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Effects of Ma-Xing-Shi-Gan-Tang on Bleomycin- Induced Lung Fibrosis in Rats

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ABSTRACT

In this study, after doses of 1 g/kg/day of the traditional Chinese formula Ma-Xing-Shi-Gan-Tang (MXSGT), significant reversal of pneumatocysts was achieved in Spague-Dawley rats with bleomycin-induced lung fibrosis. We found that MXSGT can improve the damaged condition of general cellular membranes. Greatly increased levels of lung NO were found in the bleomycin-induced group, which were then significantly reduced by MXSGT. MXSGT drastically prevented depletion of superoxide dismutase (SOD) and reduced the myeloperoxidase (MPO) activities and malondialdehyde (MDA) levels in lung tissue of rats treated with bleomycin. On the basis of the results presented in this paper, MXSGT prevents bleomycin-induced lung fibrosis, and the mechanism may be due to the inhibitory effect on nitric oxide generation in the bleomycin-induced lung-fibrosis model of rats.

Key words : Idiopathic pulmonary fibrosis, bleomycin, Ma-Xing-Gan-Shi-Tang, nitric oxide

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive chronic inflammatory interstitial lung disease of unknown etiology with potential fatal prognosis and poor response to available medical therapy(1). The American Thoracic Society (ATS) and the European Respiratory Society (ERS) have defined IPF as a distinctive type of chronic fibrotic interstitial pneumonia limited to the lungs, of unknown course, and associated with a histological pattern of usual interstitial pneumonia (UIP). The survival rate of IPF patients is low. A review of the literature revealed that clinically well defined cases of IPF have a mean survival time of 2 to 4 years after diagnosis(2). IPF can be characterized by radiographical evidence of interstitial infiltrates that mainly affect lung bases, and by progressive dyspnea and worsening of pulmonary function. No therapy is known to be clearly effective(3).

IPF has become a new critical focus of basic and clinical research both in pathogenesis and in developing novel therapeutic agents with improved efficiency. Current treatment includes corticosteroids and cytotoxic agents such as cyclophosphamide, azathioprine, and colchicines. Positive therapeutic effects from corticosteroid treatment in patients with IPF has been shown to be 10 to 30%, with most responses in patients being partial and transient(4).

In spite of the fact that the pathophysiology of pulmonary fibrosis is not clear, it has generally been hypothesized that activated inflammatory cells accumulate in the lower airways and release large amount of reactive oxygen species (ROS) that cause lung injuries and fibroblast proliferation in alveolar walls. Recently, reactive nitrogen species (RNS), including nitric oxide, peroxynitrite ($ONOO^-$), and nitrogen dioxide in the respiratory tract, have been the subject of considerable attention(5). The activated fibroblasts produce an increased amount of extracellular matrix proteins that distort the normal lung architecture and impair the vital gas exchange function of the lung(6).

Bleomycin-induced pulmonary fibrosis animal models have been developed to explore the underlying pathology and to test new therapeutic approaches(7). Gurujeyalakshmi *et al.*(8) have demonstrated an increased level of NO in bronchoalveolar lavage fluid (BALF) and overexpression of inducible nitric oxide synthase (iNOS) mRNA, and NOS protein in lungs during the course of the development of

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bleomycin (BLM)-induced pulmonary fibrosis in mice [9]. In another study, the administration of aminoguanidine by oral route, a specific inhibitor of iNOS, attenuated the bleomycin-induced lung fibrosis at a high dose without producing any systemic toxic effect[9].

Bleomycin is a compound produced by *Streptomyces verticillis*[10]. It has potent tumor killing properties, which have given it an important place in cancer chemotherapy. It leads to marrow suppression, but also to pulmonary toxicity, which is a major adverse effect. The mechanisms of cell toxicity are well described based on *in vitro* experiments on DNA. Bleomycin can also lead to cell damage independently from its effect on DNA by inducing lipid peroxidation[11,12]. Recent studies have introduced many promising agents that might counter IPF, including aminoguanidine,[9] anti-oxidants[11,12], N-acetylcysteine[13], and erdosteine[14] .

One potential agent that has not been studied in this context is Ma-Xing-Shi-Gan-Tang (MXSGT), which has been used as an effective cure by traditional Chinese medical doctors for SARS patients suffering from severe respiratory tract illness and pulmonary fibrosis[15]. It is a well-known Chinese combination formula for respiratory airway treatment, and has received much attention in recent years. MXSGT is used for the treatment of severe respiratory tract illness and pulmonary fibrosis in China; however, there is no scientific evidence or any related articles regarding its effect on pulmonary fibrosis. For these reasons, we selected this promising topic and report our results herein.

MATERIALS AND METHODS

I. *Preparation of Ma-Xing-Shi-Gan-Tang (MXSGT) Extract*

The formulation of MXSGT consists of four different medical plant ingredients as shown in Table 1. They were purchased from a traditional Chinese medicine pharmaceutical company in Taiwan. The decoction of four components was obtained from three extractions, which were combined, filtered, concentrated and lyophilized. The yield of dried extract was mixed with starch to produce a mixture of Ma-Xing-Shi-Gan-Tang. The quality of the crude materials was examined following the guidelines recorded in the Pharmacopoeia Chinese Medicine of the R.O.C. [16]

Table 1. Composition of Ma-Xing-Shi-Gan-Tang (MXSGT)

Ingredient	Weight (g)
Ephedra sinica	8.0
Prunus armeniacae	6.0
Glycyrrhizae uralensis	1.33
Gypsum	16.0
Yield of dried extract	3.9
The extract mix with starch	6.6

II. *Animal Models of Bleomycin-Induced Lung Fibrosis*

A total of 40 male Sprague-Dawley (SD: NARL) rats, 8 weeks old, weighing 250 to 300 g, were housed in Chimei Laboratory Animals Centers. All protocols were approved by the Chi-Mei IACUC in this study. A 12-h light/dark cycle was maintained and the rats had free access to water and food *ad libitum*.

Thirty-two rats were each intratracheally injected with bleomycin hydrochloride (BLM, 5 mg/kg) as described previously[5,17]. Two weeks after BLM instillation, eight rats were sacrificed as the model group to test their lung histology and biochemical analyses. The other 24 rats were randomly assigned to three groups, which receive equal volumes of saline (the control group), 5 mg/kg of prednisolone (the positive control group) or 1 g/kg of MXSGT (the treatment group), respectively, once per day for four weeks. After treatment for four weeks, the rats were anesthetized and scarified, and then lungs were removed for studying their similarity to the model group.

III. *The Lung Broncholalveolar Lavage Fluid (BALF) Analyses*

After administering anesthesia by intra-peritoneal injection of 65 mg/kg Pentothal (Abbott, USA), the rats were prepared for lavage by cannulating the trachea with a blunt needle attached by a syringe. The lung lavage was obtained by washing the lung four times with 4 mL aliquots of saline through a trachea cannula. Cell suspension was concentrated by low speed centrifugation (800 rpm for 10 min), and the cell pellet was re-suspended. Total cell counts were made by a haemocytometer. Nuclear cells differentiation was estimated from cytospine preparation by counting 300 cells stained with May-Grunwald-Giemsa.

IV. *The Lung Tissue Histology*

After sacrifice, each rat's right lung was removed and fixed by inflation with a buffered 10% formalin solution for 24 h and embedded in paraffin. Tissues were then sectioned, stained with hematoxylin and eosin (H&E), and examined for lung fibrosis.

V. *The Lung Tissue Biochemical Analyses*

The left lungs were homogenized and used for oxidative stress markers and antioxidant enzymes analyses. The blood and clot in lung tissues were removed first and then washed twice with cold saline solution. After cutting off the lungs into small pieces with a pair of scissors, the lung tissues were homogenized in a ratio of 5 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.4) per gram of wet tissues for 3 min at 16,000 rpm using a homogenizer (IKA Ultra-Turrax T25 basic homogenizer, Germany). Tissue nitrite/nitrate (NOx) levels, malondialdehyde (MDA) and myeloperoxiase (MPO) activity were determined in the homogenates. The homogenates

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were then centrifuged at 5,000 rpm for 60 min to remove debris. After the supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5/3, v/v) and centrifuged at 5,000 rpm for 60 min, the upper ethanol phase was taken and used in the superoxide dismutase (SOD) assays. The protein measurements were analyzed in homogenates, supernatant and extracted samples.

(I) *Oxidative Stress Markers Analyses*

The method for determining lung nitrite and nitrate levels was based on the Griess reaction[18]. Results were expressed in μM per gram of tissue protein.

Lung MDA levels were determined by the Wasowicz's method[19] based on the reaction of MDA with thiobarbituric acid at 95 to 100°C. Results were expressed in μM per g of wet tissue protein of the lung (μM/g protein). Myeloperoxidase (MPO) activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 with changes in absorbance recorded at 510 nm[12].

(II) *Antioxidant Enzyme Analyses*

Total superoxide dismutase (SOD) activity in the lung tissue was determined according to the method by Sun *et al*.[20,21] SOD activity was also expressed as units per gram of protein.

VI. *HPLC Comparison of the MXSGT Extract and Chemical Standards*

The MXSGT standard sample was prepared according to the Good Manufacturing Practice regulated by the Taiwanese government. HPLC analysis was performed using Shimadzu LC-10AT pumps, a SPD-10A UV-Vis detector and a Waters ODS 5 mm (250 × 4.6 mm i.d.) column. The wavelength of the UV detector was set at 254 nm and the flow rate was 1.0 mL/min. The stepwise HPLC mobile phase conditions were as follows: initial 0.2% H_3PO_4 in water/acetonitrile in composition of 5 : 95 then increased polarity to 60 : 40 in 60 min. The injection valve equipped with a 20-μL loop.

VII. *Statistical Analysis*

Data were calculated as Mean ± SD. Statistical analyses were carried out by analysis of variance (ANOVA) followed by appropriate post tests including multiple comparison tests (LSD). All analyses were made using the SPSS statistical software package and a probability value of less than 0.05 was considered as statistically significant.

RESULTS

I. *Bleomycin-Induced Lung Fibrosis Model Building and MXSGT Treatment*

Figures 1 and 2 showed lung histology. In comparison with the saline and prednisolone treatment groups, the MXSGT treatment group showed attenuation inflammatory cells infiltration in lung parenchyma and partially remodeled lung architecture. In Figure 1, treatment with bleomycin for 2 weeks induced lung fibrosis, and serious cell damage configuration of lung inflammation can be seen in the lung

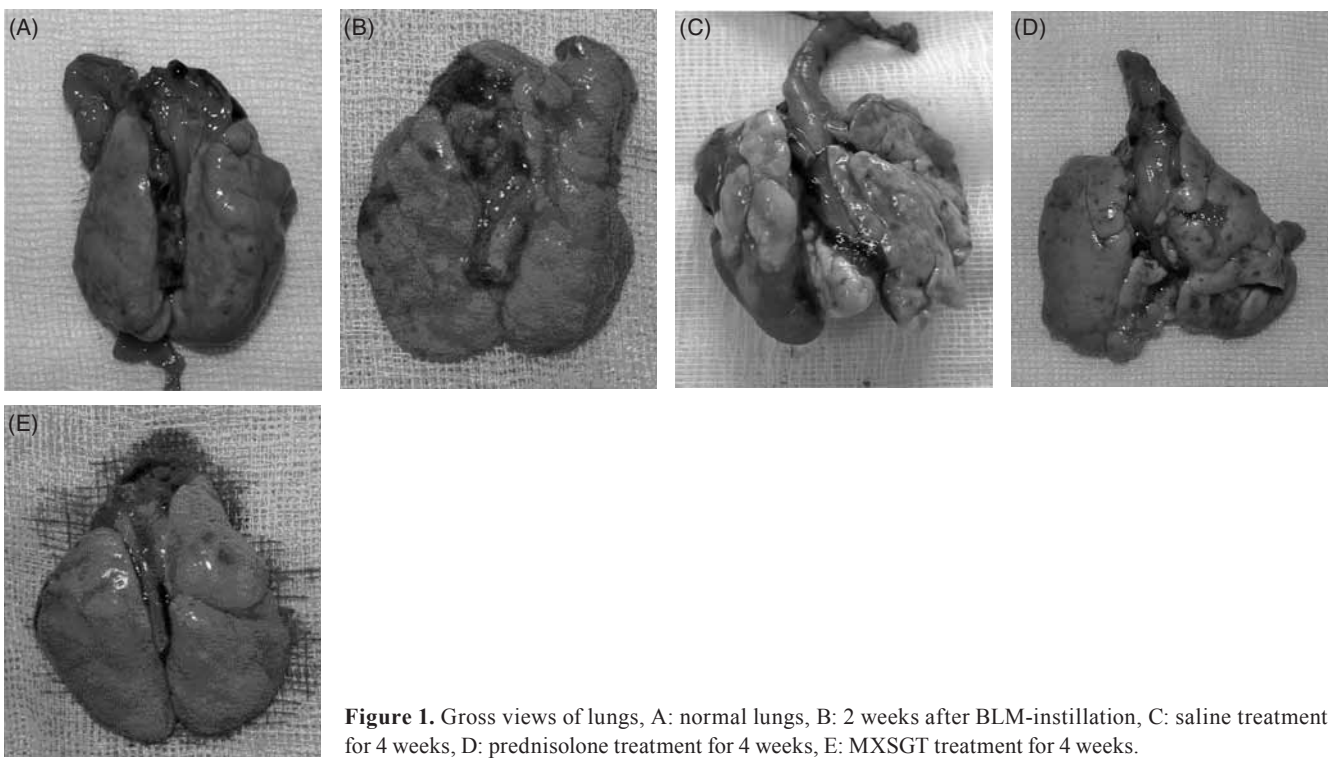

Figure 1. Gross views of lungs, A: normal lungs, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks.

organization in a gradually developed process (the model group, Figures 1B and 2B). Moreover, bleomycin caused alveolar cell damage. We can see the lung tissue with pneumatocyst decreased, implying bleomycin-induced lung fibrosis. On the 28th day, multifocal fibrosis was observed in the saline-treated group (Figures 1C and 2C). By comparison with the saline and prednisolone groups, the intervention with continuous MXSGT treatment for 4 weeks showed obvious improvements in lung morphology (Figures 1E and 2E).

II. *Effect of MXSGT on Bleomycin-Induced Changes in Total and Differential Inflammatory Cell Counts of Rats*

According to previous studies, bleomycin treatment of cancer has the side effects of leukocyte cell reduction and increased shortness of breath[11]. The alveolar wall was also thickened by edema and infiltration of fibroblasts, and some inflammatory cells including neutrophils, alveolar macrophages, and lymphocytes were observed. However, the levels of the pneumatocyst were significantly reversed by MXSGT treatment. In comparison with the saline and prednisolone groups, the inflammatory cell counts were decreased from $1.48 \pm 0.46 \times 10^6$/mL to $0.29 \pm 0.30 \times 10^6$/mL. The data detailed in Table 2. Our study show that the Chinese medicine used on the rats in bleomycin-induced pulmonary fibrosis improved and reduced related inflammatory cells. As shown in the last paragraph, lung injury was assessed by histologic evaluation. Qualitatively, histologic evidence of damage induced by bleomycin shows that the damage progressed in a similar manner to the reduction of differential inflammatory cells when compared with normal animals.

III. *Analysis of Oxidant Stress Markers*

The depletion in SOD in the tissue reflects sustained generation of free radicals produced by bleomycin administration. Intratracheal instillation of bleomycin resulted in a significant decrease in the SOD activities in the lung tissue after 2 weeks when compared with 4-week MXSGT-treated rats (already pretreated with bleomycin for two weeks) (Figure 3A).

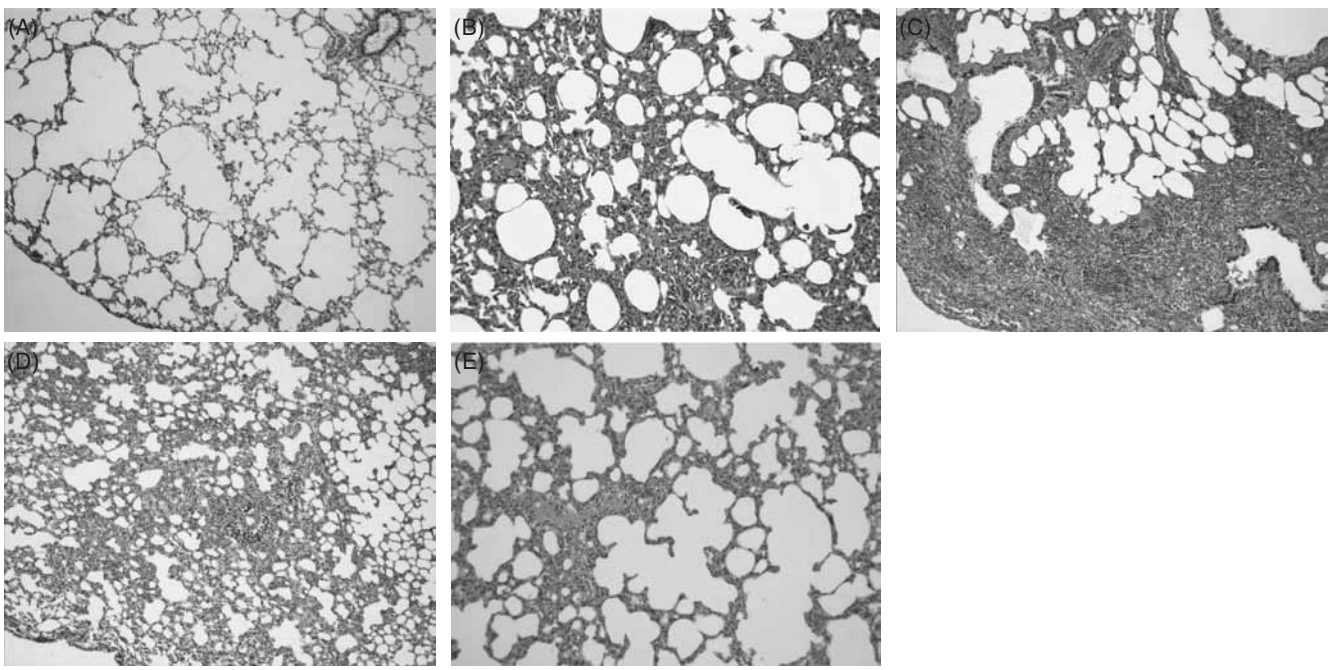

Figure 2. Lung pathology (H&E at 100X), A: normal lung, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks.

Table 2. The BALF counts and differentiation of nuclear cells in each group[a]

	Total cell number (X 10^6 / mL)	Macrophage (%)	Neutrophil (%)	Lymphocyte (%)
A: Normal	0.03 ± 0.006	91.7 ± 1.7	4.4 ± 0.8	3.9 ± 1.2
B: Model	0.84 ± 2.30	86.4 ± 5.0	3.7 ± 2.3	9.1 ± 2.2
C: Saline	1.48 ± 0.46	82.7 ± 6.0	5.2 ± 4.0	12.1 ± 3.5
D: Prednisolone	0.32 ± 0.07*	86.9 ± 2.0	3.9 ± 2.4	8.3 ± 2.3
E: MXSGT	0.29 ± 0.30*	86.5 ± 5.0	6.8 ± 1.8	6.7 ± 5.0

[a] Data were presented as mean ± SD in each group, * $p < 0.05$. The significance of statistic data was calculated by comparison of the data of the control (saline) group. A: normal lung, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks.

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In SOD activity (Figure 3A), the expression of normal rats is 1.77 ± 0.63 U/g protein. After treatment with bleomycin, the expression of the model rats is 0.16 ± 0.03 U/g protein. After 4 weeks of treatment, the effect of MXSGT (0.89 ± 0.22 U/g protein) is much better than those of the control (saline, 0.10 ± 0.02 U/g protein) and positive control (prednisolone, 0.41 ± 0.16 U/g protein) groups.

Bleomycin treatment for 2 and 3 weeks produced a significant increase in the lung tissue MDA content (47.32 ± 15.25 μM/g and 64.30 ± 18.14 μM/g protein, respectively). As shown in Figure 3B, bleomycin-induced increments in MDA content of the lung were significantly prevented by MXSGT treatment. The tissue MDA contents in the 4-week groups remained at 35.44 ± 7.71 μM/g protein.

IV. *Myeloperoxidase Analysis of Lung Tissue*

As shown in Figure 3C, MPO, a marker of neutrophils influx in tissue, showed significantly increased levels in rats treated with bleomycin alone for 2 weeks (0.08 ± 0.01 U/g protein). The increase in lung tissue MPO activity produced by bleomycin administration was significantly prevented by MXSGT treatment for 4 weeks (0.06 ± 0.02 U/g protein).

V. *Nitric Oxide Analysis of Lung Tissue*

Bleomycin produced a significant increase in lung NO level (Figure 3D) with 2 weeks of induction (14.25 ± 2.36 μM/g wet tissue). Treatment groups significantly prevented the increases in the lung tissue NO contents produced by bleomycin. The lung tissue NO contents resulted in 9.56 ± 2.56 μM/g of wet tissue by treatment with MXSGT for 4 weeks, after treatment of bleomycin for two weeks.

VI. *HPLC Analysis of MXSGT*

We further explored the HPLC chromatograms of the MXSGT extracts by comparison with standards of ephedrine (the reference compound for *Ephedra sinica*), amygdalin (the reference compound for *Prunus armeniacae*), liquiritin and glycyrrhizin (the reference compounds for *Glycyrrhizae uralensis*) in a linear gradient solvent system. The retention

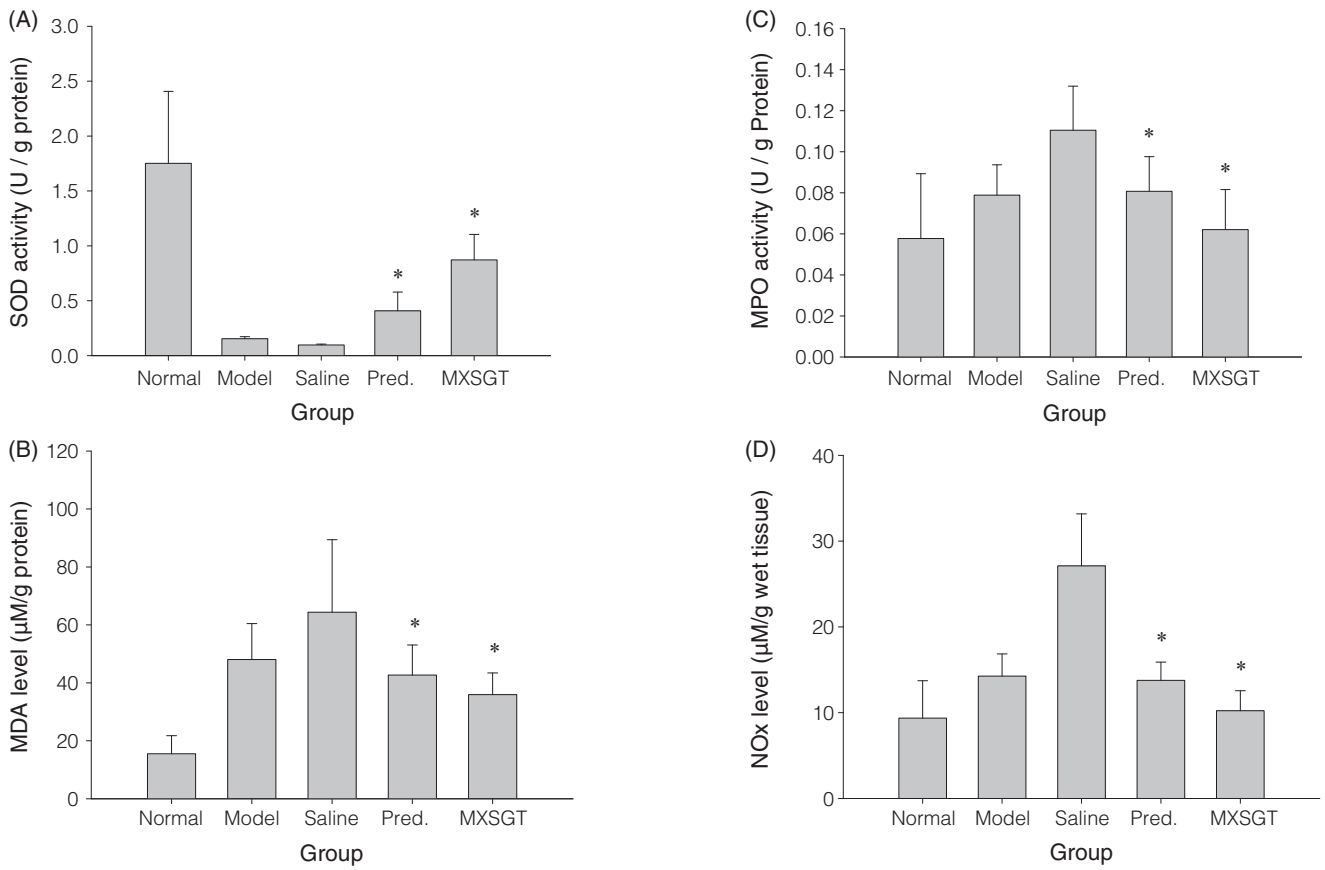

Figure 3. (A) SOD levels of lung tissue in each group. A: normal lungs, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks. Data were presented as mean ± SD in each group, * $p < 0.05$. (B) MDA levels of lung tissue in each group. A: normal lungs, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks. Data were presented as mean ± SD in each group, * $p < 0.05$. (C) MPO activity of lung tissue in each group. A: normal lungs, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks. Data were presented as mean ± SD in each group, * $p < 0.05$. (D) NOx levels of lung tissue in each group. A: normal lungs, B: 2 weeks after BLM-instillation, C: saline treatment for 4 weeks, D: prednisolone treatment for 4 weeks, E: MXSGT treatment for 4 weeks. Data were presented as mean ± SD in each group, * $p < 0.05$.

times for ephedrine, amygdalin, liquiritin and glycyrrhizin were 8.3, 13.9, 21.4 and 44.8 min, respectively. The HPLC profile of MXSGT product was also proposed. The representative HPLC chromatogram is shown in Figure 4.

DISCUSSION

In this study, MXSGT was observed to be able to prevent bleomycin-induced lung fibrosis in rats. On the basis of the improvement of the appearance of the lungs in the treatment groups and histological graphics, MXSGT may be a potential formula in the treatment of IPF. The formula used to study IPF was suggested by a traditional Chinese medicine doctor, Dr. Kuo-Chuang Mei at Hubei University of Chinese Medicine. The formula was effectively prescribed by TCM doctors during the SARS outbreak. Indeed, antioxidant therapy has been shown to block the development of lung fibrosis in animal models[22]. The experimental model shown in the current investigation is not a pre-treated or co-treated model; it is a post-treated model in which MXSGT was given after bleomycin-induced lung fibrosis for two weeks in rats. Furthermore, different inflammatory-related cells were inhibited by treatment of MXSGT. The design also presents evidence for the potential uses of the formula, MXSGT.

Inflammation and oxidative stress are a complex reaction to stimuli such as infection, trauma, or exposure to toxic drugs (bleomycin, cisplatinum and adriamycine) or irritants. ROS is considered the initial inflammation process, produced by bleomycin after its oxidation into the bleomycin-Fe (II) complex, activated by polymorphonuclear leukocytes[23,24]. Furthermore, activated macrophages have been shown to produce both NO and peroxynitrite, which are potent oxidants produced by the rapid reaction of NO. Taking into account the two reactions of nitrative and oxidative stress, two strategies may be considered to prevent the development of bleomycin-induced lung fibrosis.

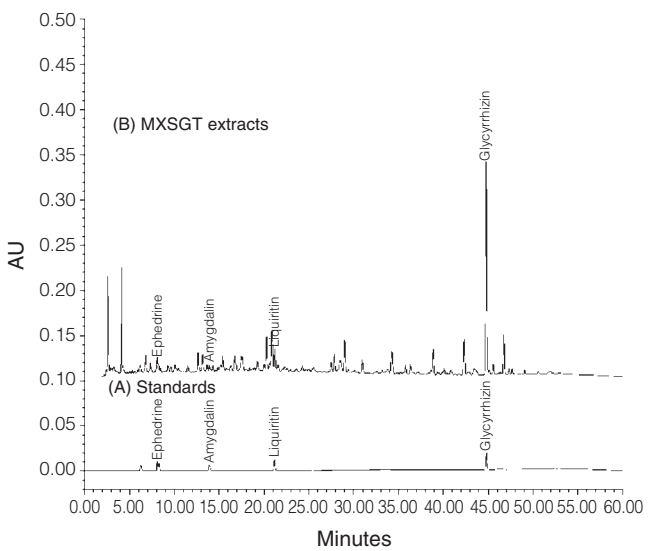

Figure 4. Four purified components, namely ephedrine, amygdalin, liquiritin and glycyrrhizin used for identification analysis of the complex MXSGT formula.

In this study, we demonstrated that intratracheal administration of a high dose (5 mg/kg) of bleomycin led to the development of lung fibrosis, using the pathologic investigation and measurements of lung NO content. Our data are consistent with the findings that alveolar macrophages, *in vitro*, produce superoxide and peroxynitrite from locally derived NO[25]. Recent studies have shown an increasingly insignificant role for NO in pathogenesis of bleomycin-induced lung fibrosis[9] and idiopathic pulmonary fibrosis[26]. Our results are also consistent with the findings by Dörger *et al*.[27], demonstrating increased nitrotyrosine formation of lungs from wild-type mice upon instillation of intratracheal asbestos fiber. ROS and RNS have been implicated in the pathogenesis of asbestos-fiber associated pulmonary fibrosis in a process similar to the pathogenesis of bleomycin-induced lung fibrosis. However, we did not determine the mechanism of tyrosine nitration in our study, but well-established induction of ROS by bleomycin leads us to speculate that peroxynitrite may be the predominantly formed reactive nitrogen species leading to strong nitrotyrosine formation in rats treated with a single dose of intratracheal bleomycin. Furthermore, this study clearly demonstrates that treatments of MXSGT significantly prevent the increase NO levels in lung tissue. Preventive effects of MXSGT on the depletion of lung tissue SOD activities and the increments in lung tissue MDA level may possibly result in their antioxidant activity[26]. In the present study, the inhibitory effects of MXSGT on the accumulation of leukocytes into the lung manifested by reducing the increase in MPO activity in lung tissue and in the neutrophils number in BAL fluids. This may contribute to further protection of the lung from free radical damage.

These findings are consistent with the protective effects of MXSGT at a dose of 1 g/kg/day against bleomycin-induced lung fibrosis. Therefore, it may be speculated that the inhibitory effect of antioxidant agents on the development of bleomycin-induced lung fibrosis in animal models is due to their preventive effect against the peroxynitrite mediated tyrosine nitration[26,28,29]. In addition, the HPLC profile of MXSGT and its four chemical standards were also studied. We hope that this preliminary *in vivo* data for MXSGT will lead to advancements in investigation and a better understanding of this TCM formula.

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