Carvacrol and PPARy agonist, pioglitazone, affects inhaled paraquat-induced lung injury in rats

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Exposed rats to normal saline and paraquat (PQ) aerosol as control and PQ group, rats exposed to PQ and treated with 20 and 80 mg/kg/day carvacrol, 5 and 10 mg/kg/day pioglitazone, low dose of pioglitazone + carvacrol and 0.03 mg/kg/day dexamethasone (Dexa) for 16 days after the end of PQ exposure were studied (n = 6 in each group). Lung pathological changes, tracheal responsiveness to methacholine and ovalbumin (OVA) as well as transforming growth factor beta (TGF-β) and interleukin (IL)-6 level in the lung tissue homogenize as well as TGF-β, IL-6, oxidant and antioxidant levels oxidant and antioxidants were increased in PQ group (p < 0.05 to p < 0.001). Lung pathological changes, tracheal responsiveness to methacholine and OVA as well as TGF-β, IL-6 oxidant and antioxidant levels were improved in all treated groups except lung pathological changes in treated group with low dose of pioglitazone (p < 0.05 to p < 0.001). The effects of low dose of pioglitazone and carvacrol alone were significantly lower than in the combination group of low dose of pioglitazone + carvacrol (p < 0.05 to p < 0.001). Carvacrol treatment improved inhaled PQ-induced lug injury similar to the effects of dexamethasone. The synergic effect of carvacrol and pioglitazone suggests PPARγ receptor mediated effects of carvacrol on inhaled PQ-induced lung injury.

Paraquat (PQ, -1, 1′-dimethyl-4, 4′-bipyridinium dichloride) is an herbicide1 for controlling various crops2, and causes high toxicity3 as well as lethal effects (30 mg/kg) in humans3. Inflammation and oxidative stress, generation of intracellular reactive oxygen species, lipid peroxidation, and redox reactions of cellular membranes4 as well as increased interleukins and tumor necrosis factor alpha (TNF-α) in the lungs have been shown for PQ poisoning5. In addition, PQ is usually absorbed in the lung tissue and causes leukocytosis, pulmonary inflammation, hypoxemia, lung fibrosis, pulmonary hypertension, heart enlargement, acute renal damage, edema, increased serum levels of amylase, glucose, and creatinine6. Chronic exposure to PQ also induces alveolitis, inflammatory cell infiltration, and collagen deposition in the lung5. Poisoning with PQ is treated by activated charcoal, anti-inflammatory, immunosuppressive, and antioxidants agents such as acetylcysteine and salicylate7 but they are not able to cure PQ poising fully, and as such new drugs are needed to prevent PQ-induced lung injury8-10.

Carvacrol, the active component of various medicinal plants11, with anti-inflammatory12-17, anti-oxidant, and immunomodulatory properties18. In addition, studies have also reported improvement of tracheal responsiveness, inflammatory mediators, and total plus differential WBC12, serum cytokines and endothelin levels13, and lung pathological changes, immunoglobulin E (IgE) and eosinophil peroxidase levels in the BALF14, serum levels of total protein, in guinea pigs model of asthma as well as various cytokine gene expressions15 and T helper cells subtypes along with their cytokine gene expression16 in the splenocytes of asthmatic mice with carvacrol treatment.

Anti-inflammatory and anti-cancer effects on lung diseases, pain, and obesity have been shown for peroxisome proliferator-activated receptors (PPARs)19,20. The regulations of cellular metabolism, cell differentiation, apoptosis, and inflammation21-23 have also reported by PPARγ ligands such as prostaglandins, leukotrienes, rosiglitazone, and pioglitazone24.

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The aims of the present study are exploring the potential effects of carvacrol and PPARγ agonist (pioglitazone) on the lung injury induced by inhaled PQ and their possible synergic effects in male Wistar rats.

**Results**

**Lung weight changes due to inhaled PQ and the effects of treatments.** The lung's wet and dry weights significantly increased in the animals exposed to inhaled PQ compared to the control group ($p<0.01$ for wet and $p<0.05$ for dry weight), (Fig. 1A, B). The lung's wet weight significantly decreased in all treated groups and lung dry weight only in groups treated with a combination of Pio-5 + C-20 and dexamethasone compared to the PQ group ($p<0.05$ to $p<0.01$), (Fig. 1A, B). The improvement in the lung's wet weight in the group treated with a combination of Pio-5 + C-20 was significantly higher than in pio-5 group ($p<0.05$), (Fig. 1A). However, the W/D ratio was not significantly different among the studied groups (Fig. 1C).

**Lung pathological changes due to inhaled PQ and the effects of treatments.** The lung's pathological changes including interstitial inflammation, edema, interstitial fibrosis, and emphysema significantly increased in the group exposed to inhaled PQ ($p<0.001$ for all cases, Fig. 2). In all treated groups, all lung pathological variables significantly improved compared to the PQ group except Pio-5 for all variables and C-20 for lung edema and interstitial fibrosis ($p<0.05$ to $p<0.001$, Fig. 2). The improvements in lung edema in C-20 and Pio-5 groups and that of interstitial fibrosis in C-20, Pio-5, and Pio-10 groups were significantly lower than in Dexa-0.03 group ($p<0.001$ for interstitial fibrosis and $p<0.05$ for other cases, Fig. 2). The improvement in all pathological changes in C-20 and Pio-5 groups were significantly lower than the combination therapy of Pio-5 + C-20 groups ($p<0.05$ to $p<0.001$, Fig. 2). A photograph specimen of the lung in each studied group is shown in Fig. 3.

**Tracheal responsiveness changes due to inhaled PQ and the effects of treatments.** Methacholine cumulative dose response curve in the group exposed to inhaled PQ was shifted to the right compared to the control group (Fig. 4A). The value of $EC_{50}$ in the group exposed to inhaled PQ was significantly lower than in the control group ($p<0.001$, Fig. 5A).

On the other hand, methacholine cumulative dose response curves in all treated groups were shifted to the right compared to the PQ exposed group (Fig. 4B). The values of $EC_{50}$ in all treated groups were significantly higher than in the PQ ($p<0.001$ for all cases, Fig. 5A). In addition, methacholine cumulative dose response curves in Pio-5 and C-20 were shifted to the left compared to that of the Pio-5 + C-20 combination group (Fig. 4C). The value of $EC_{50}$ in C-20 was also significantly lower than in the Pio-5 + C-20 combination group ($p<0.05$, Fig. 5A).

Tracheal responsiveness to OVA was significantly increased in the group exposed to inhaled PQ as compared to the control group ($p<0.001$, Fig. 5B).

In all treated groups, tracheal responsiveness to OVA was significantly improved compared to the PQ exposed group ($p<0.01$ for C-20 and Pio-5 and $p<0.001$ for other groups, Fig. 5B). Tracheal responsiveness to OVA was significantly lower in C-20 than its smaller dose (C-20) and Dex 0.05 ($p<0.01$ for both cases Fig. 5B). Tracheal responsiveness to OVA was also higher in Pio-5 than in the Pio-5 + C-20 combination group ($p<0.05$, Fig. 5A).

**Transforming growth factor beta and Interleukin 6 levels changes due to inhaled PQ and the effects of treatments.** The levels of TGF-β and Interleukin (IL)-6 in the homogenized lung tissue were increased in the group exposed to inhaled PQ compared to the control group ($p<0.001$ for both cases, Figs. 6 and 7).

In all treated groups, TGF-β level was significantly reduced compared to the PQ exposed group ($p<0.01$ for C-20 and Pio-5 and $p<0.001$ for other groups, Fig. 6). The level of TGF-β in C-20, C-80, and Pio-5 was significantly higher than in the dexamethasone treated group ($p<0.05$ to $p<0.01$, Fig. 6). In the Pio-5 group, TGF-β level was significantly higher than in the Pio-10 group ($p<0.05$, Fig. 6). The level of TGF-β in Pio-5 and C-20 groups was also higher than in the Pio-5 + C-20 combination group ($p<0.001$ for both cases, Fig. 6).

In the groups treated with the high dose of carvacrol and pioglitazone as well as dexamethasone and the combination of low dose carvacrol and pioglitazone, IL-6 level was significantly reduced compared to the PQ exposed group ($p<0.05$ to $p<0.001$, Fig. 7). The level of IL-6 was significantly higher in the C-20 group than in the Pio-5 + C-20 combination group ($p<0.01$, Fig. 7).

**Oxidative stress markers due to inhaled PQ and the effects of treatments.** The levels of all oxidant and antioxidant biomarkers were significantly deteriorated in the bronchoalveolar lavage fluid (BALF) of inhaled PQ group compared to the control group ($p<0.01$ for SOD and thiol and $p<0.001$ for other cases, Figs. 8 and 9).

The NO$_2$ level was significantly decreased in all treated groups as well as MDA level in the groups treated with the high dose of carvacrol and pioglitazone as well as dexamethasone and combination of low dose carvacrol and pioglitazone, as compared to the PQ group ($p<0.05$ to $p<0.001$, Fig. 8). However, the levels of all anti-oxidant markers (CAT, SOD and thiol) were significantly increased in the BALF of the groups treated with the high dose of carvacrol and pioglitazone as well as dexamethasone and Pio-5 + C-20 combination ($p<0.05$ to $p<0.001$, Fig. 9). The effect of high dose pioglitazone on MDA, SOD, and CAT levels was significantly higher than its low concentration ($p<0.05$ for SOD and $p<0.01$ for other cases, Figs. 8 and 9).

The values of oxidant and antioxidant biomarkers in all treated groups were significantly improved compared to the PQ group ($p<0.05$ to $p<0.001$, Figs. 8 and 9). The effect of low dose carvacrol on NO$_2$ level, the impact of low dose pioglitazone on MDA, SOD, and CAT levels, as well as the effects of both doses of carvacrol and pioglitazone were significantly lower than the dexamethasone treated group ($p<0.05$ to $p<0.001$, Figs. 8 and 9).
Figure 1. Comparisons of lung wet (A) and dry (B) weight and wet/dry weight ratio (W/D) of the lung (C) between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). *p < 0.05 compared to the PQ-54 mg/m³, #p < 0.05 compared to dexamethasone, £p < 0.05 compared to Pio-5, comparisons between Pio-5 and Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey’s multiple comparison test.
Figure 2. Comparisons of emphysema (A), interstitial inflammation (B), edema (C) and interstitial fibrosis (D) between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone +20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). ***p < 0.001 compared to the control group. +p < 0.05, ++p < 0.01, and +++p < 0.001 compared to PQ-54 mg/m³. #p < 0.05 and ###p < 0.001 compared to dexamethasone. $p < 0.05 compared to Pio-5 mg/kg/day, £p < 0.05, ££p < 0.01 and £££p < 0.001 comparison between C-20 and Pio-5 with Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey's multiple comparison test.
**Discussion**

This study was designed to evaluate the effects of carvacrol, pioglitazone, and pioglitazone + carvacrol combination on lung injury induced by inhaled PQ along with the possible mechanisms of these effects. The lung's wet and dry weight, lung histology, tracheal responsiveness to methacholine, and OVA as well as TGF-β, IL-6, oxidants, and antioxidants levels were deteriorated in the group exposed to PQ. These results indicate induction

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**Figure 3.** Lung specimens photographs of the control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg), (Magnification: 10 × 20).
Figure 4. Cumulative concentration response curves to methacholine in the control group (Ctrl) and group exposed to paraquat aerosol at doses of 54 mg/m$^3$ (PQ-54 mg/m$^3$), (A) PQ-54 mg/m$^3$, groups exposed to PQ-54 mg/m$^3$ and treated with 10 mg/kg/day pioglitazone (Pio-10 mg/kg/day), 80 mg/kg/day carvacrol (C-80 mg/kg/day) and 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day), (B) and groups exposed to PQ-54 mg/m$^3$ and treated with 5 mg/kg/day pioglitazone (Pio-5 mg/kg/day), 20 mg/kg/day carvacrol (C-20 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg), (C).
of lung injury by inhaled PQ through inflammatory and oxidative stress processes, lung pathological changes and increased tracheal responsiveness to methacholine and OVA.

The PQ dose used in the present study was chosen according the only study that had examined the effect of inhaled PQ at doses of 0.83–2.07 mg/m³ so far. Nevertheless, the exposure time period in each session decreased while the duration of exposure and PQ dose increased. The duration of the study was also chosen based on several previous studies reporting increased tracheal responsiveness to methacholine, cell count, interleukin 17 (IL-17), TGF-β levels in the BALF, and collagen deposition in the lung 15 days following PQ administration in
Figure 6. Comparison of transforming growth factor beta (TGF-β) between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). ***p < 0.001 compared to the control group, ++p < 0.01, and +++p < 0.001 compared to the PQ-54 mg/m³, #p < 0.05 and ##p < 0.01 compared to dexamethasone, $p < 0.05 compared to Pio-5 mg/kg/day, £££p < 0.001 comparison between Pio-5 and C-20 with Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey’s multiple comparison test.

Figure 7. Comparison of IL-6 level between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa 0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). ***p < 0.001 compared to the control group, ++p < 0.01, and +++p < 0.001 compared to the PQ-54 mg/m³, #p < 0.05 and ##p < 0.01 compared to dexamethasone, $p < 0.05 compared to Pio-5 mg/kg/day, £££p < 0.001 comparison between Pio-5 and C-20 with Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey’s multiple comparison test.
mice, induced lung pathological changes 28 days after PQ administration in Wistar rats, and increased W/D of lung weight ratio, lung fibrosis as well as diminished arterial oxygen partial pressure, 7, 14, 21, and 28 days following PQ administration.

Several previous studies support the effects of PQ on the lung as observed in the current study. Increased total protein of the lung tissue and serious histopathological changes in the lung tissue were shown due to administration of 20 mg/kg, i.p PQ for 3 days. Administration of PQ (15 mg/kg/mL, i.p.) increased lung fibrosis, transforming growth factor-1β (TGF-1β) and malondialdehyde (MDA) levels in the lung as well as neopterin and

Figure 8. Comparison of MDA (A) and NO2 (B) levels between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). ***p < 0.001 compared to the control group, +p < 0.05, ++p < 0.01, and +++p < 0.001 compared to the PQ-54 mg/m³. ##p < 0.01 compared to the control group. $$p < 0.01 compared to Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey's multiple comparison test.
Figure 9. Comparison of SOD (A) CAT (B) activates and tiol (C) between control group (Ctrl), group exposed to paraquat aerosol at doses of 54 mg/m³ (PQ-54 mg/m³), groups exposed to PQ-54 mg/m³ and treated with 5 and 10 mg/kg/day pioglitazone (Pio-5 mg/kg/day and Pio-10 mg/kg/day), 20 and 80 mg/kg/day carvacrol (C-20 mg/kg/day and C-80 mg/kg/day), 0.03 mg/kg/day dexamethasone (Dexa-0.03 mg/kg/day) and 5 mg/kg/day pioglitazone + 20 mg/kg/day carvacrol (Pio-5 mg/kg + C-20 mg/kg). **p < 0.01 and ***p < 0.001 compared to the control group. +p < 0.05, ++p < 0.01, and +++p < 0.001 compared to the PQ-54 mg/m³. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to dexamethasone, $$p < 0.01 compared to Pio-5 mg/kg/day. £p < 0.05, ££p < 0.01 and £££p < 0.001 comparison between Pio-5 and C-20 with Pio-5 mg/kg/day + C-20 mg/kg/day group. Data are presented as mean ± SEM (n = 6 in each group). Comparisons between different groups were made using one-way ANOVA followed by Tukey's multiple comparison test.
antioxidant levels in the serum\textsuperscript{30}. Infiltration of inflammatory cells in the lung's interstitial tissues\textsuperscript{33} and BALF, along with total and differential white blood cell (WBC)\textsuperscript{13} increased 48 h following a single dose (30 mg/kg) of PQ. Administration of PQ (10 mg/kg, i.p.) increased cellular recruitment, IL-17 and TGF-β levels in the BALF, collagen deposition in the lung and tracheal responsiveness to methacholine\textsuperscript{35}. Lung pathological changes were also reported after administration of PQ for 2 weeks in rats\textsuperscript{36}. Increased lymphocyte count, TNF-α, C-reactive protein (CRP), and retinol binding protein (RBP) levels were demonstrated in patients with acute PQ poisoning\textsuperscript{33}. However, the effect of inhaled PQ on the lung was shown as it is the common way of exposing farmers to this toxin.

The main mechanisms of the effect of PQ on the lung have been suggested as the alveolar and Clara cell membrane expression of polyamine transport system expression as well as induction of lung inflammation and oxidative stress as reported previously\textsuperscript{34}. Reduction of nicotinamide adenine dinucleotide phosphate (NADPH) levels, as membrane lipid peroxidation has also been reported to contribute to the PQ-induced lung injury\textsuperscript{35}. Treatment with both doses of pioglitazone significantly decreased the lung's wet weight, tracheal responsiveness to methacholine and OVA, as well as TGF-β and IL-6 levels, while the lung's pathological changes were induced only due to high doses of pioglitazone. The levels of oxidant biomarkers were reduced by those of antioxidants increased due to pioglitazone treatment. The effects of high dose pioglitazone on most variables were higher than the impacts of its low dose. The results of treatment of PQ exposed animals with pioglitazone demonstrated reduction of lung pathological changes and tracheal responsiveness which could be due to the ameliorating effect of pioglitazone on inflammatory and oxidative stress processes.

Anti-inflammatory and antioxidant effects of pioglitazone and other PPAR-γ agonists are well documented which support the effects of pioglitazone on lung injury due to inhaled PQ as observed in the current study. Pioglitazone (1–30 mg/kg) inhibits increased myeloperoxidase activity, as well as intestinal TNF-α protein and messenger RNA (mRNA) expression\textsuperscript{36}. Pioglitazone increased super oxide dismutase (SOD) and catalase (CAT) enzymes and inhibited ischaemia-reperfusion model\textsuperscript{37}. Increased interleukin 4 (IL-4), but decreased interferon gamma (IFN-γ), TNF-α, and interleukin 6 (IL-6) levels were reported via pioglitazone\textsuperscript{38}. Treatment with the combination of pioglitazone (15 mg/kg/day) and metformin improved lung adenoma cancer\textsuperscript{39}, reduced nitric oxide (NO) production, TNF-α, interleukin 1-beta (IL-1β), IL-6, and interleukin 8 (IL-8) but elevated IL-4 and interleukin 10 (IL-10) levels in lipopolysaccharide stimulated astrocytes (LPS-stimulated astrocytes)\textsuperscript{40}. Pioglitazone (10 mg/kg, i.p or 3 h) treatment also decreased degranulation and adhesion of neutrophils in LPS-induced lung injury\textsuperscript{41}. Pioglitazone-induced pulmonary inflammation was also diminished by atorvastatin through PPARγ receptors\textsuperscript{42}. In patients with metabolic syndrome\textsuperscript{43} and subjects with advanced diabetic nephropathy\textsuperscript{44}, pioglitazone treatment reduced WBC counts indicating its anti-inflammatory effects.

Carvacrol treatment reduced the lung's wet weight, pathological changes, tracheal responsiveness to methacholine and OVA as well as TGF-β and IL-6 levels compared to the PQ-exposed group. Carvacrol treatment also decreased oxidant biomarkers, but increased those of antioxidants. All changes were more pronounced in the group treated with the higher dose of carvacrol than its low dose. Thus, treatment of animals exposed to inhaled PQ with carvacrol decreased lung pathological changes and tracheal responsiveness to methacholine and OVA. These findings indicate the effect of carvacrol on inflammatory and oxidative stress processes induced by inhaled PQ in rats.

Anti-inflammatory, antioxidant, and immunomodulatory effects of carvacrol on lung disorders have previously been shown, which support the findings of the present study. Treatment with carvacrol improved tracheal responsiveness, inflammatory mediators, total and differential WBC\textsuperscript{45}, serum cytokine and endothelin levels\textsuperscript{13}, and lung pathological changes, immunoglobulin E (IgE) and eosinophil peroxidase levels in the BALF\textsuperscript{46}, serum levels of myeloperoxidase, phospholipase A2 (Phospholipases A2) and histamine\textsuperscript{47} in a guinea pig model of asthma as well as gene expression of various cytokines\textsuperscript{15} and T helper cells subtypes along with their cytokine gene expression\textsuperscript{48} in and splenocytes of asthmatic mice. In a guinea pig model of chronic obstructive pulmonary disease (COPD), carvacrol improved tracheal responsiveness and lung pathological changes\textsuperscript{49}, lung inflammation by oxidative stress\textsuperscript{46}, as well as systemic inflammation\textsuperscript{47}.

Carvacrol also improved wheezing, forced expiratory volume in 1 s (FEV\textsubscript{1}), and nitrite plasma levels\textsuperscript{48} as well as pulmonary function tests, respiratory symptoms, hematological indices, and high-sensitivity C-reactive protein\textsuperscript{49} in asthmatic patients. In patients with sulfur mustard-induced lung disorders, carvacrol treatment for 2 months improved hematological parameters, oxidant/antioxidant biomarkers, and pulmonary function tests\textsuperscript{50} as well as inflammatory mediators and respiratory symptoms\textsuperscript{51}. The plant extract also decreased IL-4 but increased IFN-γ level and elevated IFN-γ/IL4 ratio, indicating increased Th1/Th2 balance in in vitro and in vivo studies in animal models of asthma and peripheral blood mononuclear cells respectively\textsuperscript{52}.

Treatment with low doses of pioglitazone (5 mg/kg/day) + carvacrol (20 mg/kg/day), which was the most interesting part of the results, significantly improved all measured variables compared to PQ exposed group. Indeed, low doses of pioglitazone and carvacrol showed the lowest and in some cases non-significant effects. In addition, the effect of the combination treatment group with Pio-5 + C-20 was higher in most measured variables than the effects of low doses of pioglitazone and carvacrol alone. This finding suggests a synergic effect between pioglitazone and carvacrol which may indicate PPAR-γ receptor-mediated effects of carvacrol on lung injury induced by inhaled PQ. This suggestion was supported by the results of a study reporting on the activation of PPAR receptors and inhibition of cyclooxygenase-2 (COX-2) pathway through treatment with carvacrol\textsuperscript{53}. However, further studies examining the effect of carvacrol in the presence of PPAR-γ receptors' antagonist are required to confirm this suggestion.

Dexamethasone, a known anti-inflammatory drug used as a positive control treatment, also improved all measured variables which were not significantly different effects of high doses of pioglitazone, carvacrol, and combination of low dose of pioglitazone + low dose of carvacrol. The results of dexamethasone treated group
The wet-to-dry weight (W/D) ratio of the lungs was their wet weight were determined. The lungs were then dried at 60 °C for 48 h with their dry weighted measured.

Germany), (0.03 mg/kg KB/day)34, via gavage for 16 days after the end of PQ exposure period. The saline (5 ml) and xylazine (5 mg/kg)55 (Fig. 10).

treatment period (day 32), the rats were anesthetized through intraperitoneal injection of ketamine (50 mg/kg) and pioglitazone were prepared through diluting them in saline using few drops of tween-80. At the end of the was gavaged to the control and PQ-exposed groups on days 16–32. Appropriate concentrations of carvacrol was prepared as described previously57. Tracheal responsiveness to methacholine hydrochloride (Sigma Chemical, St. Louis, MO, USA) was evaluated using cumulative concentrations (log)-response curve to methacholine and measured34.

Animals were treated with carvacrol (purity 90%, Ji’ An HaiRui Natural Plant, China), (20 and 80 mg/kg BW/day)34, and dexamethasone (Dexa) (Sigma, St. Louis, MO, USA), (0.03 mg/kg KB/day)34, via gavage for 16 days after the end of PQ exposure period. The saline (5 ml) was gavaged to the control and PQ-exposed groups on days 16–32. Appropriate concentrations of carvacrol and pioglitazone were prepared through diluting them in saline using few drops of tween-80. At the end of the treatment period (day 33), the rats were anesthetized through intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg)34 (Fig. 10).

Lung weight measurement. At the end of the treatment period (day 33), the lungs were removed and their wet weight were determined. The lungs were then dried at 60 °C for 48 h with their dry weighted measured. The wet-to-dry weight (W/D) ratio of the lungs was calculated34.

Lung pathological evaluation. Pathological changes of the left lung (following animal scarification and removal of the lung) including interstitial inflammation, edema, interstitial fibrosis, and emphysema were examined and scored as previously described (no pathologic changes = 0, patchy changes = 1, local changes = 2, scattered changes = 3, and severe changes in most parts of the lung = 4)34.

Measurement of tracheal responsiveness to methacholine and ovalbumin. The tracheal ring was prepared as described previously34. Tracheal responsiveness to methacholine hydrochloride (Sigma Chemical Ltd, UK) was evaluated using cumulative concentrations (log)-response curve to methacholine and mea-

| Groups | Exposed agents | Dose | Treated agents | Dose |
|--------|----------------|------|----------------|------|
| Ctrl   | Saline         | –    | –              | –    |
| PQ     | Paraquat       | 54 mg/m³ | –         | –    |
| C-20   | Paraquat       | 54 mg/m³ | Carvacrol | 20 mg/kg/day |
| C-80   | Paraquat       | 54 mg/m³ | Carvacrol | 80 mg/kg/day |
| Pio-5  | Paraquat       | 54 mg/m³ | Pioglitazone | 5 mg/kg/day |
| Pio-10 | Paraquat       | 54 mg/m³ | Pioglitazone | 10 mg/kg/day |
| Pio-5+ C-20 | Paraquat | 54 mg/m³ | Pioglitazone + Carvacrol | Pio-5 mg/kg/day + C-20 mg/kg/day |
| Dexa-0.03 | Paraquat | 54 mg/m³ | Dexamethasone | 0.03 mg/kg/day |

Table 1. Different studied groups, their exposure to saline solution or paraquat aerosols as well as their treatments (n = 6 in each group). PQ paraquat, C carvacrol, Pio pioglitazone, Ctrl control.
Figure 10. Exposing animals to aerosols of inhaled PQ (54 mg/m³) 8 times (days 1, 3, 5, 7, 9, 11, 13, 15 and 16) and treatment of animals with the carvacrol, pioglitazone and dexamethasone for 16 days after the end of exposure period. Animal were sacrificed in day 32 and various variables were measured.

Cytokines measurement. Transforming growth factor beta (TGF-β) level in the lung tissue homogenize and IL-6 were measured by specific enzyme-linked immunosorbent assay (ELISA) kits (Hangzhou Eastbiopharm, Iran) according to the manufacturer’s technique.

Oxidants’ and antioxidants’ measurements. Bronchoalveolar lavage (BALF) was prepared by cannulating the trachea and through lavage of the right lung by injecting 1 ml phosphate-buffered saline (PBS) solution 5 times and aspiration after gentle lung lavage. Oxidative markers such as malondialdehyde (MDA) and nitrite (NO2) as well as antioxidant markers including superoxide dismutase (SOD) and catalase (CAT) activities plus thiol group (SH) levels were measured in BALF. For this purpose, 1.5 ml BALF was centrifuged at 2500 rpm for 10 min with the oxidant and anti-oxidants markers measured in the supernatant of BALF as previously described.

Data analysis. Data were analyzed by the one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and results were presented as mean ± SEM. Values of p < 0.05 were considered as statistically significance.

Data availability
The raw data are available by the corresponding author upon request.

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Author contributions
M.H.B. and H.R.K. designed the experiment. F.A., H.K.R., and A.M. performed the experiment. F.A., M.H.B., and A.M. wrote the manuscript. All authors discussed and contributed to the analysis of the experimental data.

Competing interests
The authors declare no competing interests.

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