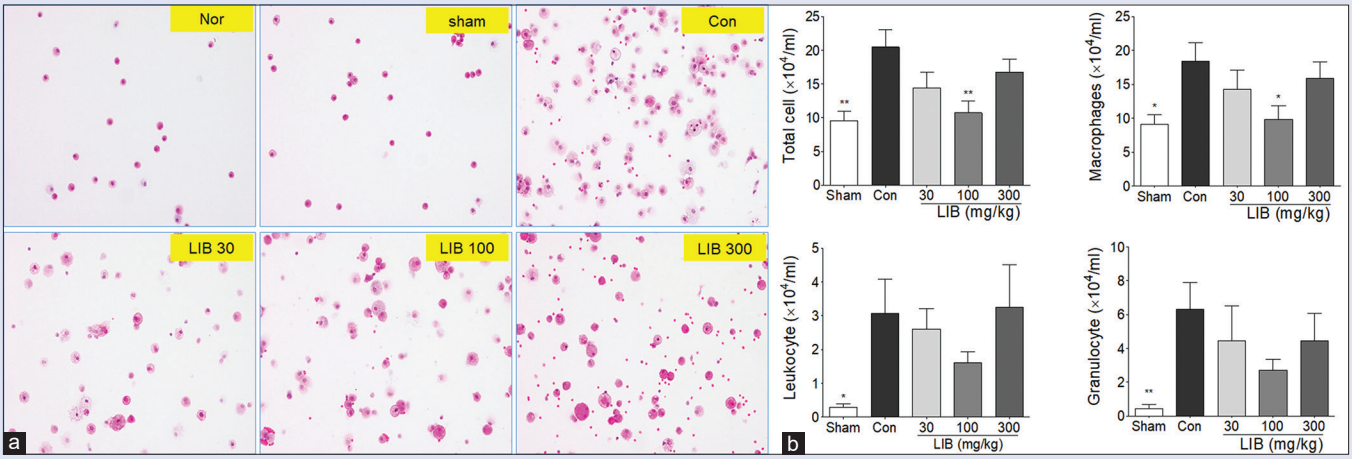

Figure 3: The effects of LIB on inflammatory cells of bronchoalveolar lavage fluid in a mouse model of pulmonary fibrosis. Data are mean ± standard error of the mean Nor: Normal group; Sham: Sham group; Con: control group; LIB: Water extract of Lilii Bulbus. *indicates a statistically significant difference from the control group (*$P < 0.05$; **$P < 0.01$)

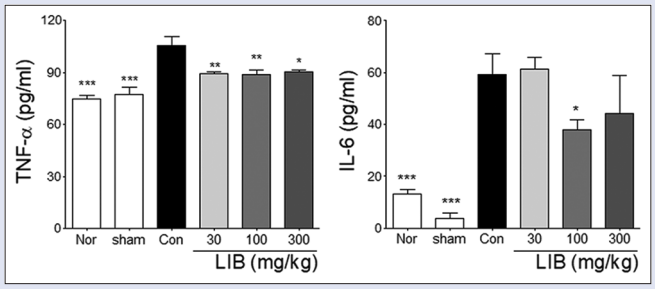

Figure 4: The effects of LIB on tumor necrosis factor-α and interleukin-6 levels of bronchoalveolar lavage fluid in a mouse model of pulmonary fibrosis. Nor: Normal group; Sham: Sham group; Con: Control group; LIB: water extract of Lilii Bulbus. * indicates a statistically significant difference from the control group (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

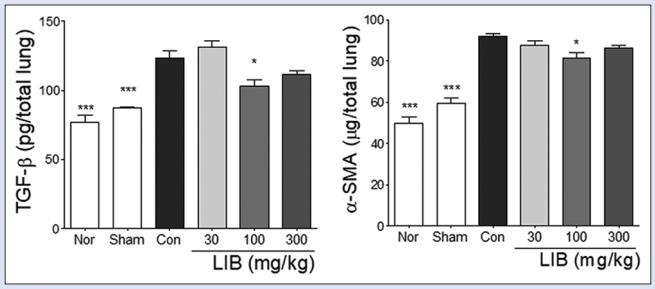

Figure 5: The effects of LIB on transforming growth factor-β and α-smooth muscle actin levels of lung tissue in a mouse model of pulmonary fibrosis. Nor: Normal group; Sham: Sham group; Con: Control group; LIB: Water extract of Lilii Bulbus. * indicates a statistically significant difference from the control group (*P < 0.05; ***P < 0.001)

corresponds to 6 g/60 kg of human weight (63.2 g/60 kg as raw material). Regarding metabolism in mice, which is approximately 10 times the rate in humans, the clinical dosage may be calculated as 6.32 g of raw material.[17]

LIB treatment demonstrated a tendency to reduce inflammatory cell production in BALF; more specifically, treatment with 100 mg/kg LIB led to a reduction in the total number of cells and macrophages. Inflammatory cells, and/or their associated cytokines in the alveoli, are well-known pathological features that are closely related to fibrotic changes in the bleomycin-induced fibrosis model.[16] The pathological roles of TNF-α and IL-6 have been well documented. TNF-α is known to be closely associated with the production of fibroblasts or the extracellular matrix, which is a key mediator of pulmonary fibrosis. IL-6 levels are known to be increased in pulmonary fibrosis by inducing the differentiation of fibroblasts into myofibroblasts, which are key cells involved in fibrosis.[18] Results of the current study suggest that the decrease in the number of inflammatory cells and cytokine levels in BALF may be closely related to the antifibrotic effect of LIB.

LIB treatment demonstrated a tendency to reduce TGF-β and α-SMA production in lung tissue. In particular, mice treated with LIB 100 mg/kg exhibited a significant and maximal effect. TGF-β has been reported to drive the differentiation of fibroblasts into myofibroblasts, which are a source of collagen and fibrogenic cytokines.[19] α-SMA is known to be a marker of myofibroblasts and plays a role in extracellular matrix accumulation in fibrotic changes.[20] Results of the current study suggest that inhibition of TGF-β and α-SMA production may be the mechanism underlying the antifibrotic effects of LIB.

CONCLUSION

LIB exerted antifibrotic effects in a mouse model of bleomycin-induced pulmonary fibrosis by inhibiting inflammatory reactions and fibrotic mediators, including TGF-β and α-SMA. Further studies are needed to investigate mechanisms related to the TGF-β/Smad3 pathway.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning NRF-2018R1A2B6009621

Financial support and sponsorship

Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning NRF-2018R1A2B6009621.

Conflicts of interest

There are no conflicts of interest.

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