Life Sciences 235 (2019) 116794

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Crocin attenuates lung inflammation and pulmonary vascular dysfunction in a rat model of bleomycin-induced pulmonary fibrosis

Marwa S. Zaghloul, Eman Said*, Ghada M. Suddek, Hatem A. Salem

Dep. of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, 35516 Mansoura, Egypt

ARTICLE INFO

Keywords:
Crocin
Halofuginone
Bleomycin
NrF2
HO-1
TGF-β1

ABSTRACT

Amongst the various forms of lung injury; pulmonary fibrosis remains the most intricate form with limited therapeutic options to both the patient and the physicians. Bleomycin (BLM) is a chemotherapeutic agent used for the treatment of various carcinomas; however, its therapeutic value is significantly limited by its associated pulmonary fibrosis. The current study highlights the prominent antioxidant, anti-inflammatory and anti-fibrotic effect of crocin against BLM-induced pulmonary fibrosis. Intratracheal BLM instillation induced significant biochemical, structural, functional and vascular pulmonary injury. BLM instillation increased oxidant load with quenching of antioxidant defenses together with increase inflammatory and fibrotic cytokines expression. Crocin significantly attenuated BLM-induced lung injury and its effect was comparable to the standard anti-fibrotic; halofuginone. The observed anti-inflammatory and anti-fibrotic and antioxidant impacts are thought to be embroiled in the therapeutic impacts of crocin. Down-regulation of TLR4, IL-10 expression is the major pathway involved in the observed anti-inflammatory effects and finally, down-regulation of tissue expression of TNF-α and TGF-β1 is the major pathways implicated in the observed anti-fibrotic activities and modulation of Nrf2 and HO-1 pathways is the main mechanism involved in the observed antioxidant effects.

1. Introduction

Pulmonary/Lung fibrosis is a chronic, progressive inflammatory lung disorder with well-established histopathological and pulmonary architectural changes. It is usually associated with significant impairment of respiratory functions [1]. Indeed, inflammatory lung disorders are eventually associated with loss of alveolar architecture, accumulation of myofibroblasts, extensive extracellular matrix (ECM) deposition and remodeling of lung parenchyma [2].

Many agents have been reported to contribute to the onset and the progression of lung fibrosis, of which, viral, radiotherapeutic and chemotherapeutic agents, aerosolized environmental toxins and certain connective tissue disorders [3].

Either alveolar epithelial injury or abnormal wound healing process involves multiple pathogenic events. Inflammation, activation of Th2 cytokines, activation of apoptotic pathways and down-regulation of re-epithelialization, myofibroblast proliferation, epithelial and excessive ECM deposition are amongst the main pathogenic events underlying the development of pulmonary fibrosis. Myofibroblasts induce fibrogenic cytokines, especially transforming growth factor-β1 (TGF-β1), increasing ECM deposition, and up-regulating tissue inhibitors metalloproteinases, with the enhancement of collagen deposition and suppression of its degradation [4].

Even though the fact that pulmonary fibrosis is a significant health problem with immense cost on the patient and his/her quality of life, it remains a disease without a definite therapy. Patients usually receive corticosteroids and cytotoxic agents. However, the undesired adverse effects significantly curb their therapeutic value [5]. Lung transplantation remains the only effective maneuver to reduce patients' suffering, but, like corticosteroids and cytotoxic agents, the surgical procedure is associated with significant risks and numerous complications [6].

Bleomycin (BLM) is an antibiotic used as a chemotherapeutic agent for the treatment of several carcinomas. BLM associated functional and biochemical changes have been linked to the onset of the development of pulmonary fibrosis [7]. Experimental models of BLM-induced pulmonary fibrosis are reported to be a valuable tool in delineating both the mechanisms of pulmonary fibrosis and the therapeutic impact of various proposed therapies. Pieces of literature have referred to the potential pathogenic pathways underlying BLM-induced pulmonary fibrosis, of which, induction of reactive oxygen species (ROS) induction of DNA damage, initiation of inflammatory and fibro-proliferative responses, depletion of endogenous antioxidants and exacerbation of oxidant-mediated tissue injury [8].

Given the negative impact of pulmonary fibrosis on the patient's life, it has become very important to search for novel therapeutic agents that can offer protection against the deleterious consequences of pulmonary fibrosis

* Corresponding author at: Dep. of Pharmacology & Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.
E-mail address: emansaid@mans.edu.eg (E. Said).

https://doi.org/10.1016/j.lfs.2019.116794
Received 18 June 2019; Received in revised form 18 August 2019; Accepted 25 August 2019
Available online 26 August 2019
0024-3205/

using well-standardized experimental models of pulmonary fibrosis.

Halofuginone has been reported to demonstrate numerous pharmacological properties as an anti-parasitic, anti-malaria, anti-inflammatory, immunomodulator, anti-fibrotic and anti-cancer agent [9]. Crocin is a nutraceutical; the main constituent isolated from the stigmas of *Crocus sativus* with immense pharmacological properties as anti-inflammatory, anticancer, antidepressant and anticonvulsant [10–12].

The current study investigated the potential therapeutic value of crocin in ameliorating BLM-induced pulmonary fibrosis in rats, comparing its effect to the standard anti-fibrotic; halofuginone and highlighting the underlying mechanism implicated in the observed anti-fibrotic impact. Anti-inflammatory, antioxidant and immuno-modulatory properties of crocin have been investigated and its impact on biochemical parameters and functional characteristics of the vascular and pulmonary functions.

2. Materials and methods

2.1. Experimental animals

Forty adult male Sprague Dawley rats (200–220 g) were provided from "Merck Research Center", Faculty of Medicine, Mansoura University. Rats were acclimatized under standard environmental and nutritional conditions for the whole duration of conduction of experimental procedures. The research protocol was approved and complied with ethical guidelines of animals experimentation endorsed by "Research Ethics Committee", Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

2.2. Drugs and chemicals

Crocin and halofuginone hydrobromide were purchased from Sigma Chemicals Co. (St. Louis, MO., USA). Bleomycin sulfate was supplied by Nippon Kayaku Co., LTD., (Japan). Crocin and halofuginone were suspended in 0.5% CMC as a vehicle for oral administration.

2.3. Experimental procedures; intratracheal instillation of BLM and pulmonary fibrosis induction

Induction of pulmonary fibrosis was conducted as described by Zaghloul et al. [13,14]. Rats were put under light thiopental sodium anesthesia (20 mg/kg, I.P.). Using a surgical scalpel, a midline neck incision was made, the trachea was bared and BLM (5 mg/kg) was instilled into the bared trachea by slow infusion. Rats were maintained on their dorsal side and swiveled many times to maintain uniform infiltration of BLM within the lungs. Then, the incision was surgically sutured and sodium fusidate (2%) was applied topically [13,14]. Rats in all the experimental groups underwent the same procedures, except the Sham control which underwent intratracheal instillation of normal saline instead.

Rats were randomized into four groups (10 rats/group) as follows: Sham control, rats received the vehicle, BLM control, rats received the vehicle, Halofuginone treated group: rats received halofuginone (0.2 mg/kg, orally) [15], Crocin treated group: rats received crocin (20 mg/kg, orally) [16]. Rats received either the vehicle or the different treatments daily for one week prior to and 4 weeks post BLM installation, for an overall of five weeks of either vehicle or drugs administration.

By the end of the duration of the experimental protocol, rats were sacrificed by deep thiopental sodium anesthesia (40 mg/kg, I.P.). Blood samples were withdrawn *via* cardiac puncture; sera were separated for biochemical assessments. After bronchoalveolar lavage fluid (BALF) collection, the trachea and pulmonary artery were excised for *in vitro* assay. Both lobes of the lungs were collected, washed in cold saline and weighed for calculation of lung/body weight index. The left lobes from all the lungs were further processed for preparation of 10% *w/v* lung homogenate as described by Daba et al. [17] and the right lobes were processed for histopathological inspection and immunohistochemical (IHC) analyses.

2.4. Collection of bronchoalveolar lavage fluid (BALF), inflammatory cells quantification, total protein content, and lactate dehydrogenase (LDH) activity determination

BALF was collected as described by Zaghloul et al. [13]. Briefly, the exposed tracheas were cannulated and the lungs were infused with 2 ml of ice-cold sterile 0.9% saline for 3 times. Approximately 50–70% of fractions were recovered. The collected BALF was centrifuged at 2000 rpm, 4 °C for 10 min and the cell sediments were re-suspended in sterile saline to quantify lung inflammatory cells. BALF's total protein content and LDH activity were determined using commercially available (Thermo Scientific, Rockford, USA) colorimetric kit and (Human diagnostics, Wiesbaden, Germany) respectively following the manufacturer's instructions.

2.5. Determination of lung malondialdehyde (MDA) and nitric oxide (NO) contents, reduced glutathione (GSH) concentration, superoxide dismutase (SOD) activity and serum total antioxidant capacity (TAC)

10% *w/v* lung homogenate was prepared as described by Daba et al. [17]. The homogenate was used for determination of oxidative/anti-oxidative stress biomarkers; MDA, NO, GSH, SOD, and serum TAC was determined using commercially available colorimetric Biodiagnostic assay kits (Giza, Egypt) as instructed by the manufacturer.

2.6. Determination of lung interleukin-10 (IL-10), Toll-Like Receptor 4 (TLR4), nuclear factor erythroid-derived-2 like protein-2 (Nrf2) contents and heme oxygenase-1 (HO-1) activity

Lung content of IL-10, TLR4 and Nrf2, and lung HO-1 activity were quantified using commercial ELISA assay kit (Cloud clone, Uscn Life Science, INC. USA.) as instructed by the manufacturer.

2.7. Determination of lung hydroxyproline and collagen content

Lung hydroxyproline content was quantified according to the procedures described by Bergman and Loxley [18] and Abdelaziz et al. [19]. Collagen content was calculated from the following equation [20],

$$\text{Lung collagen content} = \text{hydroxyproline content} \times 13.5$$

2.8. In vitro assessment of vascular reactivity of pulmonary artery to potassium chloride (KCl), phenylephrine (PE) (10^{-9}–10^{-6} M) and carbachol (10^{-8}–10^{-5} M)

Isolated pulmonary arterial (PA) rings were prepared as described by Zaghloul et al. [14]. The isolated pulmonary rings were equilibrated under 0.8 g tension for 60 min. Isometric tension was recorded using Riegestab K30 force transducer (Hugo Sachs electronic, D7806 march, Germany), and a Powerlab unit/400 linked to a PC running Chart v4.2 software (ADInstruments Pty Ltd., Australia). Contractile responses to 80 mmol/L of KCl were recorded as well. Contraction responses to semi-logarithmic concentrations of PE (10^{-9}–10^{-6} M) were recorded. Maximum effect (Emax) and concentration inducing 50% of Emax (EC_{50}) were extrapolated from the cumulative concentration-response curves and pD2 value was calculated. Relaxation responses to the semi-logarithmic concentration of carbachol (10^{-8}–10^{-5} M) were expressed as the percentage decreases of the magnitude of the contraction induced by PE (1 μM) before application of carbachol. The maximum effect (E_{max}) and inhibitory concentration 50% (IC_{50}) were determined and pD2 value was calculated. Vascular relaxation was assessed in response to carbachol (10^{-8}–10^{-5} M) after pre-contraction of pulmonary artery rings with PE (1 μM).

2.9. In vitro assessment of tracheal smooth muscles reactivity to carbachol (10^{-8}–10^{-5} M)

The tracheal zigzag was prepared according to the method described by Emmerson and Mackay [21]. The tracheas were excised and placed in Kereb's

Hensilit solution gassed with carbogen mixture and adherent extraneous connective tissue was carefully dissected. The tracheal tension was kept at 1 g throughout a stabilization period of 60 min. Isometric tension generated by the tracheal smooth muscles was recorded as previously described. To record maximal tracheal contraction, the contractile response of the tracheal zigzag to carbachol (10^{-4} M) was recorded. A cumulative concentration-response curve (10^{-8}–10^{-5} M) carbachol was established. Both E_{max} and EC_{50} were extrapolated and the responses are expressed as the percentage of maximal contraction induced by 10^{-4} M carbachol.

2.10. Histopathological examination

The right upper pulmonary lobes were fixed in 10% neutral-buffered formalin for 24 h and 2 sets of specimen were prepared; the first set stained with H&E and the second one stained with Masson's trichrome. The specimens were examined randomly in a blinded protocol. The degree of emphysema, hemorrhage, inflammatory lesions, alveolar wall thickening, and collagen deposition or fibrosis was evaluated [22]. Alveolitis and fibrosis were semi-quantitatively evaluated as described by Szapiel et al. [23].

2.11. Immunohistochemical analysis of Tumor necrosis factor- α (TNF-α) and transforming growth factor$_{β1}$ (TGF$_{β1}$) expression in lung specimen

The sections were immunostained with primary antibody rabbit polyclonal IgG to rat (TNF-α and TGF$_{β1}$) (Thermo Fisher Scientific, USA). The intensity of staining was graded semi-quantitatively and specimen were scored on a scale from 0 to 3 [24].

2.12. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses and graphing were performed using Graphpad software Prism V 5 (Graphpad Software Inc., San Diego, CA, USA). The following statistical tests were conducted: One-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparisons, Kruskal-Wallis test followed by Dunn test for parametric and non-parametric measures respectively, linear regression and non-linear regression analyses were used for the construction of standard curves and sigmoidal curves respectively. Statistical significance was accepted at $p < 0.05$.

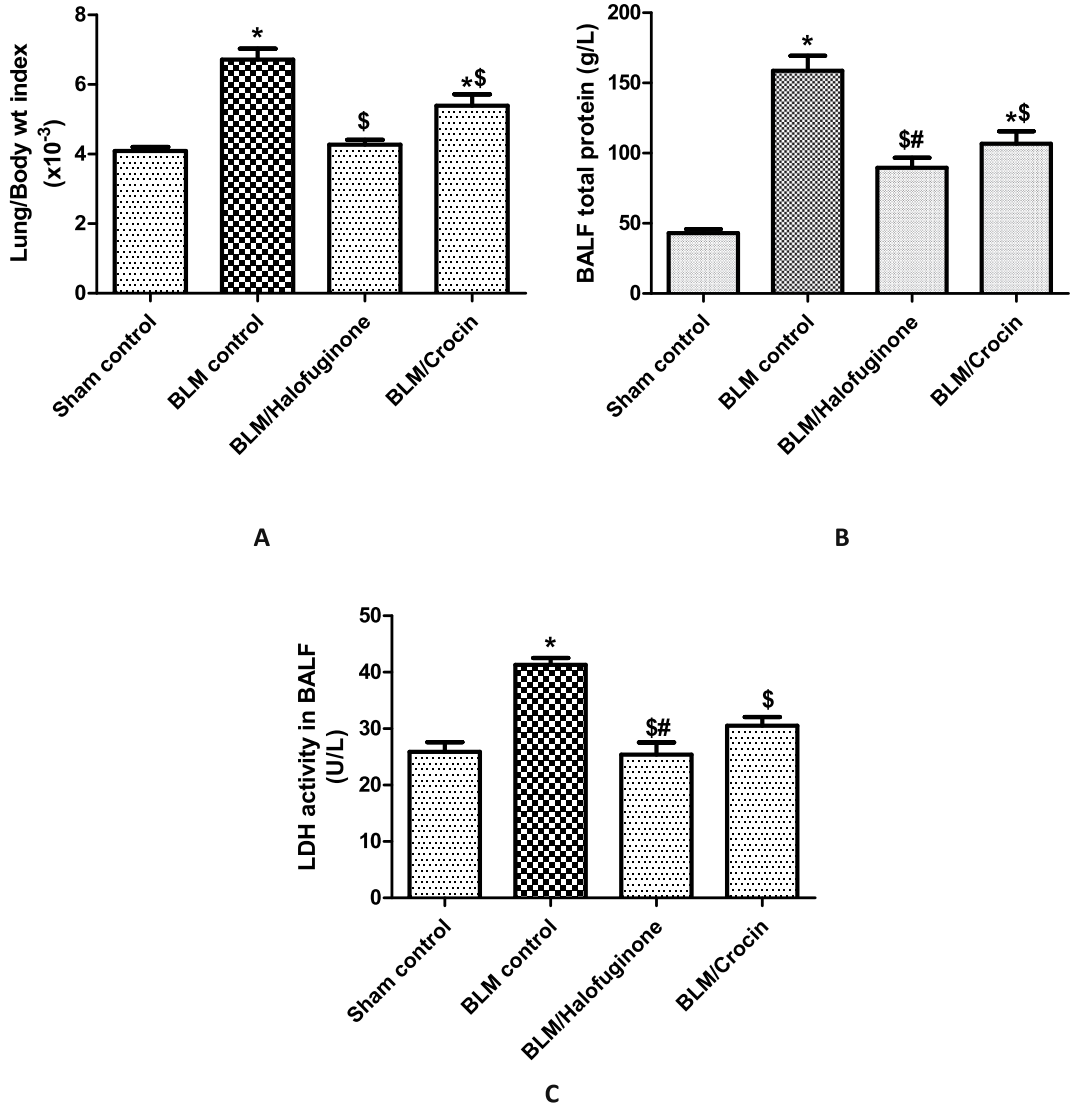


Fig. 1. Effect on (A): lung/Body weight index, (B): BALF's total protein content and (C): BALF's LDH activity.
Data are expressed as mean ± SEM, n = 6.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer's multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to normal control.
$ Significantly different in comparison to BLM control.
# Significantly different in comparison to BLM/crocin group.

Table 1
Effect of halofuginone (0.2 mg/kg) and crocin (20 mg/kg) on total lymphocytes' and neutrophils' counts, lung IL-10 and TLR4 contents.

Groups	Total cells (cell/lung × 10^6)	Lymphocytes (cell/lung × 10^6)	Neutrophils (cell/lung × 10^6)	Lung IL-10 content (pg/mg tissue)	Lung TLR4 content (ng/mg tissue)
Sham control	0.326 ± 0.068	0.194 ± 0.033	0.07 ± 0.0159	22.69 ± 1.69	0.2 ± 0.02
BLM control	1.2 ± 0.038*	0.75 ± 0.099*	0.45 ± 0.071*	41.63 ± 2.23*	2.13 ± 0.06*
BLM/halofuginone	0.57 ± 0.030*$	0.45 ± 0.089$	0.12 ± 0.032$	21.05 ± 0.92$#	0.37 ± 0.06*$
BLM/crocin	0.73 ± 0.033*$	0.45 ± 0.098$	0.28 ± 0.062$	34.34 ± 0.87*$	0.37 ± 0.02*$

Drugs' administration began one week prior to intra-tracheal instillation of BLM and persisted for further 4 weeks for an overall period of 5 weeks. BLM was intra-tracheally instilled (5 mg/kg) and the animals in the different experimental groups were sacrificed 4 weeks later.
Data are expressed as mean ± SEM, $n = 6$.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to Sham control group.
$ Significantly different in comparison to BLM control.
# Significantly different in comparison to crocin group.

3. Results

3.1. Effect on lung/body weight index

Lung/body weight index significantly increased by about 1.64 folds in BLM control rats compared to Sham control. Lung/body weight index significantly declined by about 36.5% and 20% with halofuginone and crocin treatments respectively compared to BLM control, (Fig. 1A).

3.2. Effect on lung total and differential inflammatory cell contents

Intra-tracheal BLM induced a significant augmentation in the total inflammatory cell, lymphocyte and neutrophil in BALF by 3.6, 3.8 and 6.4 folds respectively compared to Sham control. Halofuginone significantly reduced total cells', lymphocytes' and neutrophils' counts by about 52.5%, 40%, and 73% respectively compared to BLM control. Crocin significantly decreased total cells', lymphocytes' and neutrophils' counts by about 40%, 40% and 33% respectively in comparison to BLM control, (Table 1).

3.3. Effect on lung total protein content

Total lung protein content significantly increased by 3.7 folds in BLM control compared to Sham control. Both halofuginone and crocin significantly lessened lung total protein content by 43.5% and 33%, respectively in comparison to BLM control, (Fig. 1B).

3.4. Effect on lung IL-10 content

Lung IL-10 content significantly increased upon intratracheal instillation of BLM by 1.8 folds in comparison to the Sham control. Halofuginone and crocin significantly reduced lung IL-10 content by 48% and 17.5%, respectively in comparison to BLM control, (Table 1).

3.5. Effect on lung TLR4 content

Lung TLR4 content significantly escalated by 11.3 folds in BLM control in comparison to the Sham control. Lung TLR4 content significantly declined with halofuginone and crocin administration by 83% in both groups compared to BLM control, (Table 1).

3.6. Effect on LDH activity

Lung LDH activity significantly increased by 1.59 folds in BLM control compared to sham control. LDH activity significantly declined upon treatment with halofuginone and crocin by 38.5% and 26%, respectively in comparison to BLM control, (Fig. 1C).

3.7. Effect on lung oxidant/antioxidant biomarkers and serum TAC

- **Lung MDA content**: Intra-tracheal BLM significantly increased lung MDA content by approximately 2 folds compared to Sham control. Halofuginone and crocin significantly decreased lung MDA content by 35% and 34%, respectively compared to BLM control, (Table 2).
- **Lung NO content**: Intra-tracheal BLM induced a significant elevation in lung NO content by approximately 2.76 folds in comparison to the Sham control. Halofuginone and crocin significantly reduced lung NO by 45% and 22.5%, respectively in comparison to BLM control, (Table 2).
- **Lung SOD activity**: Lung SOD activity significantly declined by approximately 1.57 folds compared to Sham control. SOD activity was significantly enhanced halofuginone and crocin administration by 56% and 67%, respectively compared to BLM control, (Table 2).
- **Lung GSH concentration**: Intra-tracheal BLM significantly reduced lung GSH concentration by 1.87 folds compared to Sham control. GSH concentration significantly increased upon halofuginone and crocin administration by 61% and 47%, respectively in comparison to BLM control,

Table 2
Effect of halofuginone (0.2 mg/kg) and crocin (20 mg/kg) for 5 weeks on lung oxidant/antioxidant biomarkers, serum total antioxidant capacity (TAC) and lung nitric oxide (NO) content.

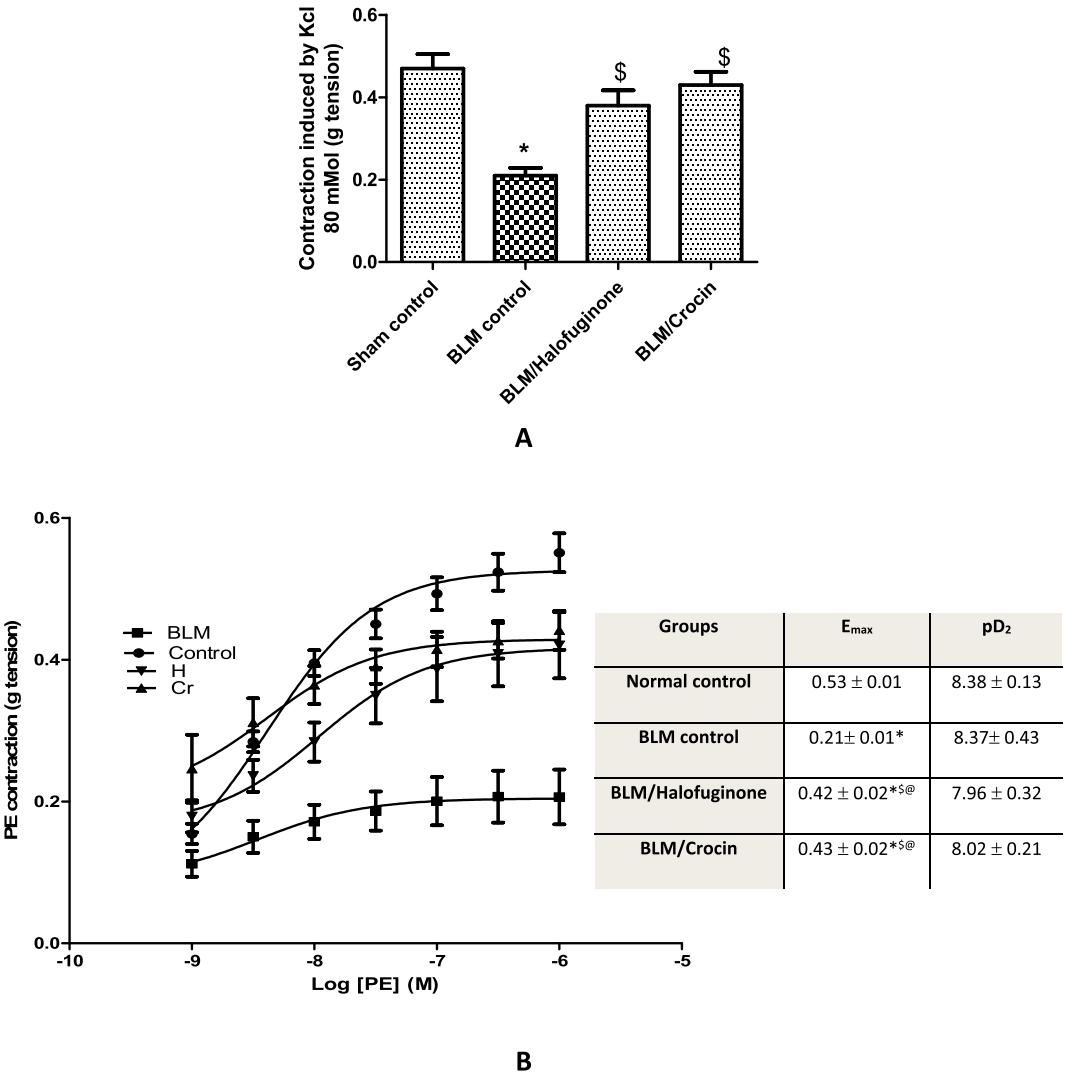
Groups	MDA (nmol/g lung)	NO (μmol/g lung)	SOD (U/g lung)	GSH (mmol/g lung)	TAC (mM/L serum)
Sham control	105.8 ± 6.09	7.48 ± 0.50	1.1 ± 0.074	1.85 ± 0.103	1.75 ± 0.059
BLM control	201.8 ± 15.76*	20.63 ± 1.82*	0.7 ± 0.047*	0.99 ± 0.04*	1.35 ± 0.073*
BLM/halofuginone	130.7 ± 12.7$	11.43 ± 0.74$#	1.09 ± 0.096$	1.59 ± 0.065$	1.69 ± 0.05$
BLM/crocin	132.6 ± 11.2$	16.0 ± 0.93*$	1.17 ± 0.085$	1.46 ± 0.079$	1.73 ± 0.047$

Drugs' administration began one week prior to intra-tracheal instillation of BLM and persisted for further 4 weeks for an overall period of 5 weeks. BLM was intra-tracheally instilled (5 mg/kg) and the animals in the different experimental groups were sacrificed 4 weeks later.
Data are expressed as mean ± SEM, $n = 6$.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to Sham control group.
$ Significantly different in comparison to BLM control group.
# Significantly different in comparison to crocin group.

Table 3
Effect of halofuginone (0.2 mg/kg) and crocin (20 mg/kg) for 5 weeks on lung Nrf2 content, HO-1 activity, hydroxyproline and collagen contents.

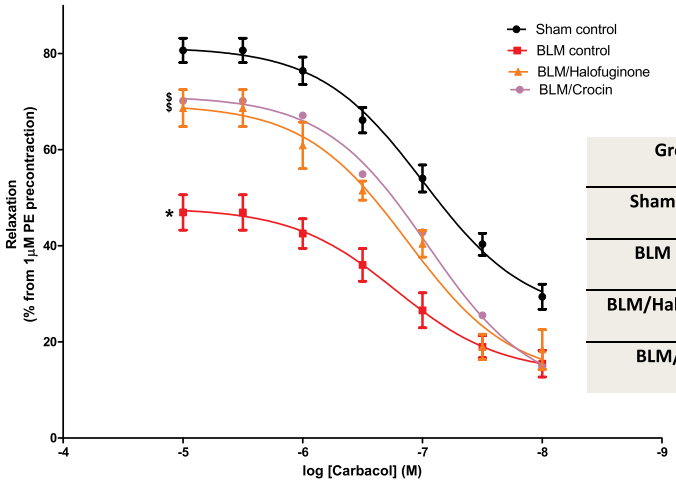
Groups	Lung Nrf2 content (pg/mg lung)	Lung HO-1 activity ($\times 10^{-2}$ ng/mg lung)	Lung hydroxyproline content (μg/g lung)	Lung collagen content (μg/g lung)
Sham control	24.11 ± 1.54	22.33 ± 1.35	56.89 ± 3.35	768.02 ± 45.22
BLM control	48.05 ± 3.29*	33.93 ± 1.24*	97.28 ± 1.54*	1313.28 ± 20.79*
BLM/halofuginone	31.48 ± 1.79$	24.82 ± 1.61$	71.13 ± 2.69*$	960.25 ± 36.32*$
BLM/crocin	28.94 ± 1.57$	23.89 ± 1.16$	81.70 ± 3.77*$	1102.95 ± 50.90*$

Drugs' administration began one week prior to intra-tracheal instillation of BLM and persisted for further 4 weeks for an overall period of 5 weeks. BLM was intra-tracheally instilled (5 mg/kg) and the animals in the different experimental groups were sacrificed 4 weeks later.
Data are expressed as mean ± SEM, $n = 6$.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to Sham control group.
$ Significantly different in comparison to BLM control group.



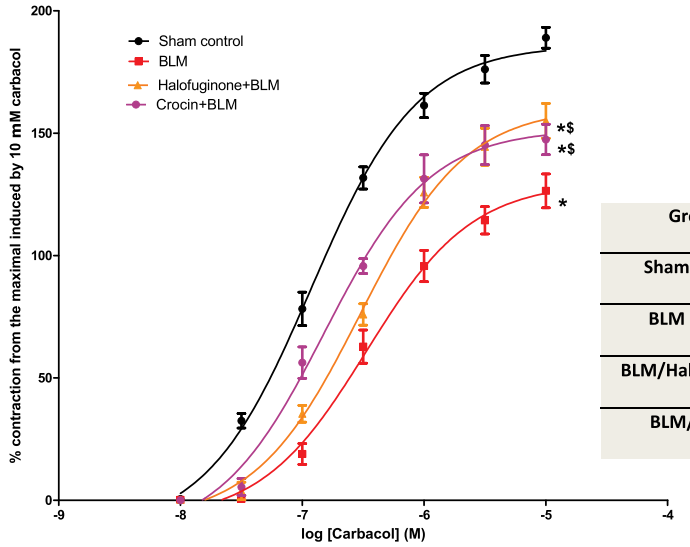
Groups	E_{max}	pD_2
Normal control	0.53 ± 0.01	8.38 ± 0.13
BLM control	0.21± 0.01*	8.37± 0.43
BLM/Halofuginone	0.42 ± 0.02*$@	7.96 ± 0.32
BLM/Crocin	0.43 ± 0.02*$@	8.02 ± 0.21

Fig. 2. Effect on (A): Kcl-induced contraction in isolated rat pulmonary arterial rings, (B): phenylephrine (PE)-induced contraction in isolated rat pulmonary arterial rings, (C): carbacol-induced relaxation of isolated PA rings pre-contracted with phenylephrine (PE) 1 μM and (D): carbachol-induced contraction in isolated tracheal zigzags and smooth muscle reactivity.
Data are expressed as mean ± SEM, $n = 6$.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer's multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to normal control.
$ Significantly different in comparison to BLM control.



Groups	E_{max}	pD_2
Sham control	81.36 ± 1.07	7.00 ± 0.10
BLM control	47.89± 2.29*	6.77± 0.19
BLM/Halofuginone	69.41 ± 2.52$	6.87 ± 0.14
BLM/Crocin	71.18 ± 1.77$	7.04 ± 0.1

C



Groups	E_{max}	pD_2
Sham control	186 ± 3.2	6.93 ± 0.05
BLM control	130 ± 4.42*	6.47± 0.08
BLM/Halofuginone	160.6 ± 4.32*$	6.52 ± 0.06
BLM/Crocin	151.7 ± 4.4*$	6.82 ± 0.08

D

Fig. 2. (*continued*)

(Table 2).

- **Serum TAC**: Serum TAC significantly declined by approximately 1.3 folds in BLM control in comparison to Sham control. Serum TAC significantly increased upon halofuginone and crocin administration by 25% and 28%, respectively compared to BLM control, (Table 2).

3.8. Effect on lung Nrf2 content

Intra-tracheal BLM significantly augmented lung Nrf2 content by 2 folds in comparison to Sham control. Lung Nrf2 content significantly declined by 34.5% and 40%, respectively compared to BLM control upon daily oral administration of halofuginone and crocin, (Table 3).

3.9. Effect on lung HO-1 activity

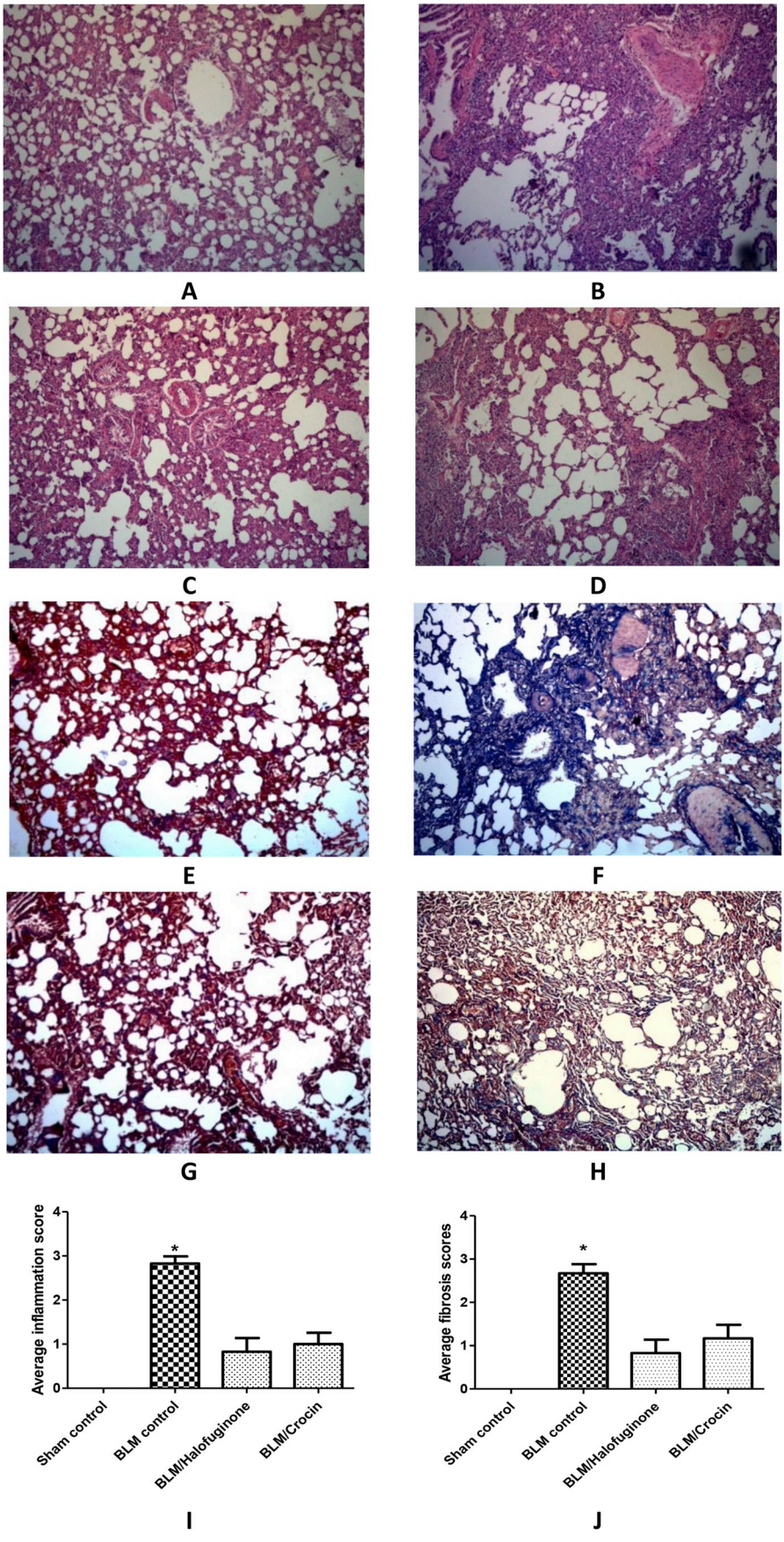
Lung HO-1 content significantly increased by 1.47 folds in BLM control in comparison to the Sham control. Halofuginone and crocin significantly reduced lung HO-1 activity by 24% and 27%, respectively in comparison to BLM control, (Table 3).

3.10. Effect on lung hydroxyproline and collagen contents

Intra-tracheal BLM induced a significant enhancement in lung hydroxyproline and collagen deposition by 1.71 folds compared to the Sham control. Halofuginone and crocin significantly reduced lung collagen contents by 27% and 16%, respectively compared to BLM control, (Table 3).

3.11. Effect on vascular reactivity of isolated rat PA rings

- **KCl-induced contraction of isolated PA rings**: BLM-instillation significantly reduced KCl-induced contraction by 2.24 folds in comparison to Sham control. KCl-induced maximal contraction significantly increased with administration of halofuginone and crocin by 81% and 105%, respectively in comparison to BLM control, (Fig. 2A).
- **PE-induced contraction in isolated PA rings**: PE-induced maximal contraction significantly declined by about 2.5 folds in BLM control in comparison to the Sham control. Halofuginone and crocin significantly increased PE-induced maximal contraction by 100% and 108%, respectively compared to BLM control, (Fig. 2B).
- **Carbachol-induced relaxation of isolated PA rings pre-contracted**



(caption on next page)

Fig. 3. Effect on lung histopathological changes in H&E stained specimens (A–D) and Masson's Trichrome stained specimen (E–H) (100×): (A), Normal control, intact lung architecture with absence of any evidence of inflammation, edema, hemorrhage, emphysema or fibrosis (B), BLM control, revealing moderate hemorrhages, emphysema, areas of increased thickening of alveolar septa, leukocytic infiltration in alveolar walls, and fibroplasia (C), BLM/Halofuginone, (D), BLM/Crocin revealing mild to moderate degree of septal thickening with few inflammatory cells infiltration, emphysematous changes and alveolar-hemorrhage, (E), Normal control, revealing absence of blue staining denoting absence of collagen deposition in interstitium as well as alveolar septae, (F), BLM control, revealing bluish discoloration of collagen deposition at interstitial space and peribronchial area, (G), BLM/Halofuginone, revealing mild to moderate bluish discoloration of collagen deposition at interstitial space, (H), BLM/Crocin, revealing mild to moderate bluish discoloration of collagen deposition at interstitial space, (I), Average fibrosis score and (J), Average fibrosis score.
Data are expressed as mean ± SEM, n = 6.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer's multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to normal control.
$ Significantly different in comparison to BLM control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with PE (1 μM): Carbachol-induced maximal relaxation in isolated rat PA rings, pre-contracted with 1 μM PE significantly declined by about 1.7 folds compared to Sham control. Carbachol-induced maximal relaxation in isolated rat PA rings significantly increased upon halofuginone and crocin treatment by 45% and 49%, respectively compared to BLM control, (Fig. 2C).

3.12. Effect on carbachol-induced contraction in isolated tracheal zigzags and smooth muscle reactivity

Carbachol-induced maximal contraction in isolated tracheal zigzag significantly declined by 1.43 folds in BLM control tracheas in comparison to Sham control. Halofuginone and crocin significantly increased carbachol-induced maximal contraction by 23.5% and 16.7% respectively compared to BLM control, (Fig. 2D).

3.13. Effect on lung histopathology

Lung specimen from the Sham control revealed ideal pulmonary architecture with no evidence of either tissue injury or fibrosis. BLM installation significantly distorted lung architecture, (Fig. 3A). Examination of specimen from BLM control revealed emphysematous changes, leukocytic infiltration in alveolar walls, severe hemorrhage, areas of increased alveolar septal thickening and fibroplasia were observed (Fig. 3B). Halofuginone (Fig. 3C) and crocin (Fig. 3D) administration significantly down-regulated BLM-induced inflammatory lesions, lung specimens showed mild septal thickening with retraction of inflammatory cells infiltration. Emphysematous distortion and alveolar hemorrhages remarkably retracted. Inflammatory lesion score significantly increased by 2.8 folds in BLM control specimen compared to Sham control. Halofuginone and crocin markedly suppressed inflammatory lesion scores by about 3.4 and 2.8 folds respectively without a statistical difference in comparison to BLM control, (Fig. 3I).

3.14. Effect on lung fibrotic changes

Lung specimen isolated from Sham control revealed no evidence of collagen deposition in either interstitium or alveolar septae, (Fig. 3E). On the other hand, BLM control revealed intense collagen deposition, compressed alveoli and extended fibrotic areas, (Fig. 3F). Collagen deposition was remarkably decreased in lung specimen from halofuginone, (Fig. 3G) and crocin, (Fig. 3H)-treated groups. The Szapiel scores of fibrosis in BLM control significantly increased by 2.7 folds in comparison to Sham control. Halofuginone and crocin groups showed a marked decrease in fibrosis grades by about 3.2 and 2.3 folds respectively without statistical difference compared to BLM control, (Fig. 3J).

3.15. Effect on lung TNF-α expression

Intra-tracheal instillation of BLM significantly augmented lung TNF-α expression by approximately 2.5 folds in comparison to Sham control, (Fig. 4A, B). Halofuginone and crocin, (Fig. 4C, D) induced a non-significant decrease in lung TNF-α expression compared to BLM control, (Fig. 4I).

3.16. Effect on lung TGF-β1 expression

Intra-tracheal instillation of BLM significantly increased lung TGF-β1 expression by 2.3 folds in comparison to the Sham control, (Fig. 4E, F). Halofuginone and crocin administration caused a non-significant decrease in lung TGF-β1 expression when compared to BLM control (Fig. 4G, H, J).

4. Discussion

Lung injury has long been considered amongst the most frequently encountered respiratory problems. Interestingly, lung fibrosis can develop secondary to different forms of lung injury or secondary to treatment with anti-cancer chemotherapeutic agents. Nevertheless, further progression into irreversible respiratory failure has been reported [25].

The association between inflammation and increased oxidant load has been reported to be involved in the pathogenesis of lung fibrosis [26]. Oxidative stress and nitrosative stress biomarkers were detected in lung specimen of pulmonary fibrosis patients. Moreover, aberrant antioxidant defenses exaggerated pulmonary fibrosis in experimentally-induced lung fibrosis [27,28].

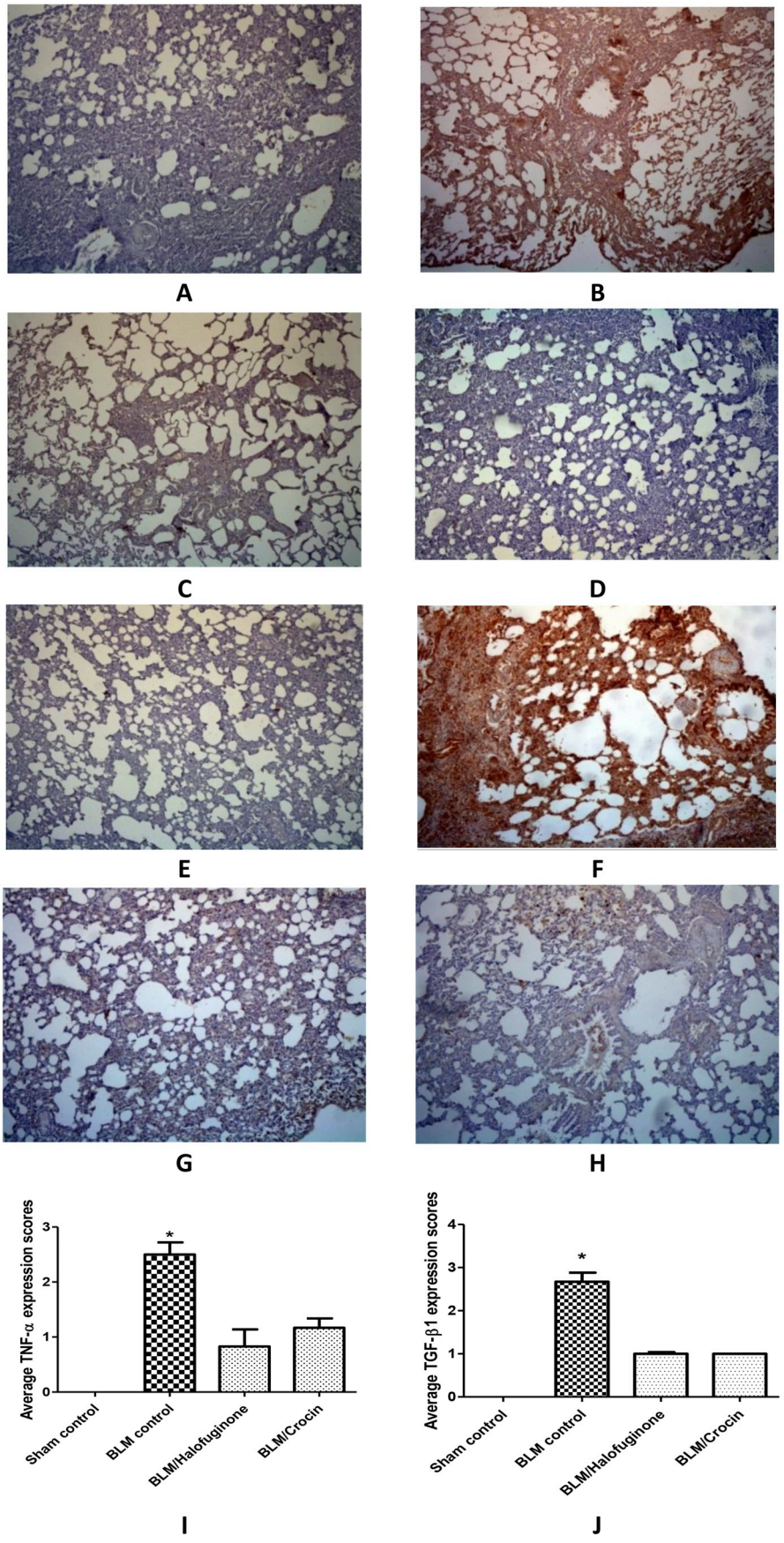
Activation of various inflammatory cells has been linked to pulmonary fibrosis progression as well. Once activated, preformed mediators are released initiating and mediating a sequel of events that promote fibrosis [29]. Thus long term untreated inflammation drives fibrotic changes, with increased deposition of collagen and myofibroblasts proliferation [30].

In the current study, intra-tracheal BLM instillation impaired oxidants/antioxidants homeostasis as evidenced by the significant escalation of lung NO and MDA contents and the paralleled reduction in GSH concentration, SOD activity, and serum TAC. Similar observations were reported by Verma et al. [22]. Indeed, BLM has been reported to bind DNA and to undergo redox cycling in the presence of oxygen with enhanced ROS generation [31]. In the current study, both halofuginone and crocin attenuated BLM-induced compromise in oxidants/antioxidants homeostasis, confirming the antioxidant impact of drugs under investigation with evident anti-inflammatory and anti-fibrotic impacts.

Abdelaziz et al. [19] have referred to the antioxidant and anti-inflammatory properties of halofuginone. The observed protective impact of crocin in the current study is in agreement with literatures reporting significant antioxidant capacity of crocin in different experimental models [10,11,16,32]. Stabilization of bio-membranes, ROS scavenging properties, and decreased peroxidation of unsaturated lipids has been reported to be amongst the mechanisms underlying the antioxidant properties of crocin [12].

The redox sensing transcription factor Nrf2 is a crucial cellular regulator of oxidant/antioxidant homeostasis. Normally, basal levels of Nrf2 and its associated target genes are expressed. However, ROS-induced electrophilic load triggers Nrf2 activation with up-regulation of its associated target genes. Indeed, dysregulated Nrf2 activity has been proposed as one of the contributing factors to lung fibrosis [33].

In agreement, BLM installation in the current study augmented Nrf2 expression and this observation is in agreement with those obtained from Chitra et al. [27]. On the other hand, both halofuginone and crocin suppressed lung Nrf2 expression. Hypothetically, these drugs have prevented BLM-induced ROS from triggering Nrf2 activation confirming the antioxidant potential of these drugs.



(caption on next page)

Fig. 4. Effect on lung tumor necrosis factor-α (TNF-α) expression (A–D) and lung transforming growth factorβ1 (TGF-β1) expression (E–F) (100×): (A), Normal control, revealing negative brown staining reaction of TNF-α in fibroblasts and pneumocytes, (B), BLM control, revealing sever brown staining denoting diffuse strong positive cytoplasmic reaction of TNF-α in fibroblasts and pneumocytes, (C), BLM/Halofuginone, revealing mild brown staining reaction of TNF-α in fibroblasts and pneumocytes, (D), BLM/Crocin, revealing mild-brown-staining-reaction-of-TNF-α-in-fibroblasts-and-pneumocytes, (E), Normal control, revealing negative brown staining reaction of TGF-β1 in fibroblasts and pneumocytes, (F) BLM control, revealing sever brown staining denoting diffuse strong positive cytoplasmic reaction of TGF-β1 in fibroblasts and pneumocytes, (G), BLM/Halofuginone, revealing mild brown staining reaction of TGF-β1 in fibroblasts and pneumocytes, (H), BLM/Crocin, revealing mild brown staining reaction of TGF-β1 in fibroblasts and pneumocytes, (I), Average TNF-α expression score and, (J), Average TGF-β1 expression score.
Data are expressed as mean ± SEM, n = 6.
Data were statistically-analyzed using One-Way ANOVA followed by Tukey-Kramer's multiple comparisons test ($p < 0.05$).
* Significantly different in comparison to normal control.
$ Significantly different in comparison to BLM control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The observed ability of halofuginone to decrease lung Nrf2 content is in line with Tsuchida et al. [34] who reported halofuginone to act as a potent Nrf2 inhibitor. In contrast to the observed ability of crocin to reduce lung Nrf2 content in the current study, Kim et al. [35] reported crocin to activate Nrf2 in cancer cell line. Also, Akbari et al. [36] reported crocin to protect rat's liver against hepatic ischemia-reperfusion-induced injury through up-regulation of protein expression of Nrf2.

Overexpression of HO-1 gene *in vivo* was associated with augmented tissue oxidative burden and lung injury [37]. In the current study, BLM instillation induced a significant increase in lung HO-1 activity. This is in line with previous studies reporting cytoprotection conferred by HO-1 inhibition against BLM-induced pulmonary fibrosis due to combined effects of the diminished generation of toxic heme metabolites and improved tissue antioxidant capacity [8]. The results of the current study also revealed a significant decrease in lung HO-1 content upon halofuginone and crocin administration. This is further confirming the antioxidant effect of these drugs and their ability to interrupt Nrf2 signaling; the major transcriptional regulator of the HO-1 gene [24].

In the current study, BLM-induced a significant increase in the total cell count with a marked increase in the proportion of neutrophils and lymphocytes in BALF following the previous reports of Sriram et al. [38] and Verma et al. [22]. Moreover, increased lung total protein content was confirmed in the current study reflecting increased vascular permeability and protein transudation from the bloodstream into lung tissues. This is in agreement with the findings of Cortijo et al. [39]. Halofuginone and crocin administration significantly decreased BALF total cell count, neutrophils and total protein, confirming anti-inflammatory activities of both drugs.

Previous literature referred to elevated plasma concentrations of IL-10 in various pathologies [40], with particular concern, experimental models of pulmonary inflammation and fibrosis [41,42] which Doughty et al. [43] accounted for to be a possible compensatory anti-inflammatory response. Meanwhile, TLR4 is expressed in a wide range of cells in the. It has been reported to be pivotal for neutrophil accumulation, TNF-α expression, and enhanced protein permeability. Nevertheless, TLR4 receptors activation has been associated with fibroblast activation by inducing TGF-β1 signaling [44].

In the current study, the association between these critical cellular events has been confirmed where BLM instillation increased lung content of IL-10, TLR4 and increased expression of lung TNF-α and TGF-β1. Such an association is in agreement with the observations of Li et al. [45].

Halofuginone and crocin administration significantly decreased lung TLR4, IL-10 contents. Jin et al. [46] reported halofuginone to decrease IL-10 level in idiopathic thrombocytopenic purpura model. Nevertheless, it suppressed cytokines production in activated T cells and suppressed their proliferation and differentiation [47]. Li et al. [48] reported crocin to suppress LPS-induced TLRs expression.

The crucial role of TNF-α and TGF-β1 in the pathogenesis of lung fibrosis is undisputed. In the current study, lung expression of TNF-α and TGF-β1 significantly escalated post BLM instillation. These results are in agreement with previous studies of Chitra et al. [27]. Halofuginone and crocin markedly suppressed BLM-induced increase in lung TNF-α and TGF-β1 expression. Halofuginone was reported to abrogate fibrotic hyperactivity in different experimental models by reducing the TGF-β1-induced expression of α-SMA, and type I collagen [49].

Crocin has been reported to reduce LPS-stimulated production of TNF-α in rat brain microglial cells and intervertebral disc [48]. Moreover, crocin inhibited colitis and colitis-associated colorectal cancer mainly through down-regulation of genetic expression of TNF-α [32,50] and attenuated thioacetamide-induced expression of TGF-β1 in mice liver [51].

The present study revealed elevated lung hydroxyproline content upon BLM instillation indicating increased collagen deposition, which was future confirmed by the significant increase in fibrosis scoring and significant elevation in lung/body weight index. Verma et al. [22] have also reported a similar increase in lung collagen content and lung/body weight index with BLM instillation.

The histological architecture was significantly restored upon halofuginone, and crocin administration. Previous literature has reported halofuginone to demonstrate a comparable effect to that of prednisolone in decreasing hydroxyproline content in esophageal and hypopharyngeal fibrosis [52]. Crocin administration led to a significant decrease in BLM-induced increase in lung hydroxyproline and collagen contents. The potent antioxidant and anti-inflammatory effects of crocin could be amongst the mechanism by which they exert this observed protective effect.

Pulmonary hypertension is a well-recognized complication of pulmonary fibrosis [53] and it is usually characterized by pulmonary arteriolar remodeling as well and this results in decreased responsiveness of pulmonary circulation to vasodilator therapies [54]. Indeed, BLM-induced lung fibrosis in the current study was associated with significant pulmonary vascular and tracheal functional impairments as seen with*in vitro* assessment of vascular reactivity of isolated pulmonary artery rings to KCL, PE and carbachol. BLM instillation resulted in a significant decrease in vascular reactivity of isolated pulmonary artery ring to Kcl and PE with impaired relaxant response to carbachol. In line with the observed biochemical and histopathological alterations, *in vitro* assessment of tracheal smooth muscle reactivity to carbachol revealed significant decrease in tracheal smooth muscle reactivity post intratracheal instillation of BLM.

On the other hand, halofuginone, and crocin through amelioration of BLM-induced dramatic damage were capable of restoring normal vascular tone and tracheal contractile response confirming evident biochemical and histopathological improvement induced by all the investigated drugs, thus extending their impact to attenuation of the complications of BLM-induced pulmonary fibrosis.

In conclusion; anti-inflammatory, anti-fibrotic and antioxidant activities of crocin are believed to be involved in the observed anti-fibrotic efficacy. Down-regulation of TLR4, IL-10 expression is the major pathway involved in the observed anti-inflammatory effects and finally, down-regulation of tissue expression of TNF-α and TGF-β1 is the major pathways implicated in the observed anti-fibrotic activities and Modulation of Nrf2 and HO-1 pathways is the main mechanism involved in the observed antioxidant effects.

Declaration of competing interest

None.

Acknowledgment

The authors of the manuscript acknowledge Dr. Ramy Ahmed Abdel-Salam, assistant lecturer of Pathology, Faculty of Medicine, Mansoura University for conducting histopathological and immunohistochemical analysis.

References

[1] D.S. Kim, Acute exacerbations in patients with idiopathic pulmonary fibrosis, Respir. Res. 14 (1) (2013) 86.
[2] T.J. Gross, G.W. Hunninghake, Idiopathic pulmonary fibrosis, New Engl. J. Med. 345 (7) (2001) 517–525.
[3] M. Selman, T.E. King, A. Pardo, Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy, Ann. Intern. Med. 134 (2001) 136–151.
[4] C. Jin, Y. Jia, C. Jin, H. Dong, P. Cheng, J. Zhou, Therapeutic effect of Halofuginone on ITP mice by regulating the differentiation of Th cell subsets, Int. Immunopharmacol. 18 (2) (2014) 213–216.
[5] S. Kayhan, A. Guzel, L. Duran, S. Tutuncu, A. Guzel, M. Gunaydin, Effects of leflunomide on inflamation and fibrosis in bleomycine induced pulmonary fibrosis in wistar albino rats, J. Thorac. Dis. 5 (5) (2013) 641–649.
[6] V. Della Latta, A. Cecchettini, S. Del Ry, M.A. Morales, Bleomycin in the setting of lung fibrosis induction: from biological mechanisms to counteractions, Pharmacol. Res. 97 (2015) 122–130.
[7] K. Yamauchi, Y. Kasuya, F. Kuroda, K. Tanaka, J. Tsuyusaki, S. Ishizaki, Attenuation of lung inflammation and fibrosis in CD69-deficient mice after intratracheal bleomycin, Respir. Res. 12 (1) (2011) 131.
[8] L. Atzori, F. Chua, S. Dunsmore, D. Willis, M. Barbarisi, R. McAnulty, Attenuation of bleomycin induced pulmonary fibrosis in mice using the heme oxygenase inhibitor Zn-deuteroporphyrin IX-2, 4-bisethylene glycol, Thorax 59 (3) (2004) 217–223.
[9] M. Pines, I. Spector, Halofuginone - the multifaceted molecule, Molecules 20 (1) (2015) 573–594.
[10] H.O. Abou-Hany, E. Said, H. Atif, H.A. ElKashef, H.A. Salem, Crocin reverses unilateral renal ischemia reperfusion injury-induced augmentation of oxidative stress and toll like receptor-4 activity, Environ. Toxicol. Pharmacol. 59 (2018) 182–189.
[11] H.O. Abou-Hany, E. Said, H. Atif, H.A. ElKashef, H.A. Salem, Crocin mediated amelioration of oxidative burden and inflammatory cascade suppresses diabetic nephropathy progression in diabetic rats, Chem. Int. 284 (2018) 90–100.
[12] H. Yosri, W.F. Elkashef, E. Said, N.M. Gameil, Crocin modulates IL-4/IL-13 signaling and ameliorates experimentally induced allergic airway asthma in a murine model, Int. Immunopharmacol. 50 (2017) 305–312.
[13] M.S. Zaghloul, R.A. Abdel-Salam, E. Said, G.M. Suddek, H.A. Salem, Attenuation of bleomycin-induced pulmonary fibrosis in rats by flavocoxid treatment, Egy. J. Basic Appl. Sci. 4 (4) (2017) 256–263.
[14] M.S. Zaghloul, R.A. Abdel-Salam, E. Said, G.M. Suddek, H.A. Salem, Flavocoxid improves bleomycin-induced respiratory dysfunction and pulmonary fibrosis; in vitro study, World J. Pharm. Pharm. Sci. 8 (4) (2017).
[15] S. Benchetrit, S. Yarkoni, M. Rathaus, M. Pines, G. Rashid, J. Bemheim, Halufuginone reduces the occurrence of renal fibrosis in 5/6 nephrectomized rats, IMAJ-RAMAT GAN 9 (1) (2007) 30.
[16] N.M. Elsherbiny, M.F. Salama, E. Said, M. El-Sherbiny, M.M. Al-Gayyar, Crocin protects against doxorubicin-induced myocardial toxicity in rats through down-regulation of inflammatory and apoptic pathways, Chem. Int. 247 (2016) 39–48.
[17] M.H. Daba, A.A. Abdel-Aziz, A.M. Moustafa, A.A. Al-Majed, O.A. Al-Shabanah, H.A. El-Kashef, Effects of L-carnitine and Ginkgo biloba extract (EGb 761) in experimental bleomycin-induced lung fibrosis, Pharmacol. Res. 45 (6) (2002) 461–467.
[18] I. Bergman, R. Loxley, Two improved and simplified methods for the spectrophotometric determination of hydroxyproline, Anal. Chem. 35 (12) (1963) 1961–1965.
[19] R.R. Abdelaziz, W.F. Elkashef, E. Said, Tadalafil reduces airway hyperactivity and protects against lung and respiratory airways dysfunction in a rat model of silicosis, Int. Immunopharmacol. 40 (2016) 530–541.
[20] C.R. Kliment, J.M. Englert, L.P. Crum, T.D. Oury, A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal, Int. J. Clin. Exp. Pathol. 4 (4) (2011) 349–355.
[21] J. Emmerson, D. Mackay, The zig-zag tracheal strip, J. Pharm. Pharmacol. 31 (11) (1979) 798.
[22] R. Verma, L. Kushwah, D. Gohel, M. Patel, T. Marvania, S. Balakrishnan, Evaluating the ameliorative potential of quercetin against the bleomycin-induced pulmonary fibrosis in wistar rats, BMC Pulm. Med. 2013 (2013) 921724.
[23] S.V. Szapiel, N.A. Elson, J.D. Fulmer, G.W. Hunninghake, R.G. Crystal, Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse, Am. Rev. Respir. Dis. 120 (4) (1979) 893–899.
[24] E.J. Adams, J.A. Green, A.H. Clark, J.H. Youngson, Comparison of different scoring systems for immunohistochemical staining, J. Clin. Pathol. 52 (1) (1999) 75–77.
[25] E. Garcia-Prieto, A. Gonzalez-Lopez, S. Cabrera, A. Astudillo, A. Gutierrez-Fernandez, M. Fanjul-Fernandez, Resistance to bleomycin-induced lung fibrosis in MMP-8 deficient mice is mediated by interleukin-10, PLoS One 5 (10) (2010) e13242.
[26] T. Divya, V. Dineshbabu, S. Soumyakrishnan, A. Sureshkumar, G. Sudhandiran, Celastrol enhances Nrf2 mediated antioxidant enzymes and exhibits anti-fibrotic effect through regulation of collagen production against bleomycin-induced pulmonary fibrosis, Chem. Biol. Interact. 246 (2016) 52–62.
[27] P. Chitra, G. Saiprasad, R. Manikandan, G. Sudhandiran, Berberine attenuates bleomycin induced pulmonary toxicity and fibrosis via suppressing NF-κB dependant TGF-β activation: a biphasic experimental study, Toxicol. Lett. 219 (2) (2013) 178–193.
[28] S. Kalayarasan, N. Sriram, G. Sudhandiran, Diallyl sulfide attenuates bleomycin-induced pulmonary fibrosis: critical role of iNOS, NF-κB, TNF-α and IL-1β, Life Sci. 82 (23) (2008) 1142–1153.
[29] L.L. Reber, F. Daubeuf, G. Pejler, M. Abrink, N. Frossard, Mast cells contribute to bleomycin-induced lung inflammation and injury in mice through a chymase/mast cell protease 4-dependent mechanism, J. Immunol. 192 (4) (2014) 1847–1854.
[30] M. Suga, K. Iyonaga, T. Okamoto, Y. Gushima, H. Miyakawa, T. Akaike, Characteristic elevation of matrix metalloproteinase activity in idiopathic interstitial pneumonias, Am. J. Respir. Crit. Care Med. 162 (5) (2000) 1949–1956.
[31] X. Liang, Q. Tian, Z. Wei, F. Liu, J. Chen, Y. Zhao, Effect of Feining on bleomycin-induced pulmonary injuries in rats, J. Ethnopharmacol. 134 (3) (2011) 971–976.
[32] A.E. Khodir, E. Said, H. Atif, H.A. ElKashef, H.A. Salem, Targeting Nrf2/HO-1 signaling by crocin: role in attenuation of AA-induced ulcerative colitis in rats, Biomed. Pharmacother. 110 (2019) 389–399.
[33] D.M. Walters, H.Y. Cho, S.R. Kleeberger, Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: a potential role for Nrf2, Antioxid. Redox Signal. 10 (2) (2008) 321–332.
[34] K. Tsuchida, T. Tsujita, M. Hayashi, A. Ojima, N. Keleku-Lukwete, F. Katsuoka, Halofuginone enhances the chemo-sensitivity of cancer cells by suppressing NRF2 accumulation, Free Radic. Biol. Med. 103 (2017) 236–247.
[35] S.H. Kim, J.M. Lee, S.C. Kim, C.B. Park, P.C. Lee, Proposed cytotoxic mechanisms of the saffron carotenoids crocin and crocetin on cancer cell lines, Biochem. Cell Biol. 92 (2) (2014) 105–111.
[36] G. Akbari, S.A. Mard, M. Dianat, E. Mansouri, The hepatoprotective and MicroRNAs downregulatory effects of Crocin following hepatic ischemia-reperfusion injury in rats, Oxidative Med. Cell. Longev. 2017 (2017) 1702967.
[37] Y.H. Weng, A. Tatarov, B.P. Bartos, C.H. Contag, P.A. Dennery, HO-1 expression in type II pneumocytes after transpulmonary gene delivery, Am. J. Physiol. Lung Cell. Mol. Physiol. 278 (6) (2000) L1273–L1279.
[38] N. Sriram, S. Kalayarasan, G. Sudhandiran, Epigallocatechin-3-gallate augments antioxidant activities and inhibits inflammation during bleomycin-induced experimental pulmonary fibrosis through Nrf2–Keap1 signaling, Pulm. Pharmacol. Ther. 22 (3) (2009) 221–236.
[39] J. Cortijo, M. Cerda-Nicolas, A. Serrano, G. Bioque, J.M. Estrela, F. Santangelo, Attenuation by oral N-acetylcysteine of bleomycin-induced lung injury in rats, Eur. Respir. J. 17 (6) (2001) 1228–1235.
[40] R.M. Sherry, J.I. Cue, J.K. Goddard, J.B. Parramore, J.T. Dipiro, Interleukin-10 is associated with the development of sepsis in trauma patients, J. Trauma 40 (4) (1996) 613–617.
[41] B.G. Kim, P.H. Lee, S.H. Lee, Y.E. Kim, M.Y. Shin, Y. Kang, Long-term effects of diesel exhaust particles on airway inflammation and remodeling in a mouse model, Allergy, Asthma Immunol. Res. 8 (3) (2016) 246–256.
[42] E.J. Park, J. Roh, S.N. Kim, M.S. Kang, Y.A. Han, Y. Kim, A single intratracheal instillation of single-walled carbon nanotubes induced early lung fibrosis and subchronic tissue damage in mice, Arch. Toxicol. 85 (9) (2011) 1121–1131.
[43] L. Doughty, J.A. Carcillo, S. Kaplan, J. Janosky, The compensatory anti-inflammatory cytokine interleukin 10 response in pediatric sepsis-induced multiple organ failure, Chest 113 (6) (1998) 1625–1631.
[44] S. Bhattacharyya, K. Kelley, D.S. Melichian, Z. Tamaki, F. Fang, Y. Su, Toll-like receptor 4 signaling augments transforming growth factor-beta responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma, Am. J. Pathol. 182 (1) (2013) 192–205.
[45] X.X. Li, D.Y. Jiang, X.X. Huang, S.L. Guo, W. Yuan, H.P. Dai, Toll-like receptor 4 promotes fibrosis in bleomycin-induced lung injury in mice, Genet. Mol. Res. 14 (4) (2015) 17391–17398.
[46] X. Jin, H. Dai, K. Ding, X. Xu, B. Pang, C. Wang, Rapamycin attenuates bleomycin-induced pulmonary fibrosis in rats and the expression of metalloproteinase-9 and tissue inhibitors of metalloproteinase-1 in lung tissue, Chin. Med. J. 127 (7) (2014) 1304–1309.
[47] G. Yavas, M. Calik, G. Calik, C. Yavas, O. Ata, H. Esme, The effect of Halofuginone in the amelioration of radiation induced-lung fibrosis, Med. Hypotheses 80 (4) (2013) 357–359.
[48] K. Li, Y. Li, Z. Ma, J. Zhao, Crocin exerts anti-inflammatory and anti-catabolic effects on rat intervertebral discs by suppressing the activation of JNK, Int. J. Mol. Med. 36 (5) (2015) 1291–1299.
[49] E.F. Nelson, C.W. Huang, J.M. Ewel, A.A. Chang, C. Yuan, Halofuginone down-regulates Smad3 expression and inhibits the TGFbeta-induced expression of fibrotic markers in human corneal fibroblasts, Mol. Vis. 18 (2012) 479–487.
[50] K. Kawabata, N.H. Tung, Y. Shoyama, S. Sugie, T. Mori, T. Tanaka, Dietary crocin inhibits colitis and colitis-associated colorectal carcinogenesis in male ICR mice, Evid. Based Complement. Alternat. Med. 2012 (2012) 820415.
[51] M.M. Algandaby, Antifibrotic effects of crocin on thioacetamide-induced liver fibrosis in mice, Saudi J. Biol. Sci. 25 (4) (2018) 747–754.
[52] H. Dabak, T. Karlidag, N. Akpolat, E. Keles, H.C. Alpay, M. Serin, et al., The effects of methylprednisolone and halofuginone on preventing esophageal and hypopharyngeal fibrosis in delivered radiotherapy, Eur. Arch. Otorhinolaryngol. 267 (9) (2010) 1429–1435.
[53] J.S. Smith, D. Gorbett, J. Mueller, R. Perez, C.J. Daniels, Pulmonary hypertension and idiopathic pulmonary fibrosis: a dastardly duo, Am J Med Sci 346 (3) (2013) 221–225.
[54] H. Christou, O.M. Reslan, V. Mam, A.F. Tanbe, S.H. Vitali, M. Touma, E. Arons, S.A. Mitsialis, S. Kourembanas, R.A. Khalil, Improved pulmonary vascular reactivity and decreased hypertrophic remodeling during nonhypercapnic acidosis in experimental pulmonary hypertension, Am. J. Phys. Lung Cell. Mol. Phys. 302 (9) (2012) L875–L890 1.