

Original Article**Saffron Protection against Bleomycin-Induced Pulmonary Fibrosis in Rats**

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ABSTRACT

Background: Bleomycin-induced lung fibrosis has been accepted as an animal model for fibrosis in rats. The aim of this study was to evaluate the effects of saffron aqueous extract on this disorder paving the way for more investigation in treating idiopathic pulmonary fibrosis in human.

Methods: Male Wistar rats (250–300 gr) were instilled a single dose of bleomycin (5 mg/kg) via intratracheal tube (n=6) in 2015. Sham group received normal saline. Saffron aqueous extract (50 mg/kg and 100 mg/kg) were given orally in two different treated groups with bleomycin for 28 days. Lung Indices was calculated at the end of this experiment. Lung segments fixed in 10% formaldehyde were used for pathological preparation with Hematoxylin & Eosin and trichrome staining.

Results: The body weight was decreased and lung Indices increased in bleomycin group ($P<0.5$). Bleomycin administration increased myeloperoxidase, malondialdehyde and finally TNF- α in lung tissue homogenates ($P<0.05$) compared with sham group. The fibrotic process and thickening of alveolar septa in treated rats with bleomycin were increased by H&E and Masson Trichrome staining. Saffron treatment (50 and 100 mg/kg) attenuated the increase in MDA (264.43 ± 10.4 nmol/g by the higher dose versus 378.4 ± 18.1 nmol/g), MPO (0.19 ± 0.03 and 0.13 ± 0.04 IU/ml versus $0.39.2\pm 0.05$ IU/ml) and TNF- α level (18.42 ± 3.7 ng/ml and 14.31 ± 3.6 ng /ml versus 35.32 ± 4.2) in lung homogenates compared to bleomycin group ($P<0.05$). It decreased collagen accumulation and alveolar destructive patterns in pulmonary fibrosis.

Conclusion: This study introduces saffron as novel anti-fibrotic agent against bleomycin-induced fibrosis due to histological examinations and preventive effects on destructive enzyme release in rats.

Keywords: Bleomycin, Fibrosis, Rat, Saffron.

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INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a poor prognostic, deteriorating and progressive lung condition in which alveolar septa are damaged and scarred. This disease is limited to this organ and identified by accumulation of macrophages and neutrophils in the respiratory tract with the fibrosis of the alveolar walls making more difficult to the lung to work correctly [1, 2]. IPF occurs predominantly in older people, more frequently men with a poor prognosis making their breathing hard. This lung disorder affecting 85000 to 100000 people in the United States is increased with age in recent years [3]. IPF as an idiopathic disorder can be caused by different exposures including cigarette

smoke, dust, gastroesophageal reflux and other environmental agents [4].

Crocus sativus L. customarily known as saffron is a traditional spice cultivated mostly in Iran and other countries like Turkey and Spain [5]. Dried red stigma with a portion of yellowish attached style is used as commercial saffron after preparation in cooking. This plant is used in many countries as an herbal medicine with different properties including oxytocic, anti-carcinogenic, anti-depressant, and anti-asthmatic effects [6, 7]. Cytotoxic and apoptotic effects of this spice in lung cancer cells via caspase-dependent activation made it suitable for treatment of respiratory malignancies. Antitussive effects of saffron and its ingredients,

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safranol, and crocin in guinea pigs was demonstrated in previous reports [8, 9]. Recent clinical trials showed disappointing results in treatment of IPF due to its complications highlighting the importance of early diagnosis and treatment of patients [10-12]. At present, it is not clear how saffron affects lung fibrosis according to its antioxidant and radical scavenging activity properties [13].

The aim of this study was to evaluate potentials of this remedy to treat fibrosis induced by intratracheal instillation of bleomycin in rats as the most widely accepted model for chemical induction of pulmonary fibrosis with similar histopathological and enzymatic changes compared to IPF in human [14, 15].

We aimed to evaluate the potential of oral saffron to attenuate the bleomycin-induced fibrosis in rats with measurement of destructive enzymes and extent of it via pathological examination in our treated groups.

MATERIALS AND METHODS

Animals

Eighty male wistar rats (250–300 g) were purchased from Animal Breeding Center, Shiraz, Iran in 2015. They were placed in groups of three in PVC cages with free access to water and normal laboratory chow in animal house of Bushehr University of Medical Sciences. Animals were kept at 20±2 °C and maintained at 12 h light-dark cycle starting at 7 a.m.

The experimental procedures were approved by the Ethics Committee for animal care of Bushehr University of Medical Sciences.

Chemical Agents

Aqueous saffron extract was kindly donated from Zardband Pharmaceutical Company in Iran. Briefly, saffron stigmas were prepared from Khorasan Province in northeast of Iran. Dried and grinded stigmas following putting the plant in shade were macerated in water for 3 days. The aqueous solution was filtered and concentrated to desired concentration (100 mg/ml) of dry weight.

Myeloperoxidase (MPO) and malondialdehyde (MDA) Elisa assay kits were purchased from ZellBio GmbH Company of life sciences (Germany). Tumor necrosis factor alpha (TNF- α) Elisa assay kit was prepared from Diaclone life company (France). Bleomycin hydrochloride (Bleo-cell) was purchased from cell-pharm GmbH (Germany).

Experimental Protocol

Male Wistar rats were anesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg) intraperitoneally and placed supine on a heated surgical table to keep the animal warm at 37 ± 1 °C, monitored through a rectal probe connected to a thermistor (Physitemp BAT-12; Texas Scientific Instruments, San Antonio, Texas, USA). The trachea was exposed following a midline incision. Rats were divided into four groups (n=6), i.e. positive control (PC), negative control (NC), low dose saffron (LS) and high dose Saffron (HS). Animals in the group NC, received normal saline intratracheally (0.2 ml) while other groups injected with 5mg/kg bleomycin (dissolved in 0.2 ml normal saline). In groups LS and HS, animals were given aqueous saffron solution orally by feeding tube (50 mg/kg and 100 mg/kg) respectively from two days before the operation until 28 d after bleomycin injection once daily according to previous articles published in literature [16-18]. Other groups received deionized water according to the same schedule. The weights of animals were recorded daily in animal room in all groups. Twenty-eight days after bleomycin injection at day 0, all rats were anesthetized and sacrificed after measuring their weights. Both lungs were removed quickly and washed with cold saline. The weight of the lungs was measured for each animal. Right lungs were kept in 10% formaldehyde solution for histological evaluation via Hematoxylin & Eosin (H&E) and Masson trichrome staining while the other lungs stored in -80 °C for biochemical assays and cytokine detection.

Enzyme measurements were performed by left lung homogenization (10%) in the normal saline on ice according to their weight. After centrifugation (2000×g for 20 min at 4°C), their supernatant was collected and stored at -20 °C. Measurements of MPO, MDA, and TNF- α were performed according to the manufacturer's protocol [19].

Histological Examination

Lung segments fixed in 10% formaldehyde for 24 h dehydrated in ethyl alcohol and embedded in paraffin. The tissue segments (5 μ m) were stained with H&E and Masson Trichrome for general morphology and detection of fibrosis respectively.

Statistical Analysis

Statistical analysis was performed using SPSS, ver. 16.0 (Chicago, IL, USA) Data are presented as mean ± SD. Statistical differences between groups

were analyzed by one-way analysis of variance, followed by post-hoc multiple comparison tests. P -value <0.05 was considered to indicate statistically significant differences.

RESULTS

Effects of Saffron on Body Weight and Lung Indices

According to Figure 1A, there was a moderate decrease in body weight of bleomycin-treated rats peaking at day 15. This effect was concentration-dependently prevented by saffron administration. Saffron treatment (50 and 100 mg per kg) led to a significant decrease in lung index (dividing lung weight by body weight and multiplying by 100) compared to bleomycin treated rats ($P<0.05$) as shown in Figure 1B.

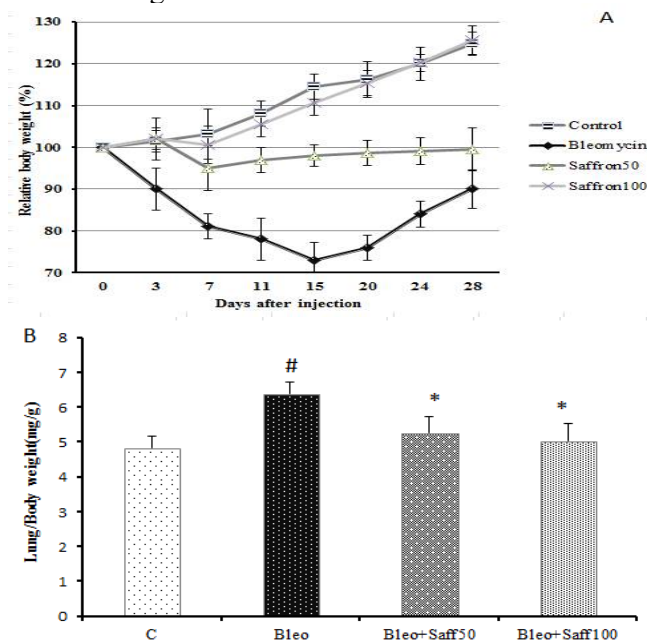


Figure 1A. Effects of Saffron aqueous extract (50 and 100 mg/kg) on weight loss in bleomycin-treated rats. Animals were randomly divided into weight-matched groups. The body weight on day of injection was considered as 100%. Data were presented as mean \pm SD ($n=6$).

B. Impact of saffron on lung indices in bleomycin-treated rats. This parameter was measured as ratio of lung weight (mg) to body weight (G) of each rat. Values were expressed as mean \pm SD ($n=6$). # $P<0.05$ versus Control group; * $P<0.05$ versus bleomycin group.

Oxidant Stress Markers

Malondialdehyde (MDA) formed from the endoperoxides breakdown during oxidation of a polyunsaturated fatty acid is the most widely used parameter in evaluation of tissue oxidative stress.

Lung MDA levels significantly increased in bleomycin treated rats (378.4 ± 18.1 nmol/g versus 238.3 ± 12.3) compared to control rats. According to Figure 2A saffron administration (100 mg per kg) once daily decreased the MDA levels (264.43 ± 10.4 nmol/g of tissues respectively) compared to bleomycin group ($P<0.05$). Figure 2B expressed that lung myeloperoxidase activity was increased significantly at day 28 following intratracheal administration of bleomycin (0.392 ± 0.05 IU/ml) compared with control (0.16 ± 0.02 IU/ml) while saffron administration in both doses (50 and 100 mg/kg) produced significant decrease (0.19 ± 0.03 and 0.13 ± 0.04 IU/ml respectively) in this oxidant marker ($P<0.05$).

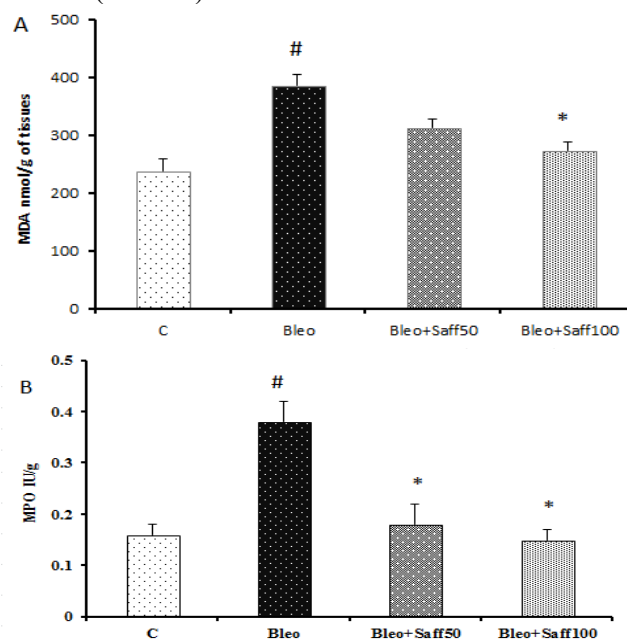


Figure 2. Influence of saffron aqueous extract (50 and 100 mg/kg) on both malondialdehyde (A) and myeloperoxidase (B) activity in the lungs of bleomycin-treated rats. Rats were sacrificed 28 days after bleomycin injection and these two products were measured in the lung homogenates according to the manufacturer's instruction. Data are presented as mean \pm SD ($n=6$). # $P<0.05$ versus Control group; * $P<0.05$ versus bleomycin group.

Effect of Saffron on Lung TNF- α

Level of TNF- α in Lung homogenates was measured in our study to determine the function of this cytokine in lung fibrosis produced by bleomycin injection. Bleomycin significantly increased it compared to control group at day 28 (35.31 ± 4.4 ng/ml versus 12.61 ± 2.3 ng/ml). As shown in Figure 3, Oral administration of saffron in 50 and 100 mg/kg in treated rats lowered this pro-inflammatory parameter dose in bleomycin treated

groups. (18.42 ± 3.7 ng/ml and 14.31 ± 3.6 ng/ml, respectively).

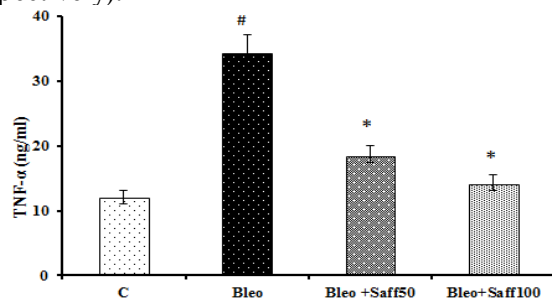


Figure 3. Effects of saffron aqueous extract (50 and 100mg/kg) on TNF- α in bleomycin- treated rats. Rats were sacrificed on day 28 after bleomycin injection. After centrifugation of homogenized lung, the supernatants were assayed for TNF- α . Values were expressed as mean \pm SD (n=6). # $P < 0.05$ versus control group. * $P < 0.05$ versus bleomycin group.

Histopathological Analysis

Rat lungs were examined histologically at day 28 in all groups. As shown in Figure 4, lung architecture in normal saline instillation as control group was intact while collapsing of alveoli, marked thickening of the septa and interstitial infiltration with inflammatory cells were present in bleomycin treated rats. Furthermore, excessive collagen deposition in bleomycin treated rats (Figure 4E) was demonstrated with masson trichrome staining compared to control (Figure 4D). Saffron treatment (100 mg/kg) following bleomycin instillation prevented significantly thickening of the septa, reduction of the alveoli and collagen infiltration as shown by masson trichrome staining (Figure 4F).

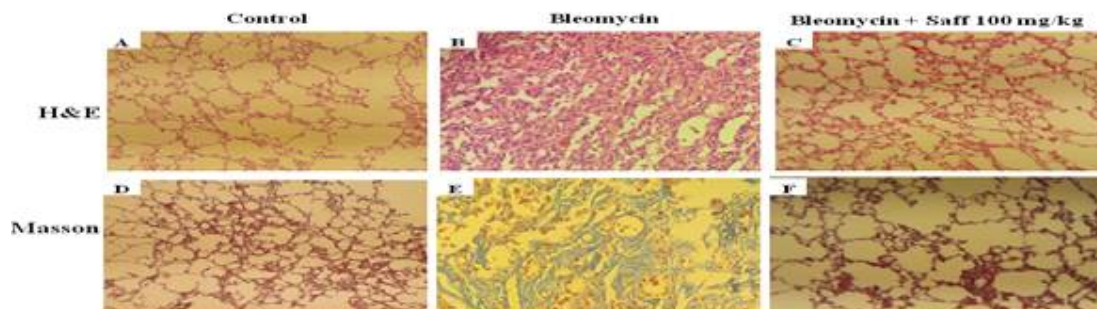


Figure 4. These histopathological figures represent Hematoxylin & Eosin (first row) and masson trichrome staining (second row) preparation of the lungs from animal in each treatment groups. Lung tissues were obtained after 28 d of bleomycin injection and viewed at a magnification of $\times 10$. Marked inflammation and fibrosis showing in-group (B) (Bleomycin) compared with group (A) (control). Significant suppression of the fibrosis induced with bleomycin-injection was seen in rat lungs in the group (C) (bleomycin + Saffron extract) in both rows. A-C. H.E stain; D-F. Masson trichrome stain. A, D. Control group; B, E. Bleomycin group; C, F. Bleomycin + 100 mg/Kg Saffron treated group.

DISCUSSION

Saffron is a spice derived from stigma of *crocus sativus* in the Middle East especially Iran. It has been used as a flavor and dye in the world. This spice has many antioxidant components making it effective in customary medicine for a wide range of conditions including assisting childbirth, urinary tract infections, antinociceptive effects and anti-inflammatory properties and so on [6, 20, 21]. There are some reports that demonstrates the effectiveness of this remedy in treating chronic coughing in guinea pigs [9].

To the extent of our knowledge, this study was the first to evaluate the beneficial effects of saffron in the fibrosis induced by intratracheal injection of

bleomycin in rats. In our study, histopathological analyses and biochemical changes were used to demonstrate the preventive properties of saffron on fibrotic phenomenon. Intratracheal injection of bleomycin is one of the most established methods that widely used for induction of pulmonary fibrosis [14]. The fibrotic stage usually begins following 14 d of inflammation induced by bleomycin injection with proliferation of fibroblasts and deposition of collagen [22, 23]. Imbalance between free radicals and antioxidants in lung tissue is the main cause of deleterious effects including irreversible fibrosis highlighting the importance of saffron as one of the most important sources of anti-oxidant components like other herbal medicine including resveratrol and curcumin [24-26]. According to Figure 1A and 1B,

saffron treatment via oral tube prevented body weight loss and increment the lung index induced with bleomycin injection like previous studies on herbal medicine reducing the severity of disorder [19, 27]. Malondialdehyde level as one of the main causes of cell membrane damage was extremely high in our study in bleomycin-induced group compared with control ($378 \pm 18.2 \text{ nmol/g}$ versus $238 \pm 8.3 \text{ nmol/g}$). The MDA level dramatically decreased in the group receiving saffron (100 mg/kg) compared with bleomycin group showing its high anti-oxidant properties of this remedy (Fig. 2A). According to Figure 2B, myeloperoxidase level contributing neutrophil activity in fibrosis phenomenon was markedly decreased by saffron treatment [28]. Inflammatory cytokines including TNF- α , IL-1 β , and IL-6 are increased following bleomycin treatment [29]. In the same manner, in our study, TNF- α as a proinflammatory cytokine was increased in bleomycin-treated group compared to control ($34.23 \pm 9.2 \text{ ng/ml}$ versus $12.43 \pm 6.4 \text{ ng/ml}$) while saffron treatment clearly prevented this event (Figure 3). The preventive effects of saffron on elevation of pro-inflammatory cytokines were previously reported on a wide range of diseases including renal ischemia, cerebral ischemia and myocardial infarction [30-32]. Significant alterations in the lung including alveolar septa thickening due to infiltration of macrophages and fibroblast proliferation is demonstrated in groups receiving bleomycin treatment (Figure 4B) comparing control group (4A) with Hematoxylin & Eosin staining. Saffron treatment like other previous anti-oxidant examinations strongly attenuated these effects in bleomycin treated rats (4C) [33, 34]. Masson staining as one of the best indicator of fibrosis, revealed it in the bleomycin-treated lung tissues (Figure 4E) compared with control (Figure 4D) while saffron treatment (100mg/kg) clearly reduced it in rat lungs with bleomycin (Figure 4F).

CONCLUSION

Oral saffron treatment with its high anti-oxidant properties will be a novel agent in protecting lung tissue against oxidative damages and fibrosis induced by bleomycin treatment in rats. This study leads the way for using this promising agent in patients suffering from IPF in human trials in the future.

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