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EXPERIMENTAL PAPERS

Carvacrol Mitigates Bleomycin-Induced Experimental Pulmonary Fibrosis

N. H. Turgut[a], H. Gungor[b,*], M. Ekici[c], M. O. Karayigit[d], and H. Kara[b]

[a] *Department of Pharmacology, Faculty of Pharmacy, Izmir Katip Celebi University, Izmir, Turkey*

[b] *Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, Turkey*

[c] *Department of Physiology, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, Turkey*

[d] *Department of Pathology, Faculty of Veterinary Medicine, Cukurova University, Adana, Turkey*
**e-mail: gungor@cumhuriyet.edu.tr*

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Abstract—Idiopathic pulmonary fibrosis (IPF) is a serious progressive pulmonary disease of unknown etiology and high mortality. Carvacrol is a natural phenolic monoterpene with various pharmacological effects, especially antioxidant and anti-inflammatory effects. Hence, the present study aimed to investigate the effect of carvacrol on bleomycin (BLM) induced pulmonary fibrosis (PF) in Wistar-albino rats. Rats were administered a single dose of BLM (5mg/kg, intratracheal) or vehicle and treated with carvacrol (100 mg/kg, p.o. for 14 days following BLM administration). For calculating the lung index, the body and lungs were weighed. The Elisa method was used to assess hydroxyproline content, anti-inflammatory, and antioxidant effects. Fibrosis score, collagen deposition and inflammation were evaluated with Hematoxylin-Eosin (HxE) and Masson's trichrome staining. Inducible nitric oxide synthase (iNOS), transforming growth factor-beta 1 (TGF-β1), and caspase 3 expressions were assessed immunohistochemically. BLM administration significantly diminished glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities and increased malondialdehyde (MDA) levels. BLM also increased tumor necrosis factor alpha (TNF-α) and collagen bundle accumulation. Carvacrol at 100 mg/kg significantly decreased collagen accumulation, MDA, TNF-α levels, iNOS, TGF-1, and caspase 3 expression, while increasing SOD and GPx activity. Histopathological examination supported the findings that carvacrol attenuated the degree of collagen deposition and inflammation. This study revealed that treatment with carvacrol (100 mg/kg) exhibits a potential healing effect on BLM-induced PF by reducing inflammatory and oxidative damages and histopathological alterations, with possible molecular targets being iNOS, TGF-β1 and caspase 3 signaling pathways.

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Keywords: carvacrol, pulmonary fibrosis, Inducible nitric oxide synthase (iNOS), transforming growth factor-beta 1 (TGF- β1), caspase 3

INTRODUCTION

Among interstitial pneumonias, idiopathic pulmonary fibrosis (IPF) is the most common lung disease. IPF is a slowly disseminated, ultimately fatal disease characterized by excessive fibroblast

proliferation, aberrant re-epithelialization, and deposition of collagen with irreversible worsening of pulmonary function [1]. IPF predominantly occurs in the elderly, and following diagnosis, patients with IPF have a median survival time of 3–5 years. The actual cause of the disease is still unknown. It is believed that pulmonary fibrosis (PF) results from many factors, such as cytokines, extracellular matrix (ECM), inflammatory chemokines, and oxidative stress [2, 3]. Corticosteroids, immunosuppressant agents, cytokine antagonists, antileukotrienes, antioxidants, and oxygen therapy are limited available medical therapies, which are incapable of reversing this devastating lung disease [4]. Thus, the development of effective and nontoxic novel treatment strategies for PF is an urgent need.

Carvacrol (2-methyl-5-isopropylphenol) is a monoterpenoid phenol, which is present in essential oils of various aromatic plants of the Lamiaceae family, like *Origanum*, *Thymus*, *Thymbra*, *Corydothymus*, and *Satureja* [5]. Carvacrol is used to add flavor to sweets and drinks. It is also used to keep food from going bad by killing bacteria [6]. It is known that essential oils containing high amounts of carvacrol have strong antioxidant properties comparable to ascorbic acid, vitamin E, and butyl hydroxyl toluene [7, 8]. Carvacrol has been reported to possess various pharmaceutical activities, including antioxidant, anti-inflammatory, antinociceptive, antiviral, antifungal, antibacterial, expectorant, antitussive, and anticancer activities [5, 6, 9, 10].

Bleomycin (BLM) is a cytotoxic chemotherapeutic antibiotic used for the treatment of different cancers such as head and neck carcinoma, testicular carcinoma, ovarian cancer, and lymphoma. One of the major and severe side effects of BLM is PF in cancer patients. BLM induced PF is a commonly used experimental model in rats, mice, and hamsters [11]. Intratracheal, a single dose of BLM administration leads to dose dependent functional modifications that induce neck carcinoma, testicular carcinoma, ovarian cancer, and lymphoma. This damage could be due to enhancing the generation of reactive oxygen species (ROS), which is related to DNA injury, followed by epithelial cell damage, inflammation and elevation of fibrotic cytokines including transforming growth factor-beta 1 (TGF-β1), interleukin-1(IL-1) and tumor necrosis factor (TNF-α). Then, with excessive deposition of ECM and collagen synthesis, it may progress to fibrosis [12].

Although many pharmacological effects of carvacrol are known, the mechanisms by which carvacrol offers protection against PF remain largely unknown. To the best of our knowledge, this study is the first to investigate the effect of carvacrol against BLM-induced PF and to explore its potential mechanisms of action. This study was designed to evaluate the effects of carvacrol on inflammation and oxidative stress against PF caused by BLM administration in rats and to assess the role of inducible nitric oxide synthase (iNOS), TGF-β1 and caspase 3 in the antifibrotic effect of carvacrol.

MATERIAL AND METHODS

Chemicals and drugs

Carvacrol was purchased from Boston USA Chemistry (Boston, Massachusetts, USA) and bleomycin (BLM) was provided by Koçak Farma Pharmaceuticals and Chemical Industry Inc. (Istanbul, Turkey). The TGF-β1, iNOS, and caspase 3 antibodies were obtained from Santa Cruz Biotechnology (Texas, USA). For glutathione peroxidase (GPX) and superoxide dismutase (SOD) Cayman Chemical Company (Michigan, USA) elisa kits were used. Bioassay Technology Laboratory (Birmingham, UK) and Andy Gene (China) provided the TNF-α and hydroxyproline elisa kits, respectively. All other chemicals and reagents used were of analytical grade.

Animals

Adult male albino Wistar rats (weighing 180–200 g, 8 weeks of age) were obtained from Sivas Cumhuriyet University, Experimental Animals Laboratory (Turkey). The rats were housed in controlled laboratory conditions (temperature: 22 ± 2°C, 40–60% humidity, 12-h light/dark cycle) and fed with standard food and water ad libitum. The study was approved by the Ethical Committee for Animal Experiments of Sivas Cumhuriyet University (HADYEK, Turkey) (Number: 65202830-050.04.04-221). The

research protocol was performed in line with the Guideline on the Care and Use of Laboratory Animals (EU Directive 2010/63/EU for animal experiments).

Induction of pulmonary fibrosis

After one week of acclimatization, rats were fasted overnight and anesthetized (ketamine 90 mg/kg + xylazine 3 mg/kg, *i.p.*). Induction of PF was achieved with a single dose of intratracheal instillation of BLM (5 mg/kg b.w.) in 0.9% NaCl solution (0.1 mL/rat). The animals in the control group received saline in the same volume instead of BLM. The neck region was shaved and sterilized, and the BLM dose was given slowly with a tracheal cannula after an incision in the midline of the neck region [13]. After administration, the incision site was closed with surgical suture and sterilized with povidone-iodine (10%) application locally. After injection, rats received an injection of ketoprofen (3 mg/kg, *i.m.*) for analgesia.

Experimental protocol

Rats were randomly distributed into five groups of six rats in each group as follows: Control group: with intratracheal administration of a single dose of saline (0.1 mL) and administration of a vehicle solution (olive oil, volume: 1 mL/kg body weight, 14 days). BLM group: with intratracheal administration of a single dose of BLM (5 mg/kg b.w) and vehicle solution for 14 days. C100 group: with intratracheal administration of a single dose of saline (0.1 mL) along with carvacrol (C) (100 mg/kg, vehicle olive oil, volume: 1 mL/kg body weight) for 14 days. Two treatment groups BLM + C50 and BLM + C100: with intratracheal administration of a single dose of BLM (5 mg/kg b.w.) along with orally administered carvacrol in a dose of 50 or 100 mg/kg, respectively, for 14 days. The BLM dose was selected from the literature of previous studies [13, 14]. Again, the selected doses of carvacrol, 50 mg/kg/day and 100 mg/kg/day were based on previous studies [15–18]. Carvacrol was applied via oral gavage one hour after BLM administration.

Rats were weighed both at the beginning and end of the study. Rats were anesthetized (xylazine + ketamine, i.p. as stated above), and blood samples were collected through cardiac punctation. Animals were then euthanized by cervical dislocation and a cannula was placed in the trachea, ligated with silk thread, and sterile saline was slowly infused into the lung 3 times (3mL) to collect bronchoalveolar lavage fluid (BALF). Total fluid recovery in each group was about 80%. For further analysis, collected samples were centrifuged (300 g × 10 min, 4°C) and frozen at −80°C. Lungs were rapidly excised out, quickly rinsed in cold saline, and weighed to calculate the lung index [(lung weight/body weight) × 100] [13, 19, 20]. Left lungs were fixed in 10% formalin for histopathological and immunohistochemical determination. The right lungs were kept at −80°C for further biochemical analysis.

Assay of lipid peoxidation

In this study, the content of malondialdehyde (MDA), as a lipid peroxidation marker, was analysed in lung tissue homogenate based on the method of Ohkawa et al [21]. The level of MDA was detected by thiobarbituric acid reactive substances (TBARS), which was related to the reaction between MDA in homogenates and thiobarbituric acid. The amount of the pink complex formed after this reaction was determined using a spectrophotometer (Perkin Elmer UV/Vis Lambda 25, USA) at 532 nm wavelength. The MDA concentration was expressed as nmol/mg protein. Standard solutions of 1,1,3,3-tetraethoxypropane (TEP) were used for comparison. For total protein content, the method of Lowry et al. [22] was used.

Activities of antioxidant enzymes and quantification of hydroxyproline

SOD and GPx enzyme activities (Cayman Chemical Company, USA), and hydroxyproline content were evaluated using the commercially available standard kits from Andy Gene (China). Tissue homogenates were prepared and analysed according to the manufacturer's instructions. Absorbances were recorded with an ELISA reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, USA) at 340 nm for GPx, 440–460 nm for SOD, and 450 nm wavelength for hydroxyproline. GPx activity was expressed as nmol/min/mg of protein, SOD activity was

expressed as U/mg of protein, and hydroxyproline as ng/mg of protein.

TNF-α analysis

TNF-α was measured using a commercially available kit from Bioassay Technology Laboratory (Birmingham, UK), and analyses were carried out in accordance with the manufacturer's instructions. For supernatant fraction analysis, BALF samples were centrifuged at 300 *g* for 10 min at 4°C. Recorded absorbance values (450 nm wavelength) were expressed as ng/L.

Histopathological examination

Fixed lung tissues in 10% formaldehyde were subjected to dehydration in increasing concentrations of alcohol before being embedded in paraffin. Sections of 5 μm were prepared from paraffin blocks using a Leica Reichert RM 2125 microtome (Germany). After all tissue sections were deparaffinized in xylene, they were rehydrated in graded alcohols. Hematoxylin-eosin (HXE) staining was applied to the tissues to determine inflammatory changes. Trichrome staining was performed in order to determine the connective tissue density. For examining the staining results, a light microscope (Nikon, YS 100, Japan) was used. For fibrosis scoring, the Ashcroft scoring system was used [23]. By applying the Ashcroft scale, the lungs were first scanned microscopically with a 10-fold objective according to Ashcroft et al. criteria [24]. As the field analyzed was very heterogeneous, we found microscopic evaluation with a 10-fold objective difficult and changed the magnification to a 20-fold objective scale. This allowed the evaluation of fine structures and provided an adequate overview. Different areas were photographed from each slide, and the fibrotic index was determined for each animal in each group. Then, it was examined whether there was a significant difference between the groups for lung fibrosis. The histopathologic assessment was conducted by an experienced pathologist, unaware of the treatment groups. Lung fibrosis grade was scored from 0 to 8. The criteria for scoring were as follows: Grade 0 = normal lung; Grade 1 = minimal fibrous thickening of the alveolar or bronchiolar walls; Grade 2–3 = moderate thickening of the walls without significant damage to lung architecture; Grade 4–5 = increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous mass; Grade 6–7 = severe deterioration of structure and large fibrous areas; Grade 8 = total fibrotic obliteration of the field.

Assessment of TGF-β1, iNOS, and caspase 3 expressions in the lung

For immunohistochemical staining, the tissues underwent antigen retrieval at 600 watts for 20 minutes, and the sections were left to cool for 20 minutes afterwards. To assess endogenous peroxidase activity the sections were treated with a 3% hydrogen peroxide solution in methanol for 15 minutes. Sections were washed 3 times for 5 minutes in phosphate buffered saline (PBS). After 10 minutes of blocking solution (Histostain-Plus Kits, California, USA) treatment, all sections were coated with anti-iNOS (1/250 dilution, Santa-Cruz Biotechnology), TGF-β1 (1/200 dilution, Santa-Cruz Biotechnology), and anti-caspase-3 (1/100 dilution, Santa-Cruz Biotechnology) antibodies and incubated at room temperature for 60 minutes. After washing the sections with PBS, biotin-labeled secondary antibody was added to the sections and left for 30 minutes, then washed 3 times with PBS for 5 minutes and incubated with streptavidin peroxidase enzyme (Histostain-Plus Kits, California, USA) for 30 minutes. At the end of this period, the tissues were washed 3 times with PBS for 5 minutes each. Finally, the sections were stained with 3-amino-9-ethylcarbazole (AEC) (Zymed AEC RED substrate kit, USA) chromogen for 10 minutes under a microscope under controlled conditions. Counterstaining was done with Gill's hematoxylin. Sections were sealed with water-based adhesive (Epredia™ Shandon™ Immu-mount, Fisher Scientific). All staining results were examined under a light microscope (Nikon, YS 100, Japan). Immunolabeling of sections with the iNOS, TGF-β1 and caspase 3 antibodies were scored semiquantitatively [25]. Visibly stained cells in the selected area were considered immune-positive. The percentage of labeled cells was evaluated using an eyepiece with grids of 100 squares from 10 contiguous fields forming a total area of 0.050 mm^2 at the last 200 magnification. Scoring for percent-

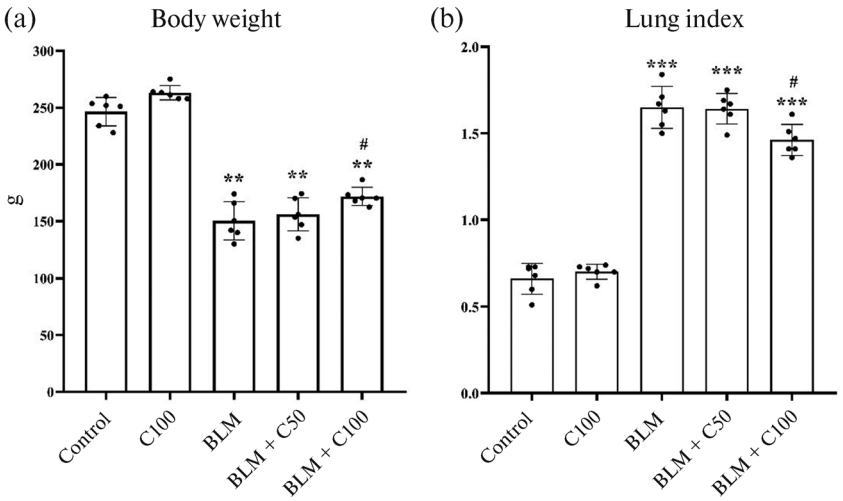

Fig. 1. Body weight and lung index in the experimental groups. Effect of carvacrol on (a) body weight and (b) lung index in rats with bleomycin-induced pulmonary fibrosis. BLM: Bleomycin, C: Carvacrol. **—$p < 0.01$, ***—$p < 0.001$, compared with the control group. #—$p < 0.05$, compared with the bleomycin group. Statistical evaluation was conducted by using one-way ANOVA, followed by Duncan multiple comparison tests.

ages of labeled cells were as follows; 0: no stain, 1: stained cell area < 10%, 2: stained cell area = 10–30%, 3: stained cell area >30%. Within a total area of 0.050 mm^2, immunolabeling intensity score (0–3) was added to the percentage score of immunolabeled tissue in order to obtain an immunoreactivity score.

Statistical analysis

All values were expressed as the mean ± standard error. The normality of data distribution was tested by using the Shapiro Wilks test. Statistical evaluation was conducted by using one-way ANOVA, followed by Duncan multiple comparison tests (SPSS software version 25, SPSS Inc. Chicago IL, USA). The Kruskall–Wallis test was used to evaluate the histopathological statistical significance of differences among the groups. The Mann–Whitney *U*-test with Bonferroni correction was used for post hoc multiple comparisons. A p value < 0.05 was assumed to be statistically significant.

RESULTS

Body weight and lung ındex

There was a notable weight loss with a single dose of BLM (5 mg/kg b.w.) administration compared to the control group ($p < 0.01$) while treatement with carvacrol did not restore it (Fig. 1a). BLM + C50 and BLM + C100 groups showed a significant reduction in body weight compared to the control group ($p < 0.01$). The BLM group also showed a significant increase in lung index compared to the control group on the 14th day ($p < 0.001$). Treatment of BLM rats with carvacrol (50 mg/kg) did not affect the lung index ($p < 0.05$) while administration of 100 mg/kg slightly, but statistically significantly, reduced it (Fig. 1b). BLM + C50 and BLM + C100 groups showed a significant increase in lung index compared to the control group ($p < 0.001$).

Hydroxyproline content

The collagen accumulation in the lung tissue was assessed by evaluating the hydroxyproline content. BLM obviously increased the hydroxyproline content compared to the control group ($p < 0.001$). Carvacrol 100 mg/kg significantly reduced lung hydroxyproline levels compared to the BLM group ($p < 0.05$) (Fig. 2a).

TNF-α levels

TNF-α, an inflammatory cytokine, was increased in BALF supernatants after BLM administration compared to the control group ($p < 0.001$). TNF-α levels in BALF samples were statistically lower after treatment with carvacrol (100 mg/kg) compared to the BLM group ($p < 0.05$). TNF-α levels in the lower carvacrol dose

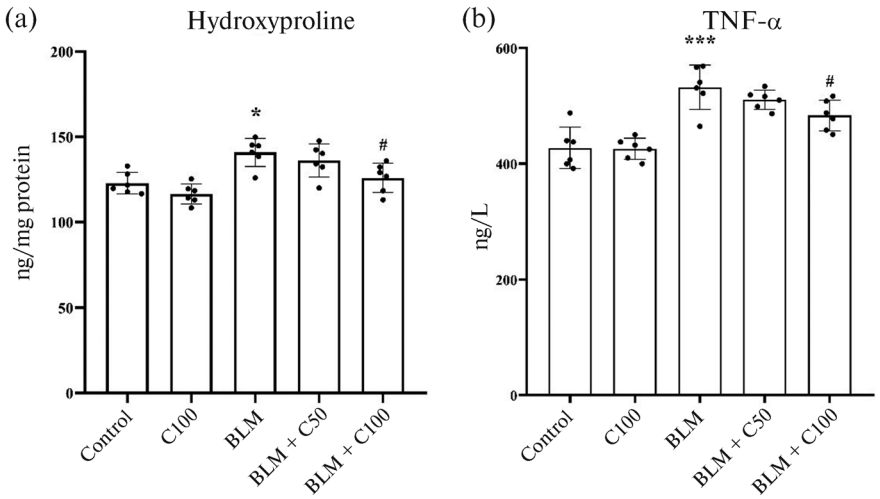

Fig. 2. Hydroxyproline and tumor necrosis factor alpha (TNF-α) levels. Effect of carvacrol on (a) hydroxyproline content in lung tissue and (b) the levels of inflammatory cytokine TNF-α in BALF samples in bleomycin-induced pulmonary fibrosis in rats. BLM: Bleomycin, C: Carvacrol. *—$p < 0.05$, ***—$p < 0.001$, compared with the control group. $^{\#}$—$p < 0.05$, compared with the bleomycin group. Statistical evaluation was conducted by using one-way ANOVA, followed by Duncan multiple comparison tests.

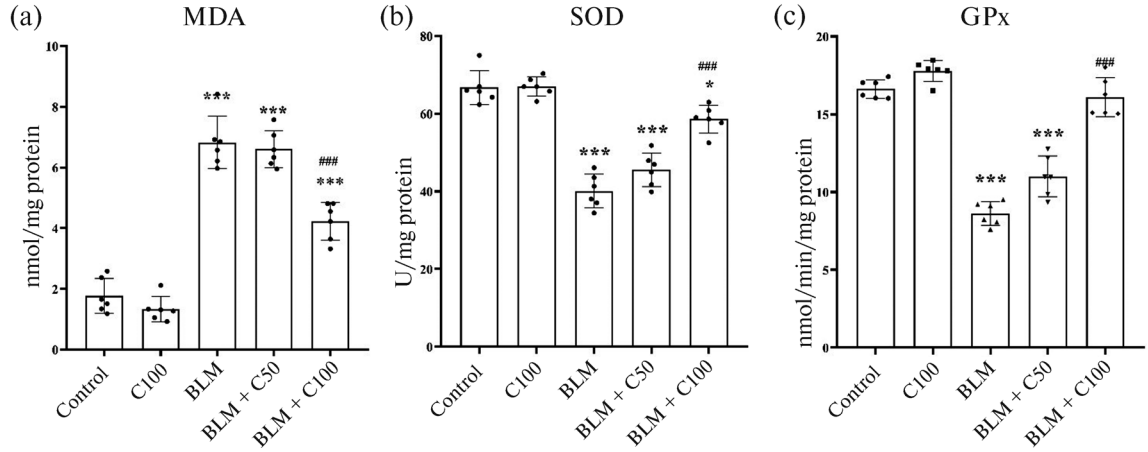

Fig. 3. Malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in rat lung tissue. Effect of carvacrol on (a) the oxidative stress marker MDA, (b) on SOD activity and (c) on GPx activity in bleomycin induced rat pulmonary fibrosis. BLM: Bleomycin, C: Carvacrol. ***—$p < 0.001$ compared with the control group; $^{\#\#\#}$—$p < 0.001$ compared with the BLM group. Statistical evaluation was conducted by using one-way ANOVA, followed by Duncan multiple comparison tests.

group (50 mg/kg) did not differ significantly from those in the BLM group (Fig. 2b).

Lipid peroxidation

Instillation of BLM led to a significant increase in MDA content in lung tissue compared to control values ($p < 0.001$). Carvacrol (100 mg/kg) administration to BLM treated rats reduced the increased MDA level in lung tissue ($p < 0.001$). There were no notable differences in the level of MDA between the BLM and BLM + C50 groups (Fig. 3a).

Effect of carvacrol on antioxidant enzymes

Lung tissue from rats treated with BLM showed a significant decrease in the activity of SOD and GPx enzymes compared to the control group ($p < 0.001$). In rats treated with BLM, carvacrol administration at a dosage of 100 mg/kg led to a significant increase in SOD activity ($p < 0.001$)

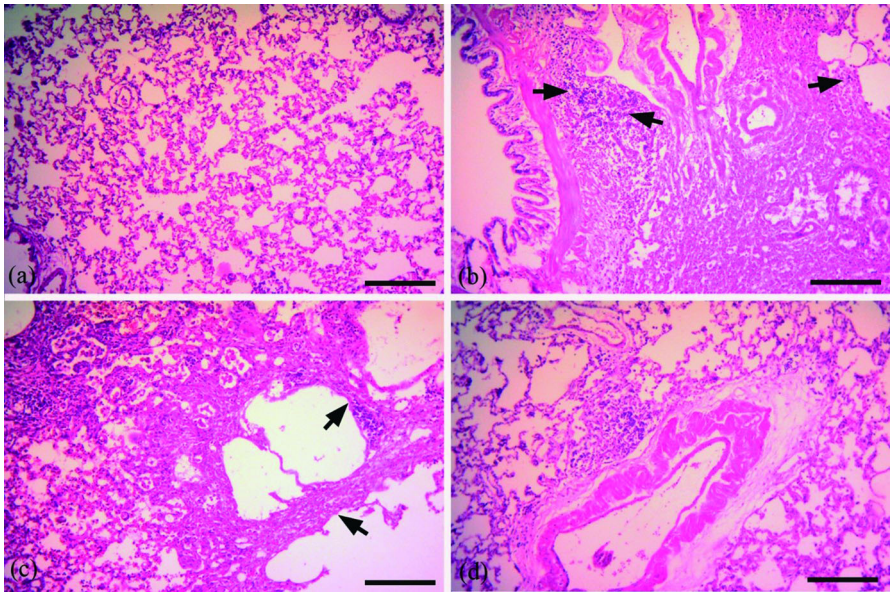

Fig. 4. Histopathological examination of lung tissue. (a) Control group, no inflammatory changes in the lumens of the alveoli, bronchi and bronchioles, and in the interstitial tissue of the lung, HE. (b) BLM group, intense inflammatory cell infiltration in the lung (arrows), HE. (c) BLM + C50 group, thickening of the alveolar septum and inflammatory cell infiltration (arrows) in the lung tissue, similar to the BLM group, HE. (d) Moderate intensity inflammatory changes in the interstitial tissue of the lung. BLM + C100, HE. Bar: 100 μm. BLM: Bleomycin, C: Carvacrol.

whereas 50 mg/kg did not cause any changes in SOD levels compared to the BLM group (Fig. 3b). As with SOD activity, there were significant variations in GPx activity in the lung tissue between the control and BLM groups. BLM treatment resulted in a significant reduction in GPx activity in rat lungs ($p < 0.001$) while administration of 100 mg/kg carvacrol reversed the decrease in GPx activity to a significant increase ($p < 0.001$) while 50 mg/kg did not have a significant effect (Fig. 3c).

Histopathological changes

In the control group, no inflammatory changes were found in the lumens of the bronchioles, bronchi, or alveoli in the interstitial tissue of the lung during histopathological examination (Fig. 4a). However, in the BLM group severe inflammatory changes in the interalveolar septum, bronchiole lumens, and in the bronchiole as well as a marked thickening of the interalveolar septum were observed (Fig. 4b). No significant difference was observed in terms of histopathological lesions (Fig. 4c) in the BLM + C50 group compared with the BLM group. Using hematoxylin-eosin staining, it was observed that in the lungs of the rats from the BLM group there was severe inflammation in the interalveolar septum, bronchi, bronchioles and alveolar lumens while administration of 100 mg/kg of carvacrol led to a significant decrease and moderate regress of inflammation (Fig. 4d).

Using triple staining, in the control group and in the C100 group only normal connective tissue around the bronchioles, bronchi, and vessels, without any fibrosis could be seen (Grade 0) (Figs. 5a, 5b). Nevertheless, in the lungs of rats from the BLM group a dense accumulation of fibrosis was noted (Grade 7). This difference between the control and BLM groups was found to be statistically significant ($p < 0.01$) (Figs. 5c, 5d). No statistically significant difference was observed in triple staining when the BLM + C50 group was compared with the BLM group. However, in the BLM + C100 group, fibrosis around the bronchi, bronchioles and vessels was significantly reduced (Figs. 5e, 5f) compared with the BLM group (Grade 3) ($p < 0.01$) (Table 1).

Immunohistochemical changes

Applying immunohistochemistry (IHC) staining for iNOS, no expression of this protein was

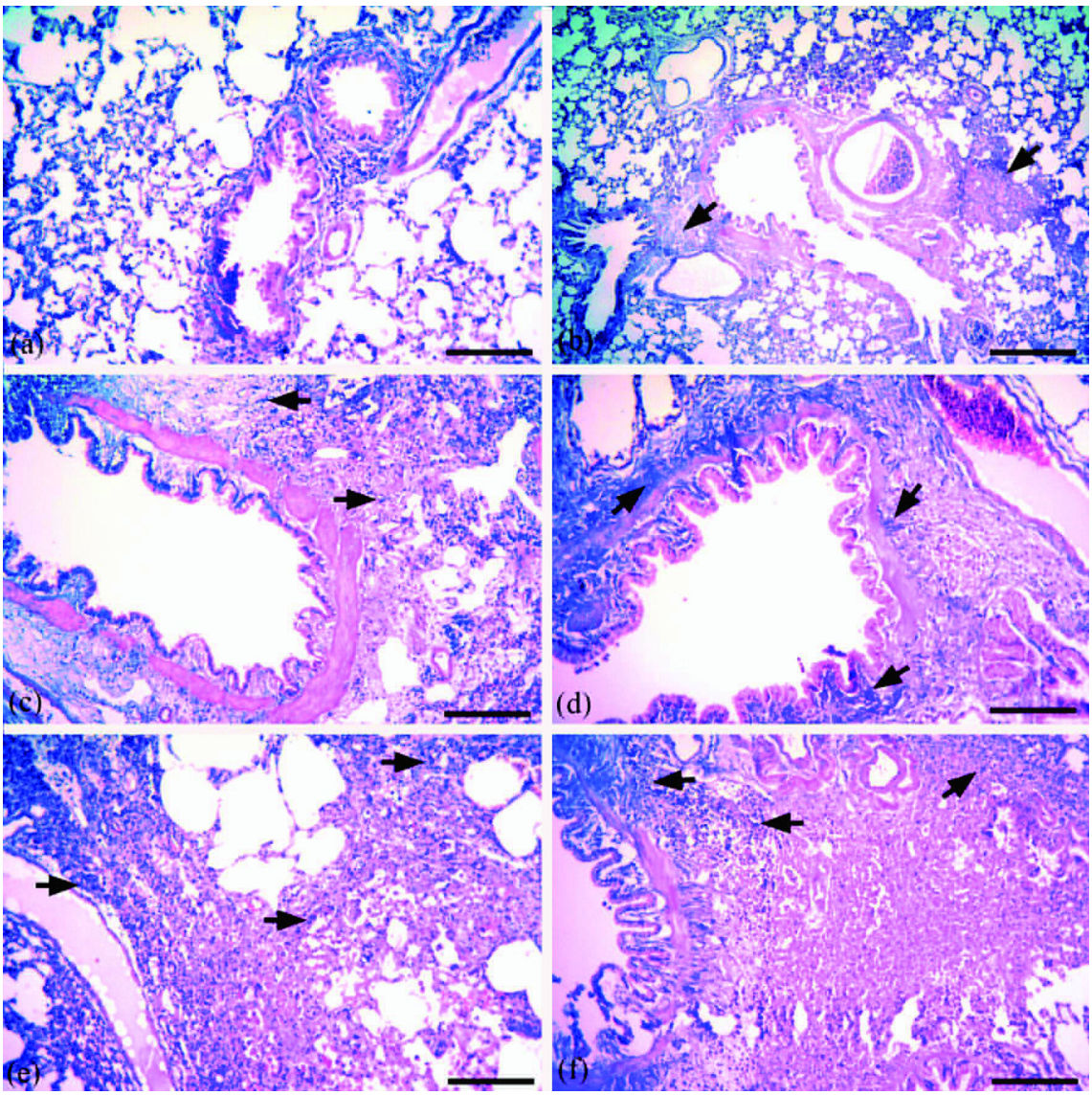

Fig. 5. Histopathological examination of fibrosis in lung tissue using trichrome staining. The grades (0–7) were given using Ashcroft scoring (see Methods). (a) Control group—no fibrosis in lung tissue, only normal connective tissue can be seen around the vessels, bronchi, and bronchioles. Alveolar septa: no fibrotic burden in alveolar walls. Lung structure: normal lung, Grade: 0. (b) BLM + C100 group—significantly reduced fibrosis formation in the interalveolar septum (arrows). Alveolar septa: moderate thickening of bronchiolar walls. Lung structure: alveoli partly enlarged and rarefied, but no fibrotic masses, Grade: 3. (c–d) BLM group—intense fibrosis (arrows) in the peri-bronchial region and interalveolar septum in the lung. Alveolar septa: non-existent. Lung structure: alveoli nearly obliterated with fibrous masses but still up to five air bubbles, Grade: 7. (e–f) BLM + C50 group—intense fibrosis (arrows) in the peri-bronchial region and interalveolar septum in the lung. There are fibrotic changes in the lung tissue similar to the BLM group. Grade: 7. Bars: 100 μm. BLM: Bleomycin, C: Carvacrol.

observed in the control lung tissue (Fig. 5a), while it was found to be intensely expressed by inflammatory cells, especially from alveolar macrophages, in the BLM group (Fig. 6b, Table 2, $p < 0.01$). In the iNOS immunostaining in the BLM + C50 group, no difference was observed compared to the BLM group (Fig. 6c) while in the BLM + C100 group we observed statistically lower intensity of staining ($p < 0.01$) (Fig. 6d, Table 2).

TGF-β1 antibody staining of control lung tissues revealed only mild expression of this protein in the bronchial and bronchiolar epithelium (Fig. 7a). However, in the BLM group significant

Table 1. Effect of Carvacrol (50 and 100 mg/kg) on fibrosis scores in histopathological examination of lung tissue. The difference between groups is statistically significant. Fibrosis scoring was performed using the Ashcroft scale

	Control	C100	BLM	BLM + C50	BLM + C100
	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM
Average fibrosis score	0.00 (0.00) ± 0.00	0.13 (0.00) ± 0.13	7.13 (7.00) ± 0.13**	7.13 (7.00) ± 0.13	2.75 (3.00) ± 0.25$^{\#\#}$

**—$p < 0.01$ to Control group, $^{\#\#}$—$p < 0.01$ compared to the BLM group using the Kruskal–Wallis test following the Mann–Whitney *U*-test with Bonferroni correction.

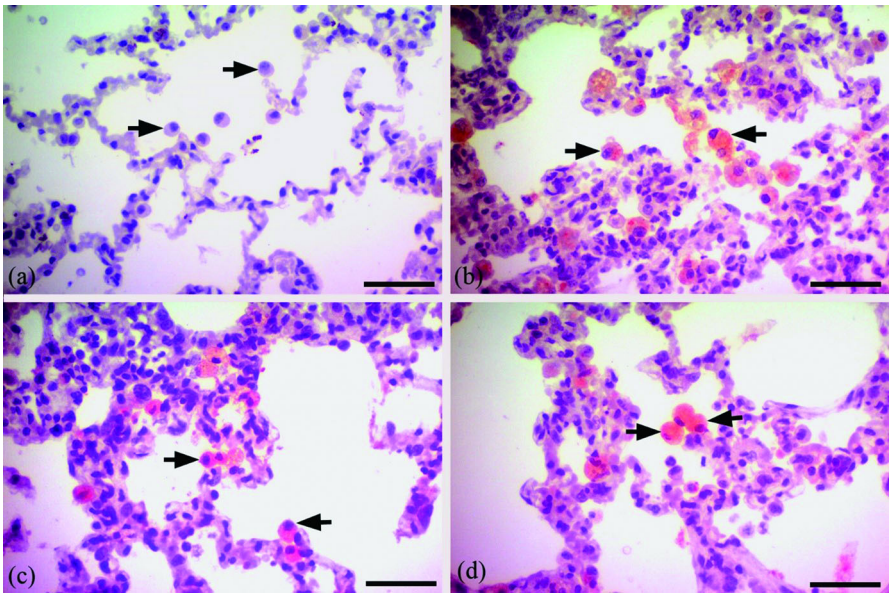

Fig. 6. Inducible nitric oxide synthase (iNOS) immunostaining. (a) Control group, mild iNOS signal in bronchiole epithelium in lung tissue. (b) BLM group, intense iNOS expression in bronchiolar epithelium. (c) BLM + C50 group, intense iNOS expression in bronchial epithelia similar to the bleomycin group (arrows). (d) BLM + C100 group, decreased iNOS expression in bronchial epithelium compared to the bleomycin group (arrow). IHC, Bar: 100 μm. BLM: Bleomycin, C: Carvacrol.

Table 2. Effect of Carvacrol (50 and 100 mg/kg) on immunolabeling intensity score in immunohistochemical examination of lung tissue. The difference between groups is statistically significant

	Control	C100	BLM	BLM + C50	BLM + C100
	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM	Mean (Median) ± SEM
iNOS	0.33 (0.00) ± 0.21	0.17 (0.00) ± 0.17	2.83 (3.00) ±0.17**	2.60 (3.00) ± 0.21	0.33 (0.00) ± 0.21$^{\#\#}$
Caspase-3	0.33 (0.00) ± 0.21	0.33 (0.00) ± 0.21	2.66 (3.00) ± 0.33*	2.50 (2.50) ± 0.22	0.50 (0.50) ± 0.22$^{\#}$
TGF-1β	0.33 (0.00) ± 0.21	0.33 (0.00) ± 0.21	2.66 (3.00) ±0.21**	2.33 (3.00) ± 0.42	0.50 (0.50) ± 0.22$^{\#\#}$

*—$p < 0.05$, **—$p < 0.01$ to Control group; $^{\#}$—$p < 0.05$, $^{\#\#}$—$p < 0.01$ compared to the BLM group using the Kruskal–Wallis test following the Mann–Whitney *U*-test with Bonferroni correction.

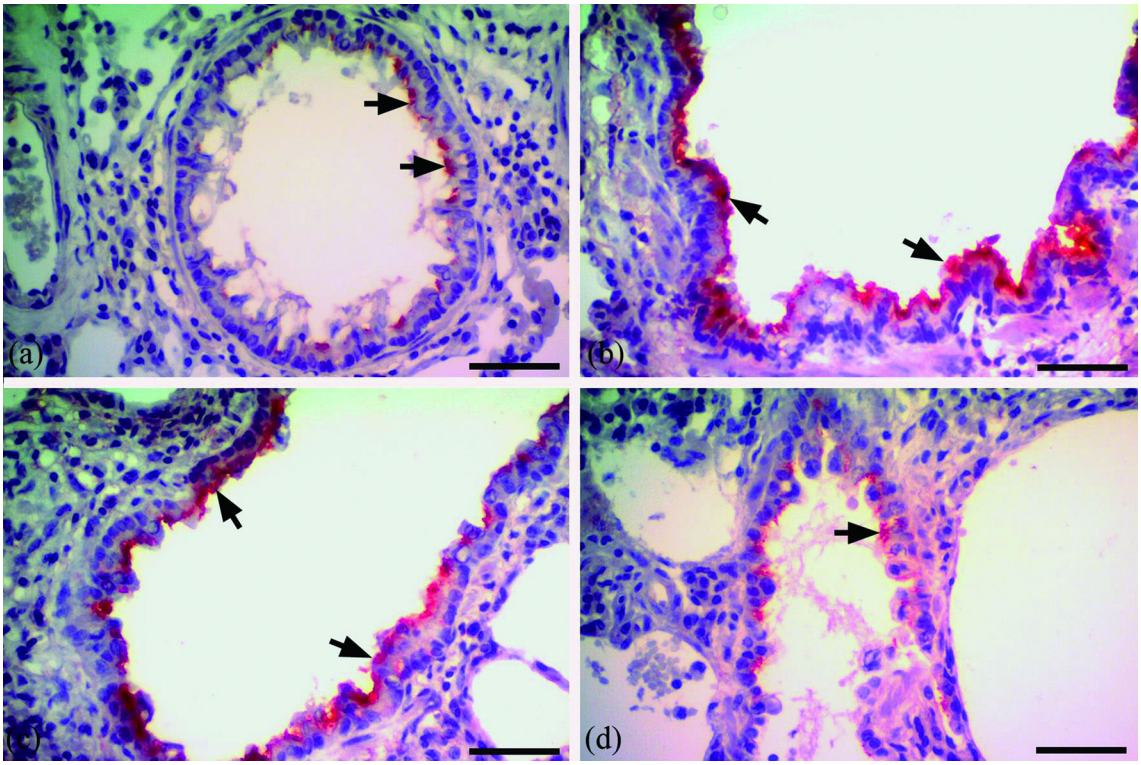

Fig. 7. Transforming growth factor-beta 1 (TGF-β1) immunostaining. (a) Control group, mild TGF1-β immunohistochemical staining in bronchiole epithelium in lung tissue. (b) BLM group, intense TGF1-β expression in bronchiolar epithelium. (c) BLM + C50 group, intense TGF1-β expression similar to the bleomycin group in bronchial epithelia (arrows). (d) BLM + C100 group, decreased TGF1-β expression in bronchial epithelium compared to the bleomycin group (arrow). IHC, Bar: 100 µm. BLM: Bleomycin, C: Carvacrol.

levels of TGF-β1 expression and severe fibrosis were observed in the bronchi and bronchiole epithelium (Fig. 7b, Table 2, $p < 0.01$). While similar TGF-β1 expression was observed in the BLM and BLM + C50 groups (Fig. 7c), treatment with 100 mg/kg of carvacrol resulted in its decrease and regression in the number of inflammatory cells in the bronchial and bronchiole epithelium (Fig. 7d) with the difference between the BLM group and the BLM + C100 group being statistically significant ($p < 0.01$) (Table 2).

The alveolar epithelium, bronchiole epithelium and alveolar macrophages were also stained with anti-caspase-3 antibody which revealed mild expression of this protein in the control lungs (Fig. 8a), but very high caspase-3 expression was noted in the BLM group (Fig. 8b, Table 2, $p < 0.05$). The levels of caspase-3 expression were similar in the BLM and BLM + C50 groups (Fig. 8c) while in the BLM + C100 group less intense immunopositivity was observed (Fig. 8d, $p < 0.05$). No changes were observed in the group given only carvacrol (Table 2).

DISCUSSION

Pulmonary fibrosis (PF) is a chronic progressive lung disease with a fatal prognosis. As PF is a disease of unknown cause, there is a need to develop new animal models in order to elucidate underlying mechanisms and suggest treatment strategies. BLM, a chemotherapeutic agent used in different cancers, primarily exerts its cytotoxic effect in the lungs with chronic inflammation and fibrotic reactions. Rats treated with BLM show similar histological signs in their lungs to patients with idiopathic pulmonary fibrosis (IPF) [26]. Therefore, the BLM-induced PF rat model is useful for exploring the effects of novel agents and underlying mechanisms of IPF in humans. Although possible ways to reduce PF have been reported for many years, the effect of carvacrol on

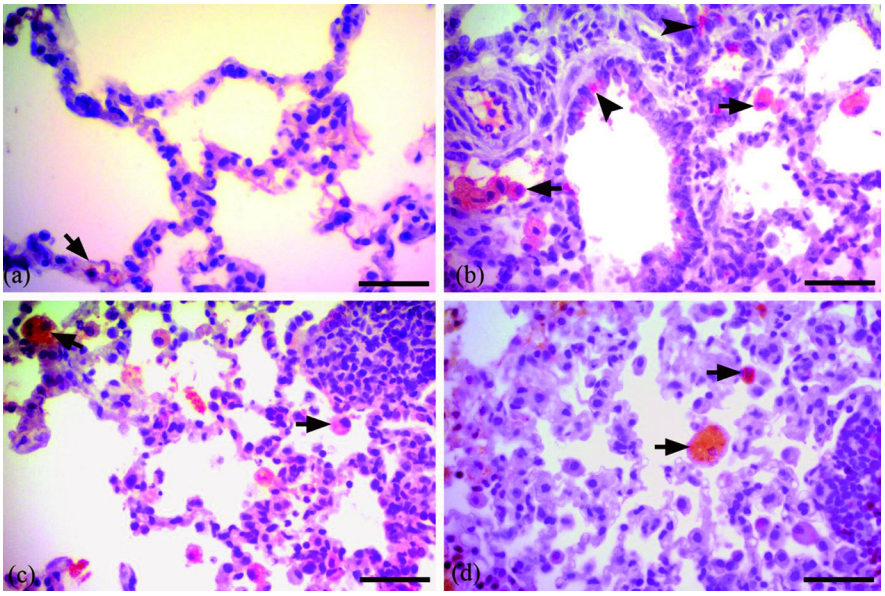

Fig. 8. Caspase-3 immunostaining. (a) Control group, mild caspase-3 immunohistochemical staining in alveolar epithelium in lung tissue (arrow). (b) BLM group, intense caspase-3 expression (arrows) in bronchiole epithelium (arrowheads) and alveolar macrophages. (c) BLM + C50 group, intense caspase-3 expression in alveolar macrophages, similar to the bleomycin group. (d) BLM + C100 group, decreased caspase-3 expression in alveolar macrophages compared to the bleomycin group (arrows). IHC, Bar: 25 μm. BLM: Bleomycin, C: Carvacrol.

BLM induced PF has not yet been reported. The present study was conducted to investigate the effect of orally administered carvacrol in a BLM-induced PF rat model. The mechanisms underlying the ameliorative effect of carvacrol on the lungs were explored by analyzing oxidative stress markers as well as of iNOS, TGF-β1 and caspase-3 levels.

Body weight loss and decreased food intake was observed in rodents treated with BLM [26]. In our study, BLM administration caused a significant reduction in body weight and a significant elevation in lung weight and lung index. BLM induced body weight loss may be due to the toxicity of BLM and/or a decrease in food consumption [27]. Treatment with carvacrol improved the decrease in body weight and reduced the increased lung index, confirming the protective effect of carvacrol against BLM-induced PF.

BLM causes toxic effects by damaging DNA due to the presence of cofactors, molecular oxygen and iron ion (Fe^{2+}) in the DNA double-strand structure. BLM binding to these elements produces complexes which can attack the DNA, leading to an increase in nitrogen and oxygen radicals. These radicals elevate inflammation, cytokine levels and lead to the formation of fibrosis. The assessment of lipid peroxidation through MDA measurement is an appropriate way to monitor tissue damage caused by oxidative stress [27]. According to the literature, BLM administration has been found to cause oxidative stress in the lung tissue of rodents, leading to a significant increase in MDA levels [29–31]. The current study also showed that BLM administration resulted in a rise in MDA levels, which is in line with previous data on BLM-induced PF studies [30, 32]. The potent antioxidant properties of carvacrol have been attributed to its ability to scavenge highly reactive free radicals [33]. Undeger et al. have shown that carvacrol at low levels exerts antioxidant activity in lung fibroblast cells [34]. Pretreatment with carvacrol extract has been shown to reduce lung oxidative damage in methotrexate-induced lung injury in rat [35]. The results of our study demonstrate that carvacrol has the ability to decrease lipid peroxidation by reducing BLM-induced MDA production in lung tissue.

Free radicals are detoxified by endogenous antioxidant defense systems including SOD, GPx, and catalase (CAT) activity under normal cellular conditions. Excessive ROS deposition

leads to lipid peroxidation, which can result in tissue damage. BLM administration has been found to significantly lower the activities of major antioxidant enzymes in rat lung tissue [36, 37]. Consistent with previous studies, we also observed a marked decrease in GPx and SOD levels following BLM administration. The effects of carvacrol on oxidative stress have been investigated in several studies [38–40]. In our work a decreased enzyme activity in lung tissue of rats treated with BLM was reversed after treatment with 100 mg/kg of carvacrol daily for 14 days. The increased activity of GPx and SOD in the lungs of rats treated with this dose of carvacrol may be responsible for the reduced levels of MDA and oxidative stress. The effect was dose-dependent since we have not observed significant changes in enzyme activities with carvacrol administration at a dose of 50 mg/kg. Our study indicates that carvacrol treatment at a dose of 100 mg/kg may provide protective benefits by normalizing enzyme activities and their ROS scavenging actions.

It has been reported that cytokine activity has a crucial role in the pathogenesis of PF as cytokines regulate matrix and fibroblast production. Increased levels of TNF-α, a key player in the pathogenesis of inflammation, was shown to elevate collagen deposition, leading to pulmonary injury [41]. In our study, the marked increase in production of MDA with BLM application probably led to the elevation in the synthesis of TNF-α. The anti-inflammatory effects of carvacrol were suggested to be due to suppressing COX-2 expression and also diminishing the release of such proinflammatory mediators as PGE2, TNF-α, IL-1β [9, 10]. Carvacrol treatment has been shown to prevent inflammation in benzo(a)pyrene induced lung toxicity in mice by downregulating the protein expressions of COX-2, iNOS and NF-κB [9]. These anti-inflammatory benefits of carvacrol have also raised the question of whether it could be a supportive therapy option for reducing the harmful effects of Covid-19-related lung pathology [42]. Carvacrol, when administered to mice modelling lipopolysaccharide-induced lung injury, has demonstrated a protective effect by mitigating inflammation. It has been reported that the ability of carvacrol to inhibit MAPKs and NF-κB signaling pathways is likely to underlie the mechanisms of its anti-inflammatory effects, leading to the suppression of production of inflammatory cytokines such as IL-6, TNF-α, and IL-1β [43]. Carvacrol has been shown to reduce oxidative stress and inflammation in rats with lung damage caused by breathing in paraquat suggesting PPAR-γ receptor mediated effects of carvacrol [44]. It has also been reported that carvacrol treatment for two months, 3 times/day improves oxidative stress markers, cytokine levels, and pulmonary function tests in asthmatic patients [45]. Treatment with carvacrol for two months has reduced oxidant biomarkers, inflammation, and improved pulmonary function tests in patients with sulfur mustard induced lung disorders [46]. Again, in sulfur mustard exposed patients, two months treatment with carvacrol has been shown to improve respiratory symptoms by modulating serum cytokine levels (IL-2, IL-4, IL-6 and IL-8), interferon-gamma levels and pulmonary function tests [47]. The current study found that carvacrol administration in a dose of 100 mg/kg slightly reduced the level of the proinflammatory cytokine TNF-α in BLM-treated rats, which might add to other beneficial effects of this compound in suppression of inflammation associated with BLM-induced PF.

Nitric oxide (NO) has been demonstrated to play a major role in the pathogenesis of lung diseases. iNOS takes part in the production of NO and is the main mediator of inflammation and any increase in iNOS expression results in the overproduction of NO. It has been reported that iNOS expression increases in the lung tissue of PF patients, while there was no change in other isoforms. In addition to ROS reactive nitrogen species (RNS) have an important role in the transition of inflammation to fibrosis. RNS generated by BLM in the lung were shown to induce lipid peroxidation, DNA injury and elevation in collagen deposition [48]. Several previous studies with BLM administration reported a significant increase in iNOS expression [49, 50] which was also observed in our study. The immunohistochemical analysis showed that carvacrol (100 mg/kg) treatment significantly suppressed the expression of iNOS in the lung tissue of BLM treated rats indicating that it may attenuate inflammation by suppressing iNOS expression in BLM-induced PF.

TGF-β1 plays a critical role in BLM-induced PF therefore, it is regarded as a potential therapeutic target [51, 52]. TGF-β1 promotes transformation and proliferation of lung fibroblasts, collagen synthesis, and excessive ECM deposition [53]. The histological examinations in the current study showed that BLM administration led to collagen deposition and significant thickening of the interalveolar septum which correlates with the results of other researchers [54]. Other studies also demonstrated that the level of TGF-β1 in the BLM-induced PF model in rodents is significantly increased [55, 56]. IHC examination performed in our study showed a significant increase in TGF-β1 levels in BLM-treated rats compared to controls, while carvacrol treatment effectively reduced it. Since hydroxyproline concentration was shown to correlate with collagen levels it is regarded as another indicator of the severity of fibrosis [57]. In the present study, carvacrol (100 mg/kg) significantly reduced hydroxyproline levels increased by BLM administration. These findings indicate that carvacrol may exert an anti-fibrotic effect by inhibiting fibroblast activation and TGF-β expression, thereby modulating collagen synthesis in BLM-induced PF.

Studies have shown an increased number of apoptotic epithelial cells in the lung tissues of IPF patients, in part related to the increased expression of pro-apoptotic proteins [58, 59]. Animal experiments also revealed epithelial apoptosis in BLM-induced rodent models [60, 61] and BLM treatment has been found to shift the balance between antiapoptotic and proapoptotic proteins in favor of the proapoptotic pathway. Activation of caspase-3, a main apoptosis mediator, leads to mitochondrial fragmentation and cell death [62]. Therefore, caspase activation in lung cells is thought to be one of the cellular events leading to apoptosis in BLM-induced PF. Antioxidants exert a protective effect by reducing activation of caspase-3 [63]. Consistent with the results reported previously, immunohistochemical staining revealed activation of caspase-3 expression in BLM-injured lung tissue. In previous reports, it has been shown that carvacrol prevents cell death in a dose dependent manner [64, 65]. Our study demonstrated that treatment with 100 mg/kg of carvacrol decreased caspase-3 immunopositive staining, indicating that it can regulate apoptosis by inhibiting caspase-3 expression in BLM-induced lung tissue damage.

Analyzing all the effects of two different doses of carvacrol (50 and 100 mg/kg) we have shown that statistically significant improvements in the lung tissue of BLM-treated rats were only observed in the BLM + C100 group which suggests that the lower concentrations of carvacrol might not be effective in PF therapy.

CONCLUSİON

Developing therapies designed to counteract the detrimental effects of oxidative stress, inflammation, and apoptosis may be beneficial to treat PF. The outcomes of this study in BLM-induced PF provided evidence for the anti-fibrotic efficacy of carvacrol, a natural phenolic monoterpene. The findings of this research demonstrate that the administration of carvacrol shows promising effects in healing pulmonary fibrosis induced by BLM. It achieves this by reducing inflammation, oxidative damage, and histopathological changes in lung tissue. The study also suggests that the effect of carvacrol involves iNOS, TGF-β1, and caspase 3 signaling pathways which could be potential molecular targets for its therapeutic action. Our results suggest that for treatment of PF these protective properties of carvacrol might be used in combination therapy in clinics.

AUTHORS' CONTRIBUTION

Nergiz Hacer Turgut: Conceptualization, model generation, experimentation, data collection and analysis, data presentation, writing and editing. Mehmet Ekici: Experimentation and data collection and analysis, data presentation. Mehmet Onder Karayigit: Experimentation and data collection and analysis, data presentation. Haki Kara: Conceptualization, review, editing, and revision. Huseyin Gungor: Conceptualization, experimentation, data collection and analysis, data presentation, project administration.

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ETHIC STATEMENT

The study was approved by the Ethical Committee for Animal Experiments of Sivas Cumhuriyet University (HADYEK, Turkey) (No. 65202830-050.04.04-221).

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest.

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