Original Article

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Protective Effects of Hydroalcoholic Extract of Hypericum Perforatum Against Bleomycin-Induced Pulmonary Fibrosis in Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Pulmonary fibrosis is a chronic interstitial lung disease caused by parenchymal lung damage due to inflammatory factors and fibrosis. Hypericum perforatum contains various flavonoids with antioxidant properties. This study aimed to evaluate protective effects of hydroalcoholic extract of Hypericum against bleomycin-induced pulmonary fibrosis in rats.

METHODS: This empirical study was conducted on 30 Wistar rats weighing 150-180 grams. Animals were randomly divided into five groups of six. Group one received a single dose of normal saline intraperitoneally, and group two was administered with bleomycin (7.5 units per kg) intratracheally. Other groups received daily doses of Hypericum extract (50, 100 and 200 mg/kg) via intraperitoneal injection one week before and two weeks after bleomycin administration. After 21 days, animals were sacrificed, and blood and lungs were collected for histopathological examinations, and measurement of plasma malondialdehyde (MDA) and lung hydroxyproline (HP).

FINDINGS: In this study, lung index, HP and plasma MDA in normal saline group were respectively 7.09±0.32 mg per kilogram of body weight, 1.80±0.23 mg per gram of lung tissue, and 1.32±0.27 micromoles per liter of plasma. In rats administered with bleomycin, these values were 9.75±0.90, 5.43±0.7 and 3.04±0.42, respectively. Treatment with Hypericum extract, especially at dosage of 200 mg/kg, resulted in a significant reduction in the aforementioned parameters compared to bleomycin group ($p<0.05$).

CONCLUSION: According to the results of this study, hydroalcoholic extract of Hypericum perforatum could exert protective effects against bleomycin-induced pulmonary fibrosis.

KEY WORDS: *Bleomycin, Hypericum extract, Pulmonary fibrosis, Rat.*

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Introduction

Pulmonary fibrosis is a chronic interstitial lung disease, which is caused by parenchymal lung damage due to inflammatory factors and fibrosis. This disease is associated with high mortality rate and is resistant to medical treatments. Survival rate of patients with pulmonary fibrosis has been estimated at 2-3 years (1). At present, causes and pathogenesis of pulmonary fibrosis remain unclear, while several factors such as reactive oxygen species, growth factors, inflammatory cells (e.g., lymphocytes and neutrophils), cytokines and chemokines may directly or indirectly involve in the process of fibrosis (2, 3).

Some of the contributing factors to this disease are infections caused by herpes viruses and bacteria, damages caused by mineral compounds (e.g., asbestos and silica) and side effects of chemical drugs, such as bleomycin and methotrexate (4). Bleomycin is a potent antimicrobial antibiotic with therapeutic effects on squamous cell carcinomas of head and neck, testicular cancer, soft tissue sarcomas and lymphogranuloma (5). Through extensive immunologic reactions, bleomycin may damage alveolar cells, increase accumulation of inflammatory cells and collagen tissues in airbags, and stimulate proliferation of fibroblasts in interstitial airbags. This process will eventually lead to fibrosis. Furthermore, it has been reported that bleomycin changes the metabolism of eicosanoids, and if these changes target prostaglandins and thromboxane, fibrosis is likely to occur (6).

On the other hand, bleomycin contributes to the production of transforming growth factor beta protein, which is considered as a tumor growth factor and plays a pivotal role in the proliferation of fibroblasts and development of pulmonary fibrosis (7). Despite extensive research in this area, major causes and pathogenesis of pulmonary fibrosis remain unclear. In previous studies, various compounds have been suggested for the prevention and treatment of pulmonary fibrosis through different mechanisms (4, 8). However, no specific medication has been introduced for definitive treatment of pulmonary fibrosis (9-11). One of the proposed treatment methods involves the use of antioxidants in order to eliminate free radicals and prevent inflammatory processes (12).

Today, use of herbal medications has drawn the attention of many researchers. Hypericum perforatum is a glabrous plant with average height of 10-110 cm and yellow glowing flowers without fluff. H. perforatum is mostly used for its blossoming branches and flowers (13). According to phytochemical studies, H. perforatum is a rich source of flavonoids and anthocyanins with remarkable antioxidant properties (14). Quercetin and luteolin are the most important flavonoids in this plant. Amount of flavonoids in flowers of H. perforatum is 11.7% and it is 7.4% in the leaves and branches (15). This medicinal herb has been widely used for the treatment of depression and neurological disorders (16, 17).

According to the literature, flavonoids and hyperforin from H. perforatum are involved in anti-inflammatory, analgesic, antibacterial and antiviral properties of this plant. Among other functions of these two compounds are changes in capillary permeability, anti-arrhythmia effects, coronary vasodilation, anti-spasmodic effects and changes in the intensity and velocity of cardiac muscle contractions (18).

Pulmonary fibrosis is a progressive inflammatory disease starting with acute lung inflammation, which gradually becomes chronic and leads to fibrotic changes in the alveoli of lungs. Hypericum extract has been shown to have anti-inflammatory properties, as well as flavonoids such as quercetin (8). Considering the side effects and high treatment costs of chemical drugs prescribed for patients with pulmonary fibrosis, this study aimed to evaluate the protective effects of hydroalcoholic extract of Hypericum against bleomycin-induced pulmonary fibrosis.

Methods

Preparation of herbal extract: In this study, Hypericum perforatum was purchased from Goldarou Company in Isfahan, Iran. The plant was harvested in June and identified in Isfahan Center for Research of Agriculture Science and Natural Resources (herbarium number: 11427). To prepare the hydroalcoholic extract of Hypericum, we used the soaking method (19, 20). Initially, twigs of the plant were quickly dried in shade and scattered into small pieces (2-3 cm).

Afterwards, twigs were soaked in 70% ethanol solution (30% water, 70% ethanol) for 72 hours. After three days, the extract was filtered, and 70% ethanol was poured on the remaining waste, which was added to the initial extract.

Following that, obtained extract was passed through filter paper, and the filtered solution was concentrated using a rotary device. Dried extract was prepared after it was placed in an oven at temperature of 40-30°C (19, 21-23).

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Study animals: This empirical study was performed on 30 healthy male adult Wistar rats weighing 150-180 grams. Animals were purchased from Laboratory Animal Production Center of Isfahan University of Medical Sciences. They were kept in polycarbonate cages at temperature of 24±4°C within the photocycle of 12 hours of light and 12 hours of darkness. In addition, they had free access to special compressed food and tap water. In order to adjust with laboratory environment, animals were preserved in test conditions one week prior to the study. Afterwards, they were divided into five groups of six, as follows:

1.Group one (Normal saline) received physiologic saline solution via intraperitoneal injection for 21 consecutive days.

2.Group two (Bleomycin) received drug carrier (normal saline) with an equivalent volume for seven days before and 14 days after receiving a single dose of bleomycin (7.5 IU/Kg).

3.Groups three, four and five received 50, 100 and 200 mg/kg of Hypericum extract intraperitoneally for seven consecutive days before and 14 consecutive days after receiving a single dose of bleomycin (7.5 IU/Kg) intratracheally (21, 24, 25).

Dosages used in this study were based on the findings of previous studies, which confirmed the preventive and protective effects of this herbal extract on the inflammation and pain caused by formalin (pulmonary fibrosis is a progressive inflammatory disease). At this stage, we also considered studies that evaluated effects of similar plants on pulmonary fibrosis induced by bleomycin (26). In the review of literature, there were no reports of toxic or risky side effects due to the applied doses of Hypericum extract. Moreover, previous research has confirmed the protective effects of this medicinal herb on liver and kidneys against drug poisoning (27).

At the end of experiment and after weighing the animals, they were anesthetized via ketamine injection. Afterwards, chests were cut open, and lungs were carefully removed and weighed. After cleaning the lungs with cold normal saline, the left lung was separated for histological examinations, and one section of the lung was placed in 10% formalin solution. For histopathological examination, six slides were provided from each group (one slide per each animal), and five fields were assessed for each slide. Remainder of the lungs were preserved in special containers in a freezer (-70°C) for biochemical tests.

Measurement of lung hydroxyproline (HP): For measuring HP, 1% homogenized tissue was obtained in hydrochloric acid 6.0N from the right lung of animals, and lung HP was measured using the colorimetric method of Edwards and O'Brien. Moreover, we used Ehrlich's reagent with Chloramine T and hydroxyproline standards (28-31).

Measurement of lipid peroxidation (Malondialdehyde): In this study, we used Satoh's method with slight alterations in order to measure lipid peroxidation (32). Accordingly, 500 microliters of plasma was added to 1.5 milliliters of 10% trichloroacetic acid and centrifuged for 10 minutes at 4000 rpm. Afterwards, 1.5 milliliters of the supernatant was removed, and 2 milliliters of 0.67% thiobarbituric acid was added to it to boil for 30 minutes in a bain-marie.

After cooling, 2 millilitres of n-Butanol was added, and the compound was thoroughly mixed and centrifuged for 15 minutes at 4000 rpm. Following that, the pink supernatant was removed, and its absorption was expressed using a spectrophotometer at wavelength of 532 nm. In order to draw malondialdehyde (MDA) standard curve, various densities of tetra ethoxy propane were prepared in nanomoles.

Statistical analysis: Data analysis was performed in SPSS V.16, using one-way analysis of variance (ANOVA) and Tukey's test, and $p<0.05$ was considered significant.

Results

In this study, level of lung tissue HP in normal saline and bleomycin groups was 1.80±0.23 and 5.43±0.7 mg per gram of lung tissue, respectively ($p<0.05$). In addition, level of lung tissue HP in groups receiving 50, 100 and 200 mg/kg of Hypericum extract was 4.66±0.61, 3.45±0.29 and 2.59±0.40 mg of lung per kg of body weight, respectively (fig 1).

Level of plasma MDA in normal saline and bleomycin groups was 1.32±0.27 and 3.04±0.42 micromoles per liter of plasma, respectively ($p<0.05$). Moreover, MDA levels in groups receiving 50, 100 and 200 mg/kg of Hypericum extract were 2.74±0.21, 2.24±0.16 and 1.74±0.29, respectively (fig 2). With respect to lung index, values in normal saline and bleomycin groups were 7.09±0.32 and 9.75±0.90 mg per kg of body weight, respectively ($p<0.05$). Moreover, lung index in groups receiving 50, 100 and 200 mg/kg of Hypericum extract was 9.28±0.51,

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8.91±0.83 and 8.29±0.55 micromoles per liter of plasma, respectively (fig 3).

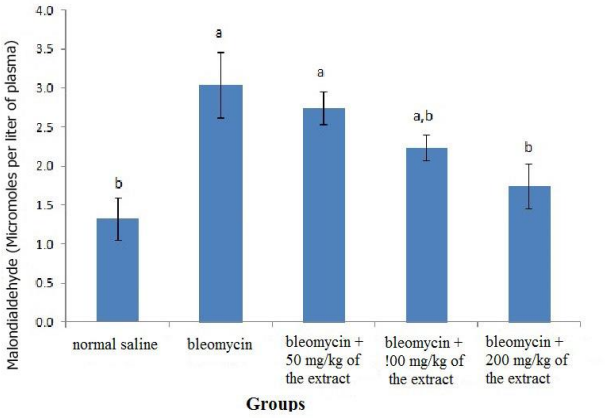

Figure 1. Effects of hydroalcoholic extract of hypericum perforatum on plasma malondialdehyde; a) Significant difference with normal saline (p<0.05); b) Significant difference with bleomycin (p<0.05)

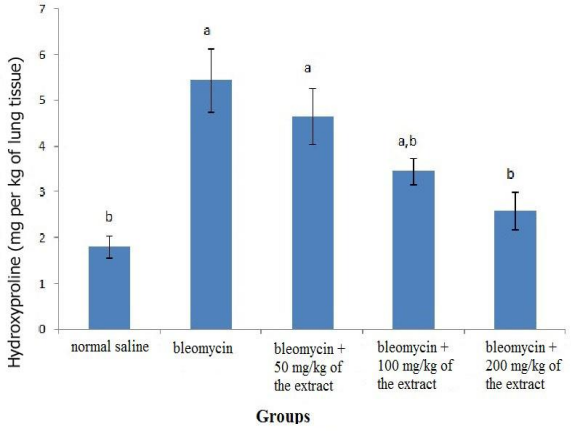

Figure 2. Effects of hydroalcoholic extract of hypericum perforatum on lung tissue hydroxyproline; a)Significant difference with normal saline (p<0.05); b) Significant difference with bleomycin (p<0.05)

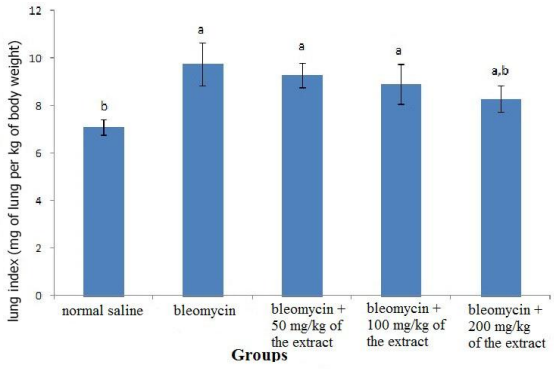

Figure 3. Effects of hypericum perforatum extract on lung index; a) Significant difference with normal saline (p<0.05);b)Significant difference with bleomycin (p<0.05)

Microscopic observation of lung tissues in different groups of animals based on hematoxylin and eosin stain indicated that rats in normal saline group had lung tissues with normal color, as well as normal alveolar and interstitial tissues with no damage. In addition, accumulation of inflammatory cells and collagen fibers and/or fibrosis was not detected in these subjects (fig 4A).

In bleomycin group, microscopic examinations revealed accumulation of connective tissue, fibroblasts in alveolar spaces, thickening of alveolar walls, massive alveolar destruction and accumulation of fibroblasts and collagen fibers in some areas of the lungs. As such, hemosiderin pigment deposition was indicative of previous bleeding (fig 4B).

According to the other results of this study, inflammatory and fibrotic lesions had a more significant decrease in animals receiving bleomycin with 50 mg/kg of Hypericum extract compared to bleomycin-only group. However, thickness of alveolar walls and inflammatory cells were detected in several areas of the lung tissue (fig 4C).

In the group receiving bleomycin with 100 mg/kg of Hypericum extract, lung damage was reduced through inhibition of inflammatory cell infiltration, and thickness of alveolar septal walls was detected as well (fig 4D). On the other hand, most sections of the tissue had a normal structure in animals receiving bleomycin with 200 mg/kg of Hypericum extract, while lung tissue damage significantly reduced (fig 4E).

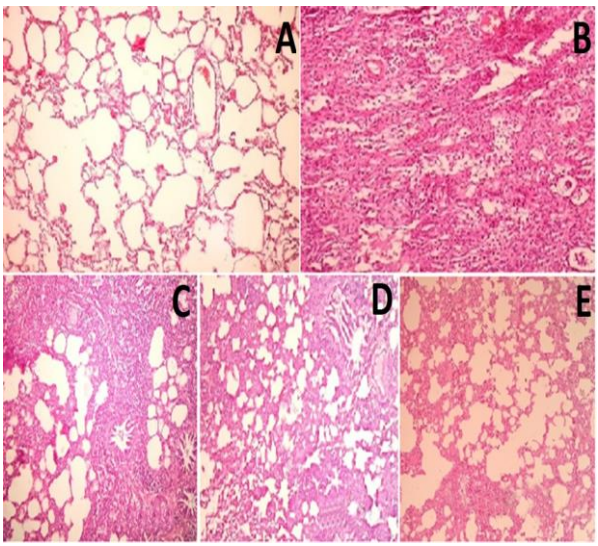

Figure 1. Image of tissue sections obtained from lungs of rats in different groups (A-E); hematoxylin and eosin staining with 400x magnification

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Discussion

According to the results of this study, the highest mean values for plasma MDA, lung tissue HP and lung index were observed in the group receiving bleomycin, while the lowest amounts were observed in normal saline group. Increased levels of plasma MDA in animals of positive control group was due to the development and progression of pulmonary inflammatory responses. In the event of pulmonary inflammatory diseases, some of the oxidants produced in lungs cross through the cell membrane, enter the bloodstream and oxidize unsaturated fats.

In one study, Nohrstedt et al. reported that H. perforatum contains a variety of active components, including flavonol glycosides, phenyl-propane, biflavones, tannins, pro-anti cyanidins, xanthines and flavonoids (33). In the present study, different components from the hydroalcoholic extract of H. perforatum significantly reduced oxidative compounds in the blood of ill rats, and level of plasma MDA also decreased in protective groups one, two and three by 9.8%, 26.4% and 42.9%, respectively compared to bleomycin-only group.

It seems that flavonoids such as quercetin, rutin and anthocyanoside that are found in H. perforatum extract contributed to the reduction of plasma MDA levels in protective groups. These flavonoids are considered as the most important substances in H. perforatum with preventive and inhibitory mechanisms against pulmonary fibrosis induced by bleomycin. Flavonoids are able to collect reactive oxygen species, chelate iron ions and inhibit pro-oxidative lipids (34). Previous studies suggest that flavonoids could decrease inflammation through reducing tumor necrosis factor alpha (TNF-α) and interleukin-1 cytokines (35). Furthermore, by affecting the synthesis of arachidonic acid and inhibition of eicosanoid production, flavonoids are able to reduce inflammatory response (36). On the other hand, flavonoids could act as antioxidant agents and protect cells against depletion of reduced glutathione by increasing enzymatic capacity of glutathione, glutathione reductase, glutathione peroxidase and catalase (35). Also, they could exert positive effects through chelating iron, which is a major contributing factor to the formation of free radicals (37).

Through reducing non-removable leukocytes and inhibiting degranulation of neutrophils, flavonoids could prevent the fixation and adhesion of leukocytes to the endothelial wall, as well as the formation of oxygen free radicals and inflammatory intermediaries, which result in higher complementary activation. Moreover, flavonoids could decrease inflammation by restricting the activity of NF-κB protein (35, 36). Hydroalcoholic extract of Hypericum perforatum contains numerous phenolic compounds, such as flavonoids and phenolic acids, which indicate the antioxidant properties of this medicinal herb (38).

In one study, Verma et al. claimed that quercetin exerts protective effects on lungs against pulmonary fibrosis by reducing TNF-α, increasing inflammatory cells in bronchoalveolar lavage fluid, decreasing superoxide dismutase activity and reducing the level of MDA and HP (8).

According to the results of the current study, doses of 100 and 200 mg/kg of H. perforatum extract could definitely inhibit inflammation and eventually prevent progressive fibrosis induced by bleomycin with remarkable antioxidant properties. In a review study, Russo et al. evaluated the pharmacological effects of Hypericum and reported that hyperforin from the plants of phloroglucinol family (e.g., Hypericum perforatum) affects molecular inhibition of NF-κB protein, which plays a pivotal role in the reduction of inflammation and inflammatory angiogenesis. Recent studies have highlighted this effect as a common mechanism of medicinal herbs for the prevention of pulmonary fibrosis induced by bleomycin (39). Xanthine is another major compound found in Hypericum. In a study by Libroski et al., xanthenes were reported to have potent anti-inflammatory and analgesic properties, which appear through the activation of anti-inflammatory cytokines and inhibition of pro-inflammatory cytokines, such as TNF-α (40).

Pulmonary fibrosis and inflammation are directly correlated, while inflammatory and anti-inflammatory cytokines also have a significant role in the progression of pulmonary fibrosis. Preventive effects of Hypericum extract against pulmonary fibrosis are partly associated with the presence of xanthines in this medicinal herb. In one study performed in Portugal to evaluate antioxidant features of Hypericum methanol extract, IC_{50} was calculated at 21 micrograms of equivalent biomass of dry weight per milliliter. Final results of the study were indicative of high antioxidant activities from Hypericum perforatum (41).

Pulmonary fibrosis is accompanied by accumulation of inflammatory cells in the alveolar space, increased thickness of alveolar walls and development of fibrotic lesions (10, 11). Results of the

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present study revealed that hydroalcoholic extract of H. perforatum decreases the thickness of alveolar walls and restricts the progression of fibrosis in rats administered with bleomycin. Pulmonary fibrosis is caused by penetration of extracellular matrices, collagen deposition and proliferation of cells in interstitial tissues. Large proportions of these cells account for fibroblasts and myofibroblasts, which increase the production of pulmonary collagen leading to pulmonary disability (42).

In the current study, collagen content of lung tissues in protective groups one, two and three was respectively 14.3%, 36.6% and 52.3% lower than bleomycin group. Moreover, collagen content at the highest dose was close to the values in animals of the control group, and no significant difference was observed between the study groups in this regard. According to the other findings of the present study, although prescription of antioxidants cannot prevent pulmonary fibrosis induced by bleomycin, it could delay the progression of the disease. In conclusion, apart from free radicals and reactive oxygen species, several other factors are involved in the occurrence of initial inflammatory response in pulmonary fibrosis. According to the results of this study, hydroalcoholic extract of Hypericum perforatum could inhibit collagen deposition and lipid peroxidation, and decrease plasma MDA levels. These functions will ultimately prevent the occurrence of pulmonary fibrosis. It is recommended that future studies be conducted as to determine the positive effects of Hypericum herbal extract on diseases that start and progress with inflammatory processes, such as pulmonary fibrosis.

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