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# Protective effects of febuxostat against paraquat-induced lung toxicity in rats: Impact on RAGE/PI3K/Akt pathway and downstream inflammatory cascades

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ABSTRACT

*Aims:* The herbicide paraquat causes fatal lung toxicity by induction of xanthine oxidase, production of free radicals and inflammation. Febuxostat, a xanthine oxidase inhibitor and anti-gout has recently shown anti-inflammatory activity. Accordingly, this study was carried out to investigate whether febuxostat may attenuate paraquat-induced lung toxicity and to explore the possible underlying mechanisms.

*Main methods:* Rats were administered either vehicle, a single dose of paraquat (30 mg/kg, i.p.), febuxostat (15 mg/kg, oral), or both for 14 successive days. Serum LDH and sRAGE were estimated. Lung tissue xanthine oxidase activity, SOD, TAC, MDA, and RAGE, HMGB1 gene expression, PI3K/Akt and β-catenin protein expression, MMP-9, IL-8, VEGF and COX-2 gene expression were estimated.

*Key findings:* Results showed that paraquat induced lung injury characterized by enhanced oxidative stress and inflammation, upregulated RAGE, HMGB1 gene expression, PI3K/Akt and β-catenin protein expression. Administration of febuxostat inhibited the deleterious effects of paraquat on lung through inhibition of xanthine oxidase activity and related oxidative stress, downregulation of RAGE/PI3K/Akt pathway, and suppression of β-catenin protein expression and its downstream inflammatory mediators.

*Significance:* The present study showed that febuxostat may abrogate paraquat-induced lung toxicity and demonstrated a novel mechanism for its ameliorative effects.

## 1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is a rapidly acting herbicide that is widely used in developing world for weed control. However, there are increasing incidences of human poisoning and mortalities following suicidal or accidental exposure to paraquat secondary to multiple organ failure including heart, liver, kidney and nervous system [1]. Interestingly, paraquat is accumulated primarily in the lung tissue, therefore respiratory failure and lung fibrosis appear to be the major causes of paraquat-induced fatalities [2].

Paraquat-induced lung injury is characterized by alveolitis, edema, inflammatory cell infiltration, and collagen deposition [2]. Albeit it is evident that oxidative stress and lipid peroxidation play pivotal role, the exact mechanism underlying paraquat-induced pulmonary toxicity remains to be elucidated [3]. Superoxide radicals are not only produced during paraquat biotransformation, but also paraquat induces xanthine oxidase activity. This enzyme catalyzes the conversion of xanthine and hypoxanthine into uric acid and superoxide radicals in lung tissue [4,5].

Uric acid was shown to enhance the expression of receptor for advanced glycation end-product (RAGE), while superoxide radicals exert excessive cellular oxidative stress resulting in depletion of antioxidants, lipid peroxidation, membrane disruption, and tissue necrosis [6,7]. Interestingly, paraquat was found to elevate serum uric acid level in human subjects, while suppression of xanthine oxidase activity attenuated paraquat-induced cytotoxicity in cultured endothelial cells [8,9]. Though previous research studies reported the protective effects of some antioxidants and anti-inflammatory agents against paraquat-induced lung damage, still no robust antidote has been proposed up till now to counteract paraquat-induced toxicity [7,10]. Extracts of natural plants such as *Berberis vulgaris* and Marticaria chamomilla have been found to suppress inflammatory markers and enhance antioxidant enzymes activity in the lungs of paraquat-intoxicated rats [11,12]. Selenium has shown similar protective effects by virtue of its antioxidant effect [13]. Recent studies reported the ameliorative effects of statins

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and rapamycin on paraquat-induced lung toxicity via inhibition of NF-κBp65 activity, and stimulation of Nrf2 signaling pathway, respectively [14,15].

Febuxostat, on the other hand, is a potent and selective xanthine oxidase inhibitor that is used to treat gout. In addition, it showed antioxidant and anti-inflammatory effects in different experimental models [4,16].

Interestingly, xanthine oxidase-induced reactive oxygen species was found to enhance necrosis in tissues. The latter was reported to release high mobility group box-1 (HMGB1) transcription factor. It is well known that HMGB1 plays a pivotal role in inflammation and chemotaxis. Binding of HMGB1 to RAGE results in activation of inflammatory pathways and further tissue damage [17]. Lately, febuxostat showed inhibitory effects on HMGB1 expression, Akt activation, and collagen deposition in renal tissues [18,19].

Since the protective effect of febuxostat against paraquat-induced lung toxicity has not been explored before, therefore this study was carried out to investigate the possible protective effects of febuxostat in paraquat-induced lung toxicity in rats and to reveal the underlying mechanistic pathways.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar albino rats weighing 180–200 g were used in this study. They were kept in clean plastic cages, on a 12 h light/dark cycle and controlled temperature of 23 ± 1 °C. The animals were allowed standard chow pellets and water ad libitum. Rats were allowed one week for acclimatization to minimize physiological responses to handling. All the experiments were carried out in accordance with the ARRIVE guidelines (Animal Research: Reporting of In-Vivo Experiments) and carried out in accordance with the NIH (National Institutes of Health) guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and with the approval of the local institutional Research Ethics Committee.

### 2.2. Chemicals

Febuxostat was obtained as a gift from Hikma Pharmaceutical Co. (Egypt). Paraquat was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

### 2.3. Experimental design

Male Wistar albino rats were randomly distributed into four groups of 10 animals each. Rats of the normal control group received vehicle (i.p., and oral) for 14 successive days. The second group was administered 0.9% normal saline with 2 drops of Tween 80 orally for 14 successive days with a single dose of paraquat (30 mg/kg, i.p.) on the seventh day [20]. The third group was administered febuxostat (15 mg/kg, oral) dissolved in 0.9% normal saline and 2 drops of Tween 80 for 14 successive days [4]. The combination group received febuxostat (15 mg/kg, oral) for 14 successive days with a single dose of paraquat (30 mg/kg, i.p.) on the seventh day.

### 2.4. Collection of samples

On day 14, 1 h after the last febuxostat dose, all rats were sacrificed by decapitation. Blood was collected, and serum was separated by centrifugation. Lungs were isolated immediately and weighed. All samples were stored at −70 °C until properly processed.

## 3. Methods

### 3.1. Determination of serum lactate dehydrogenase (LDH), lung tissue superoxide dismutase (SOD) and xanthine oxidase activities, malondialdehyde (MDA), hydroxyproline and total antioxidant capacity (TAC) contents

Serum LDH activity was assayed by Stanbio colorimetric kit (USA). In brief, the principle of the kit depends on the ability of LDH to specifically catalyze the oxidation of lactate to pyruvate with the subsequent reduction of NAD to NADH. The rate at which NADH is formed is proportional to LDH activity. The increase in NADH absorbance per minute at 340 nm was recorded. SOD and XO activities were estimated in lung tissue homogenate by colorimetric kits from Biovision (USA). SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The SOD assay kit utilizes a chromagen that produces a water-soluble formazan dye upon reduction by superoxide anions. The inhibition of chromagen reduction is used to determine the activity of SOD. Xanthine oxidase assay kit is based on the ability of xanthine oxidase to oxidize xanthine to hydrogen peroxide which reacts with OxiRed Probe to generate a color that can be detected at absorbance of 570 nm. MDA, hydroxyproline, and TAC were assessed by colorimetric kits from Abcam (USA). The MDA assay kit is based on the reaction of MDA in the sample with thiobarbituric acid to generate a MDA-TBA adduct that was quantified colorimetrically at absorbance of 532 nm. Hydroxyproline assay depends on its oxidation to form a brightly-colored chromophore that can be easily detected at OD 560 nm. On the other hand, TAC assay kit depends on the reduction of $Cu^{2+}$ into $Cu^{+}$ by small molecule antioxidants e.g. GSH, vitamin E, ascorbate. The reduced $Cu^{+}$ ion was chelated with a colorimetric probe giving an absorbance peak at OD 570 nm, proportional to the total antioxidant capacity.

### 3.2. Determination of serum level of soluble receptor for advanced glycation end-product (sRAGE), lung tissue content of matrix metalloproteinase-9 (MMP-9), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF)

Serum level of sRAGE, lung tissue content of MMP-9, IL-8, and VEGF were estimated using ELISA kits (MyBioSource Inc., San Diego, CA, USA). The procedures were followed exactly as per the provided manuals. In brief, the ELISA kits employed the quantitative sandwich enzyme immunoassay technique. Monoclonal antibodies specific for rat sRAGE, MMP-9, IL-8 and VEGF were pre-coated onto a microplate. Standards and samples were pipetted into the wells, and sRAGE, MMP-9, IL-8 and VEGF were bound by the immobilized antibodies. After washing away unbound substances, enzyme-linked polyclonal specific antibodies were added to the wells. Wells were washed to remove unbound antibody-enzyme reagent, then a substrate solution was added to the wells. Color development was in proportion to the amount of the bound protein. The intensity of the color was measured using a microplate reader adjusted to 450 nm. Total protein in the samples was assayed colorimetrically (Cayman, USA).

### 3.3. Determination of receptor for advanced glycation end-products (RAGE), high mobility group box-1 (HMGB1) and cycloxygenase (COX-2) mRNA expression in the lung tissue of rats by real time PCR (RT–PCR)

RNeasy Purification Reagent (Qiagen, USA) was used to extract total RNA from brain tissue. The purity and concentration of RNA was detected spectrophotometrically (A260/A280 ratio) (Gene Quant 1300, Uppsala, Sweden). RNA quality was confirmed by gel electrophoresis. Oligo-(dT)-12–18 primer and Superscript™ II RNase Reverse Transcriptase (SuperScript Choice System, Life Technologies, Netherlands) were used to make the first-strand cDNA. The mixture was incubated for 1 h at 42 °C. Real time PCR was done using 10 μl of the

amplification mixture that contained 3 µl of cDNA, Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and 300 nM of primers. Quantification and analysis of data was done by ABI Prism Sequence Detection Software (v.1.7, PE Biosystems, USA). All values were normalized to GAPDH gene. Relative expressions of the investigated genes were calculated by the comparative threshold cycle method [21]. The PCR reactions comprised 1 cycle at 95 °C for 10 min, followed by another one at 94 °C for 15 s, then 40 cycles at 60 °C for 1 min. The gene sequences of the PCR primer pairs were as follows:

RAGE
Forward 5′-GCTCTGACCGAAGCGTGA-3′
Reverse 5′-CCTTCAGGCTCAACCAACAG-3′
HMGB1
Forward 5′-TGTCCACACACCCTGCATATT-3
Reverse 5′-CAAGCTCCCCCTCTTTTTCA-3′
COX-2
Forward 5′-CAGAAGAGGCTAAGACCGCCT-3′
Reverse 5′-TCTGGTCTTTGTGTTCCTCTGTCA-3′
GAPDH
Forward 5′-CTCCCATTCTTCCACCTTTG-3′
Reverse 5′-CTTGCTCTCAGTATCCTTGC-3′

### 3.4. Determination of phosphatidyl inositol-3-kinase (PI3K), protein kinase B (Akt) and β-catenin protein expression in the lung tissue of rats by western blot analysis

Following a previously described method [22], lung tissue homogenate was centrifuged, the supernatant was collected, and total protein concentration was determined according to Micro BCA protein assay kit (Thermo Scientific, USA). Protein samples were electrophoresed on 8% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, USA). Blocking of non-specific binding sites was done by incubation in TBST for 2 h at room temperature (0.05% Tween 20 in Tris buffered saline) and 7.5% (w/v) non-fat dry milk. Membranes were then rinsed for 10 min with TBST. This step was followed by overnight incubation at 4 °C with the primary specific antibodies against PI3K, Akt and β-catenin at 1:1000 dilution (Cell Signaling Technology, USA). Membranes were washed, then incubated at room temperature for 1 h with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody) at 1:25,000 dilution (Bio-Rad, USA). This was followed by additional washing. Immunocomplexes visualization was done by enhanced chemiluminescence ECL Plus System (Amersham Biosciences, USA). Quantification was carried out using densitometry and Molecular Analyst Software (Bio-Rad, USA). PI3K, Akt, and β-catenin protein expression was determined relative to β-actin.

### 3.5. Statistical analysis

Data were expressed as mean ± SEM (n = 10). Statistical comparisons between different groups were performed by ANOVA followed by Tukey's post hoc test with the aid of Graph Pad Prism software v.3 (San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

## 4. Results

### 4.1. Effect of paraquat, febuxostat and their combination on serum lactate dehydrogenase (LDH) activity and soluble receptor for advanced glycation end-product (sRAGE) serum level in rats

As graphically illustrated in Figs. 1 and 2, administration of paraquat (30 mg/kg, i.p.) induced a significant increase in serum LDH activity and a significant decrease in serum sRAGE level by 222.58% ($p < 0.001$) and 55.33% ($p < 0.001$) as compared to the control group, respectively. Administration of febuxostat (15 mg/kg, oral) to paraquat-intoxicated rats induced a significant decrease in serum LDH activity by 67.01% ($p < 0.001$) and a significant increase in serum sRAGE level by 91.35% ($p < 0.01$) as compared to paraquat group, respectively.

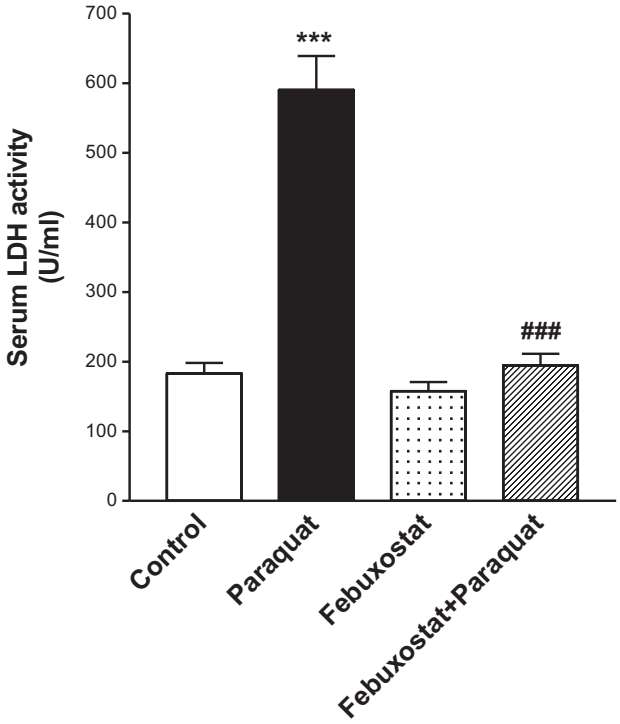


**Fig. 1.** Effect of febuxostat, paraquat and their combination on serum lactate dehydrogenase (LDH) activity in rats.
Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

### 4.2. Effect of paraquat, febuxostat and their combination on oxidative stress markers; xanthine oxidase activity (XO), superoxide dismutase activity (SOD), total antioxidant capacity (TAC), malondialdehyde content (MDA) and hydroxyproline content in the lung tissue of rats

As shown in Table 1, administration of a single dose of paraquat (30 mg/kg, i.p.) enhanced XO and reduced SOD activities in the lung tissue of rats by 131.68% ($p < 0.001$) and 45.37% ($p < 0.05$) as compared to the control group, respectively. The same dose of paraquat induced a significant decrease in TAC by 54.84% ($p < 0.01$) and a significant increase in MDA and hydroxyproline contents in the lung tissue of rats by 220.35% ($p < 0.001$) and 62.08% ($p < 0.05$) as compared to the control group, respectively.

On the other hand, administration of febuxostat (15 mg/kg, oral) for 7 days before and 7 days after paraquat single dose significantly inhibited XO and enhanced SOD activities in the lung tissue of rats by 35.18% ($p < 0.01$) and 99.19% ($p < 0.01$) as compared to paraquat group, respectively. Moreover, in the combination group, febuxostat induced a significant increase in TAC by 90.83% ($p < 0.05$), and a significant decrease in MDA and hydroxyproline lung contents by 42.12% ($p < 0.001$) and 34.36% ($p < 0.05$) as compared to paraquat group, respectively.

### 4.3. Effect of paraquat, febuxostat and their combination on receptor for advanced glycation end-product (RAGE) and high mobility group box-1 (HMGB1) gene expression in the lung tissue of rats

As shown in Figs. 3 and 4, paraquat significantly increased RAGE

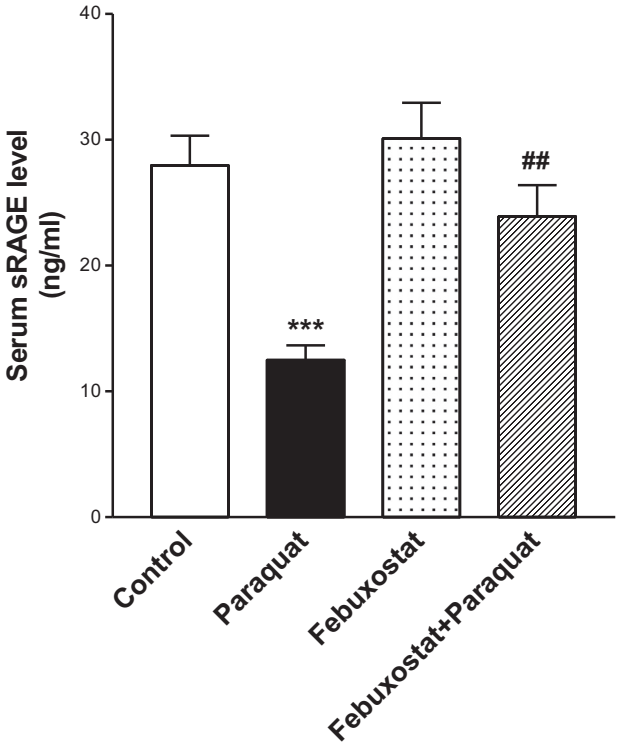


**Fig. 2.** Effect of febuxostat, paraquat and their combination on soluble receptor for advanced glycation end-product (sRAGE) serum level in rats.
Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ## Significantly different from the paraquat group at $p < 0.01$.

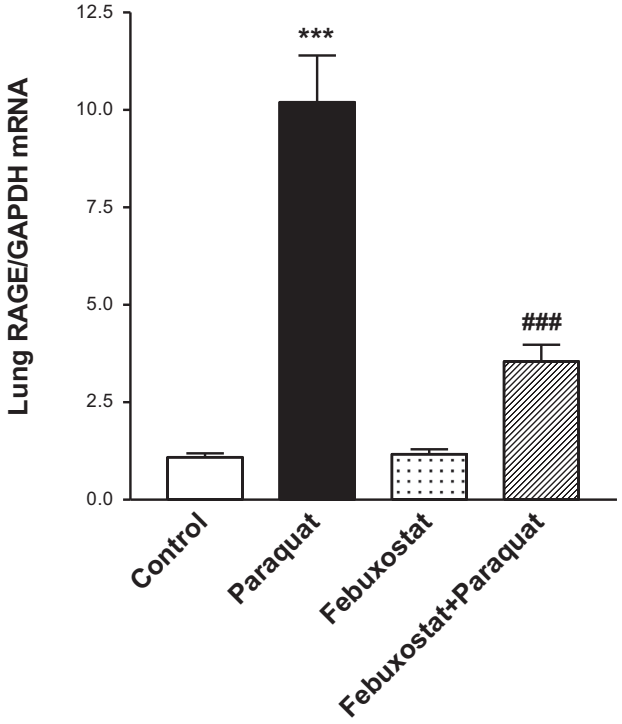


**Fig. 3.** Effect of febuxostat, paraquat and their combination on receptor for advanced glycation end-product (RAGE) gene expression in the lung tissue of rats.
Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

and HMGB1 gene expression in the lung tissue of rats by 833.21% ($p < 0.001$), and 1061.42% ($p < 0.001$) as compared to the control group, respectively. On the other hand, febuxostat administration to paraquat-treated rats significantly reduced RAGE and HMGB1 gene expression by 65.26% ($p < 0.001$) and 71.84% ($p < 0.001$) as compared to paraquat group, respectively.

### *4.4. Effect of paraquat, febuxostat and their combination on phosphatidyl inositol-3-kinase (PI3K), protein kinase B (Akt) and β-catenin protein expression in the lung tissue of rats*

As shown in Figs. 5, 6 and 7, Paraquat-administered rats showed significant increase in PI3K, Akt, and β-catenin protein expression in the lung tissue of rats by 856.95% ($p < 0.001$), 772.27% ($p < 0.001$), and 883.35% ($p < 0.001$) as compared to the control group, respectively. On the other hand, administration of febuxostat to paraquat-intoxicated rats showed significant decrease in PI3K, Akt and β-catenin protein expression in the lung tissue of rats by 71.27% ($p < 0.001$), 73.93% ($p < 0.001$) and 66.75% ($p < 0.001$) as compared to paraquat group, respectively.

### *4.5. Effect of paraquat, febuxostat and their combination on matrix metalloproteinase-9 (MMP-9), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) content and cycloxygenase-2 (COX-2) gene expression in the lung tissue of rats*

As shown in Table 2, paraquat induced significant rise in rat lung tissue content of MMP-9, IL-8 and VEGF by 129.77% ($p < 0.001$), 107.33% ($p < 0.001$) and 93.73% ($p < 0.01$) as compared to the control group, respectively. In addition, paraquat group showed a significant upregulation of COX-2 gene expression in the lung tissue of rats by 822.64% ($p < 0.001$) as compared to the control group. Meanwhile, febuxostat administration to paraquat-treated rats significantly reduced lung tissue content of MMP-9, IL-8 and VEGF by 29.49% ($p < 0.05$),

**Table 1**
Effect of paraquat, febuxostat and their combination on oxidative stress markers in the lung tissue of rats.

| | Control | Paraquat | Febuxostat | Febuxostat + paraquat |
|---|---|---|---|---|
| X.O. (U/mg wet tissue) | 43.94 ± 4.04 | 101.80 ± 11.50*** | 28.19 ± 2.60 | 65.99 ± 6.75## |
| SOD (U/mg wet tissue) | 2.27 ± 0.23 | 1.24 ± 0.19* | 2.86 ± 0.10 | 2.47 ± 0.31## |
| TAC (nmol/mg wet tissue) | 34.79 ± 3.86 | 15.71 ± 1.93** | 41.13 ± 3.56 | 29.98 ± 3.29# |
| MDA (nmol/mg wet tissue) | 3.98 ± 0.364 | 12.75 ± 1.39*** | 3.53 ± 0.41 | 7.38 ± 0.87### |
| Hydroxyproline (ng/mg wet tissue) | 23.63 ± 2.64 | 38.30 ± 4.75* | 27.80 ± 3.06 | 25.14 ± 2.64# |

Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *, **, *** Significantly different from the control group at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. #, ##, ### Significantly different from the paraquat group at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. X.O. (xanthine oxidase), SOD (superoxide dismutase), TAC (total antioxidant capacity), MDA (malondialdehyde).

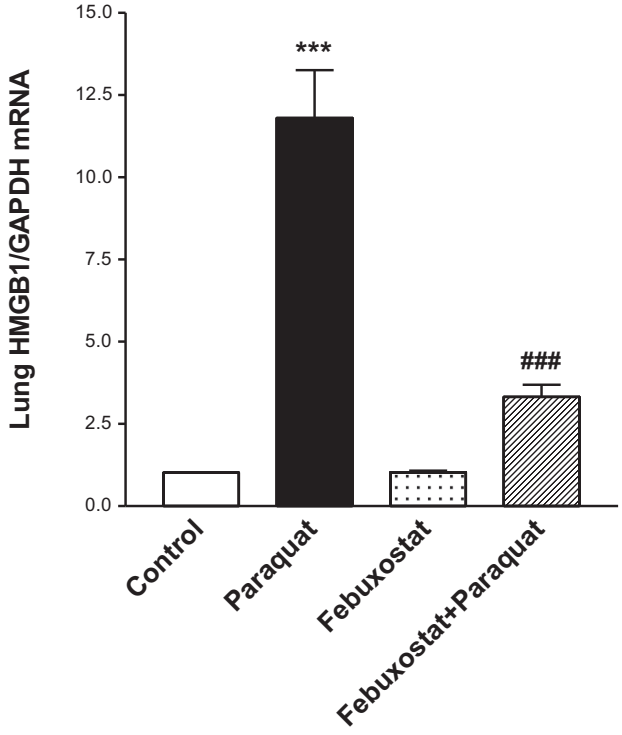


**Fig. 4.** Effect of febuxostat, paraquat and their combination on high mobility group box-1 (HMGB1) gene expression in the lung tissue of rats.
Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

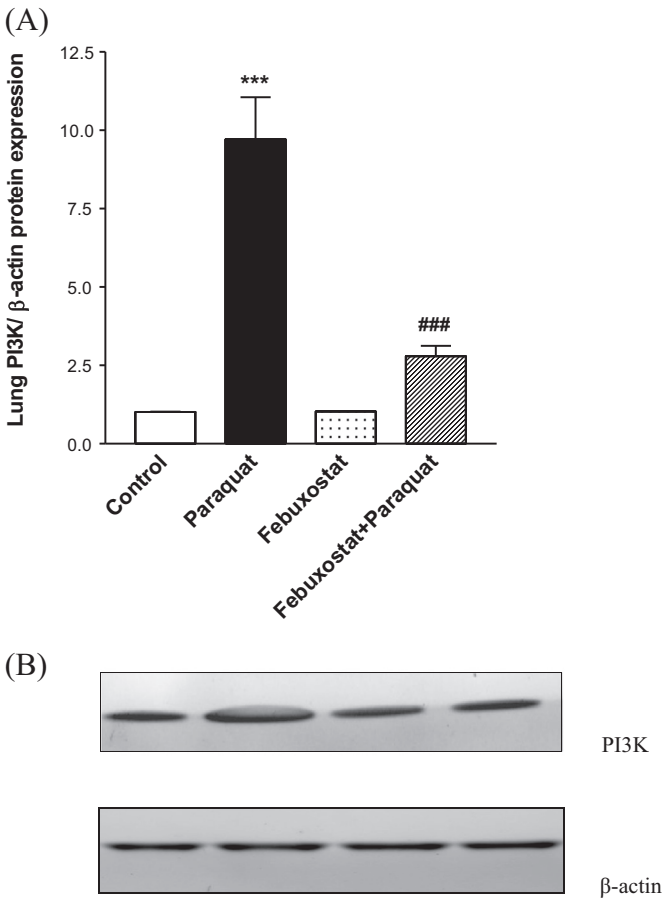


**Fig. 5.** Effect of febuxostat, paraquat and their combination on phosphatidyl inositol-3-kinase (PI3K) protein expression in the lung tissue of rats.
(A): Graphical representation of lung PI3K protein expression, (B): a representative western blot of lung PI3K protein (the sequence of the groups is the same as the graph). Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

39.88% ($p < 0.01$) and 31.75% ($p < 0.05$) as compared to paraquat group, respectively. Moreover, the combination group showed a significant downregulation of COX-2 gene expression in the lung tissue of rats by 70.35% ($p < 0.001$) as compared to the control group.

## 5. Discussion

In the present study administration of paraquat to rats induced lung toxicity characterized by elevated serum activity of lactate dehydrogenase (LDH) and increased levels of soluble receptor for advanced glycation end-product (s-RAGE). Similar deleterious effects of paraquat on the lung were previously reported [2,23]. Enhanced leakage of intracellular enzymes such as LDH and subsequent increase in its serum activity is considered a marker of tissue necrosis following exposure to free radicals [24]. Serum sRAGE is considered a biomarker for acute lung injury and pulmonary inflammation [25]. sRAGE can bind to circulating pro-inflammatory molecules and thereby preventing their binding to and activation of transmembrane RAGE with ultimate inhibition of RAGE pathway and its downstream inflammatory responses [26]. The inhibitory activity of febuxostat on serum LDH and sRAGE in this study may be attributed to the ameliorative effect of febuxostat on paraquat-induced lung injury. Similar inhibitory effects of febuxostat on LDH activity was previously observed in lipopolysaccharide-induced lung injury in rats [4].

The current study demonstrated the ability of paraquat to increase pulmonary tissue content of oxidative stress markers namely lipid peroxides and hydroxyproline. Enhanced xanthine oxidase and suppressed SOD activities in addition to reduced total antioxidant capacities were also observed. Previous studies showed that reactive oxygen species such as hydrogen peroxide and superoxide radicals were produced during the metabolism of paraquat by NADPH-cytochrome-P-450 reductase enzyme [7]. Moreover, paraquat-induced enhancement of xanthine oxidase activity may lead to excessive transformation of xanthine and oxygen into uric acid and superoxide radicals [4]. The latter are normally extinguished by superoxide dismutase. However, the obtained results showed inhibitory effect of paraquat on superoxide dismutase activity resulting eventually in the accumulation of superoxide radicals [10]. Accumulated superoxide radicals may interact with lung tissues, enhance lipid peroxidation, diminish total antioxidant capacity and stimulate the formation of inflammatory cytokines [27]. The latter may induce oxidative stress thereby initiating a vicious cycle [28,35]. On the other hand, elevated lung content of hydroxyproline in the present study may be regarded as an early marker of fibrosis since hydroxyproline is a main constituent of collagen [29].

In the current study, Febuxostat, a xanthine oxidase inhibitor, has shown protective effects against paraquat-induced oxidative stress in the lung tissue of rats. This was demonstrated as suppression in serum LDH activity and re-balance of redox status. Similarly, allopurinol, another xanthine oxidase inhibitor, was previously reported to inhibit free radical production and diminish paraquat toxicity in cultured endothelial cells [8]. It can be anticipated that inhibition of xanthine oxidase activity by febuxostat in the present study interfered with paraquat-induced accumulation of superoxide radical and thereby normalized superoxide dismutase activity, inhibited lipid peroxidation

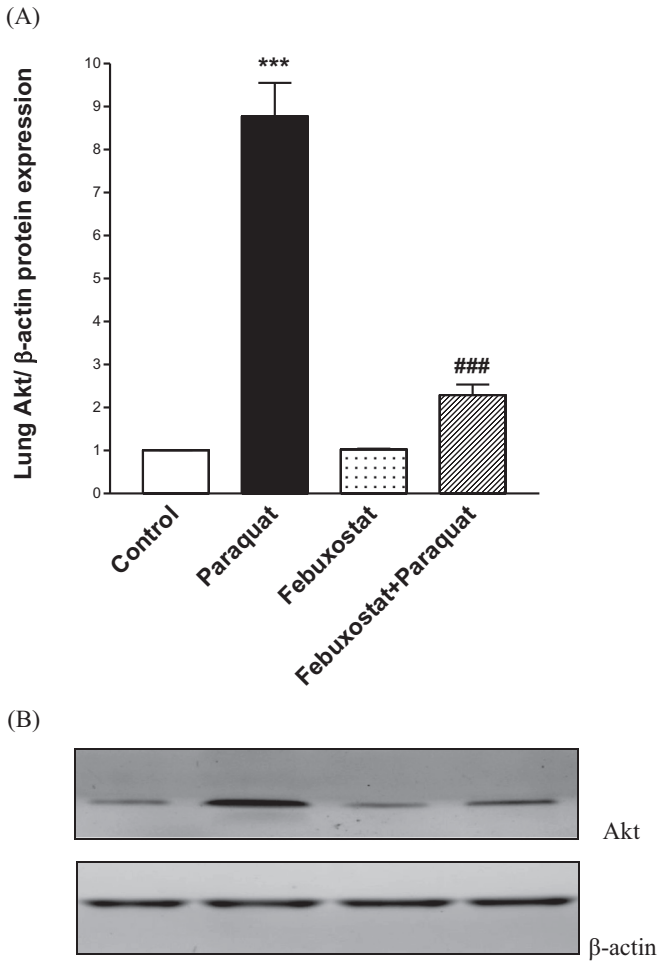


**Fig. 6.** Effect of febuxostat, paraquat and their combination on protein kinase B (Akt) protein expression in the lung tissue of rats.
(A): Graphical representation of lung Akt protein expression, (B): a representative western blot of lung Akt protein (the sequence of the groups is the same as the graph). Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

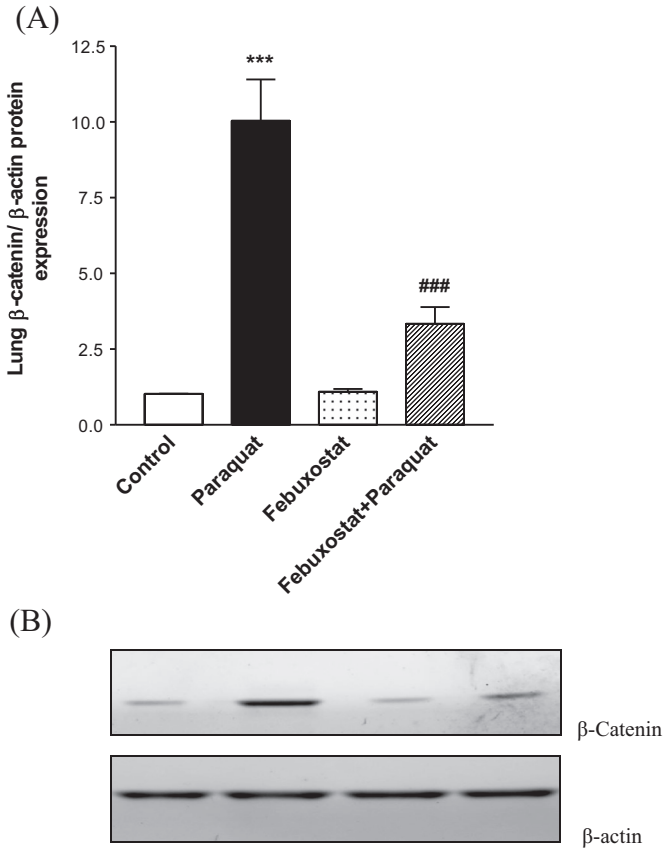


**Fig. 7.** Effect of febuxostat, paraquat and their combination on β-catenin protein expression in the lung tissue of rats.
(A): Graphical representation of lung β-catenin protein expression, (B): a representative western blot of lung β-catenin protein (the sequence of the groups is the same as the graph). Paraquat (30 mg/kg, i.p., single dose), febuxostat (15 mg/kg, oral). Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from the control group at $p < 0.001$. ### Significantly different from the paraquat group at $p < 0.001$.

and elevated total antioxidant capacity. This in turn resulted in suppression of oxidative stress-induced inflammation and fibrosis [35]. Similar antioxidant and anti-inflammatory effects of febuxostat were previously reported [4].

The present study showed that paraquat upregulated HMGB1 and RAGE mRNA, PI3K/Akt and β-catenin protein expression in the lung tissue of rats. These effects were significantly abolished by febuxostat. The high mobility group box-1 (HMGB1) is an intracellular transcription factor released by immune cells and necrotized tissue to act as an inflammatory mediator and chemoattractant to initiate immune responses resulting in enhanced release of inflammatory cytokines and interleukins [30]. HMGB1 level was negatively correlated with pulmonary function. It may even indicate promoted fibroblasts migration and enhanced fibrosis. Conversely, some studies showed that inhibition of HMGB1 expression in lung decreased airway inflammation [31,32]. Interestingly, allopurinol, another xanthine oxidase inhibitor, inhibited renal and cerebral HMGB1 expression and inflammation in rat [33,34].

On the other hand, the receptor for advanced glycation end-product (RAGE) is a membrane-bound receptor which is highly expressed in pulmonary tissues and regarded as an important mediator in inflammatory responses [35]. Oxidative stress and uric acid may upregulate HMGB1 and RAGE mRNA and protein expression [36,37]. Remarkably, reactive oxygen species and inflammatory cytokines may enhance binding of RAGE to its ligand HMGB1 [38]. Binding of RAGE to HMGB1 leads to the production of more reactive oxygen species, activation of NF-κB and propagation of inflammatory cascades [17]. Interestingly, suppression of uric acid and superoxide radicals following xanthine oxidase inhibition by febuxostat may account for the unprecedented current observation of febuxostat-induced down-regulation of RAGE expression.

Activation of RAGE by paraquat initiates a cascade of intracellular signaling pathways, including phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt), which is known to be related to inflammation and collagen synthesis [31]. Paraquat was previously reported to enhance Akt activation [39]. On the contrary, inhibitors of PI3K/Akt hindered lipopolysaccharide-induced production of inflammatory mediators both in cell lines and mice [40,41]. Akt may activate β-catenin either by direct phosphorylation or through a cross-talk with Wnt pathway [42,43]. This was evidenced by downregulation of Wnt/β-catenin signaling pathway upon inhibition of the kinase Akt [44]. β-catenin signaling has been reported to be involved in fibrotic tissue repair after tissue injury [45]. Therefore, it can be postulated that the protective effects of febuxostat in the present study against paraquat-induced lung inflammation may be achieved by the inhibition of PI3K/Akt pathway and its downstream molecules.

Moreover, paraquat-induced elevation in the lung tissue content of

**Table 2**
Effect of paraquat, febuxostat and their combination on inflammatory markers in the lung tissue of rats.

| | Control | Paraquat | Febuxostat | Febuxostat + paraquat |
|---|---|---|---|---|
| MMP-9 (ng/mg protein) | 23.21 ± 2.15 | 53.33 ± 5.64*** | 28.34 ± 3.07 | 37.60 ± 3.88# |
| IL-8 (ng/mg protein) | 58.41 ± 6.61 | 121.10 ± 11.11*** | 61.10 ± 5.95 | 72.80 ± 6.98## |
| VEGF (ng/mg protein) | 64.16 ± 7.35 | 124.30 ± 15.53** | 75.81 ± 6.03 | 84.83 ± 7.78# |
| COX-2 (COX-2/GAPDH mRNA) | 1.06 ± 0.02 | 9.78 ± 1.15*** | 1.11 ± 0.09 | 2.90 ± 0.26### |

Data were expressed as mean ± S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. **, *** Significantly different from the control group at $p < 0.01$ and $p < 0.001$ respectively. #, ##, ### Significantly different from the paraquat group at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. MMP-9 (matrix metalloproteinase-9), IL-8 (interleukin-8), VEGF (vascular endothelial growth factor), COX-2 (cycloxygenase-2).

MMP-9 may be attributed to enhanced expression of β-catenin; a transcription factor that was reported to target the expression of metalloproteinases [46]. Interestingly, it was observed that bleomycin enhanced β-catenin expression in an animal model of pulmonary fibrosis. The authors showed that blockade of β-catenin pathway could attenuate bleomycin-induced matrix metalloproteinases expression, collagen synthesis, and pulmonary fibrosis in the lungs of mice [47]. The protease MMP-9 is known to be released from activated neutrophils and macrophages. It is capable of digestion of elastin, collagen and other structural proteins resulting in destruction of the basement membrane and extracellular matrix. This leads to increased permeability of alveolar capillaries, exudation, alveolar damage and impaired gas exchange [10]. MMP-9 increases predominantly in early stages of pulmonary fibrosis and plays an essential role in T cell migration into lung tissue through basement membrane and interstitial collagen in pulmonary inflammation [48]. MMP-9 may also enhance the release of TGF-β and TNF-α leading to accelerated pulmonary fibrosis [49].

The anti-inflammatory effect of febuxostat against paraquat-induced pulmonary injury may be mediated partly through inhibition of paraquat-induced oxidative stress with consequent alleviation of free radicals-mediated endothelial damage and vascular permeability. In addition, febuxostat-induced inhibition of PI3K/Akt pathway and its downstream molecule β-catenin may abrogate the stimulatory effect of paraquat on pulmonary MMP-9 content leading to preservation of alveolar integrity. In a similar manner, it was previously reported that inhibition of MMP-9 by naringinin attenuated paraquat-induced lung injury in rats [10].

The increased content of the inflammatory mediators; IL-8 and VEGF and the overexpression of COX-2 mRNA in the lungs of rats treated with paraquat in this study may be attributed to enhanced PI3K/Akt/β-catenin pathway. Moreover, paraquat-induced RAGE may play a role. RAGE stimulates the transcription factor NF-κB, which promotes the production of interleukins and other inflammatory mediators [50]. In harmony with the present study, previous reports elucidated the ability of paraquat to induce IL-8 and other inflammatory mediators in the lung tissue of rodents [51,52]. IL-8 is a potent neutrophil activator and chemotactic which plays essential role in lung inflammation, alveolitis, migration of fibroblasts and pulmonary fibrosis [53]. Febuxostat-induced decrease in pulmonary IL-8 may result from inhibition of β-catenin expression. Knocking down β-catenin attenuated lipopolysaccharides-induced IL-8 and other inflammatory cytokines expression in bronchial epithelial cells [54].

In accordance with the present study, paraquat was previously found to induce VEGF in acute lung injury [55]. Vascular endothelial growth factor is a cytokine that plays many roles. It is normally expressed in alveolar and bronchial epithelium and is considered a marker of endothelial cell injury [56]. It controls endothelial cell proliferation and survival, vascular permeability, angiogenesis and monocytes recruitment [57]. It may promote inflammation by induction of NF-κB translocation [58]. Paraquat-induced endothelial injury may prompt the release of large quantities of VEGF. VEGF increases vascular permeability and allows inflammatory cell infiltration to the lung tissue leading to excessive damage. Drugs that hinder endothelial cell damage can decrease VEGF concentration and reduce vascular permeability [55]. In the current study, upregulation of β-catenin by paraquat may participate in the induction of the downstream molecule VEGF. Interestingly, VEGF gene promoter shows binding sites for β-catenin, and hence a direct correlation was found between β-catenin activation and upregulation of VEGF expression [59]. β-catenin may even upregulate VEGF receptor expression and enhance Akt phosphorylation leading to positive reinforcement of VEGF production [59]. The present data revealed that febuxostat inhibited paraquat-induced increase in lung content of VEGF. This effect may be mediated by downregulation of PI3K/Akt pathway and β-catenin expression [58].

Cycloxygenase-2 (COX-2) is an inducible pro-inflammatory enzyme that is normally present in low amounts in alveolar epithelia, and bronchial smooth muscles. It transforms arachidonic acid into prostaglandins and mediates inflammation, apoptosis and fibrosis in lung in response to oxidative stress [60]. Induction of COX-2 by paraquat was previously reported [61]. Enhanced COX-2 expression is accompanied by macrophage infiltration, which promotes β-catenin translocation from the cytoplasm to the nucleus [64]. Induction of PI3K/Akt signaling pathway upregulates COX-2 expression [62]. COX-2, in turn, enhances PI3K/Akt/Wnt/β-catenin pathway [63,64]. Febuxostat-induced inhibition of COX-2 activity in the current study may contribute to its protective activity against paraquat-induced lung inflammation. Interestingly, a recent in-vitro study showed that downregulation of COX-2 protected against bleomycin-induced inflammation [65]. Similar inhibitory effects on COX-2 activity by febuxostat was previously reported in diabetic renal injury model [66].

In conclusion, the present study showed that paraquat may induce lung injury by enhancement of xanthine oxidase activity and consequent accumulation of uric acid and superoxide radicals leading to upregulation of HMGB1 and RAGE expression. Binding of RAGE to HMGB1 leads to the activation of PI3K/Akt pathway, β-catenin and a milieu of inflammatory mediators. Febuxostat offered a novel intervention to protect against paraquat-induced lung toxicity by downregulation of RAGE, PI3K/Akt pathway, β-catenin expression and inhibition of the downstream inflammatory cascades.

Quantitative histopathological studies by specialists are recommended to demonstrate the cellular damage accompanied by paraquat pulmonary toxicity. Clinical trials are encouraged to investigate the lung protective effects of febuxostat in paraquat-intoxicated patients.

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## Conflict of interest

The authors declare equal contribution to every part of this research study and deny any conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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