RESEARCH ARTICLE
DOI: 10.4274/cjms.2025.2024-93

The Therapeutic Effect of Flavan-3-Ols from Organic Extracts of Juniperus drupacea Fruit against Elastase-Induced Chronic Obstructive Pulmonary Disease (COPD) in Rats

Akbulut et al. Effect of extracts of *J. drupacea* fruit against COPD in rats

Hatice Feyza Akbulut[1], Hüsamettin Vatansev[2], Bayram Çolak[3], Hülya Özdemir[4], Zeliha Esin Çelik[5], Mehmet Akbulut

[1]Department of Medicinal and Aromatic Plants, Selçuk University, Çumra Vocational School, Konya, Türkiye
[2]Department of Medicinal Biochemistry, Selçuk University Faculty of Medicine, Konya, Türkiye
[3]Department of General Surgery, İzmir Bakırçay University Faculty of Medicine, İzmir, Türkiye
[4]Department of Medicinal Biology, Selçuk University Faculty of Medicine, Konya, Türkiye
[5]Department of Patology, Selçuk University Faculty of Medicine, Konya, Türkiye
[6]Department of Food Engineering, Selçuk University Faculty of Agriculture, Konya, Türkiye

Abstract
BACKGROUND/AIMS: Chronic obstructive pulmonary disease (COPD) is a common chronic airway disease with acute exacerbations of varying frequency that is the main cause of disease morbidity and mortality. The aim of this study was to investigate the utility of extracts of flavanol-3-ols rich (%85-92) Juniperus drupacea fruit in the treatment of rats with PPE-induced COPD.
MATERIALS AND METHODS: Thirty female rats of Wistar Albino breed were randomly divided into four groups: control, porcine pancreatic elastase (PPE), PPE + Methanol Extract (ME), PPE + Water Extracts (WE). The emphysema in the lung tissues of rats and lymphocyte (B cells, Cytotoxic T lymphocyte (CTLs), natural killer (NK) cells), cytokines (Interleukin-8 (IL-8), Interleukin-6 (IL-6), and Tumor Necrosis Factor-Alpha (TNF-α)) and blood gas values in blood samples were analyzed.
RESULTS: It was observed that the emphysema occurred in the lungs of rats after PPE exposure, and the number of inflammatory cells, except for NK cells, and IL-6, IL-8 and TNF- α cytokines in their blood increased. Among the blood gas values, PaCO2 increased with emphysema, and PaO2 decreased. The rats with PPE-induced COPD showed a decrease in the number of B cells and NK cells as a result of treatment with J. drupacea fruit extracts.
CONCLUSION: Our results showed that PPE application causes COPD and water and methanol extracts of flavan-3-ols rich J. drupacea fruit can protect from the development of elastase-induced lung injuries as an anti-inflammatory and antioxidant factor.
Keywords: COPD, flavan-3-ols, Juniperus drupacea, elastase, cytokines, lymphocytes

To cite this article: Akbulut HF, Vatansev H, Çolak B, Özdemir H, Çelik ZE, Akbulut M. The Therapeutic Effect of Flavan-3-Ols from Organic Extracts of Juniperus drupacea Fruit against Elastase-Induced Chronic Obstructive Pulmonary Disease (COPD) in Rats.

ORCID IDs of the authors: H.F.A. 0000-0001-6798-0953; H.V. 0000-0002-0230-3414; B.Ç. 0000-0003-1403-6963; H.Ö. 0000-0002-0806-9470; Z.E.Ç. 0000-0002-3220-7845; M.A. 0000-0001-5621-8293.

Hatice Feyza Akbulut
haticefeyza@selcuk.edu.tr
orcid.org/0000-0001-6798-0953

14.10.2024
09.01.2025

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a prevalent, avoidable, and manageable ailment characterized by persistent respiratory symptoms and restricted airflow due to respiratory or alveolar tract abnormalities, primarily caused by substantial exposure to harmful particles or gases.[1] COPD constitutes a range of advancing lung disorders, notably emphysema and chronic bronchitis, with many individuals experiencing both.[2]. Emphysema gradually damages lung air sacs, impeding outward air movement.[3] Chronic bronchitis induces inflammation and constriction of bronchial tubes, resulting in mucus accumulation.[4] COPD is among the important causes of chronic morbidity and mortality worldwide. COPD is a major and growing global health problem, estimated to be the third most common cause of death in the world and the fifth most common cause of disability by 2020.[5] The goals of COPD treatment are to improve symptoms, prevent disease progression, improve quality of life, increase exercise tolerance, prevent, and treat complications, and reduce mortality. To achieve these goals, reducing the risk factors that cause decrease in lung functions, providing the diagnosis of COPD and education of patient is provided with pharmacological and non-pharmacological treatment.[1] Worldwide, there may be a large number of herbs for which there is no detailed record of usage in traditional complementary medicine practices to relieve chronic pulmonary obstructive disease conditions. In the last few years, many herbs have been reported in scientific literature. *Juniperus drupacea* fruits are widely used as folk medicine in traditional medicine in Turkey.[6] For example, *J. drupacea* fruits have been used in the treatment of helminth infections and abdominal pain.[7,8] and against hemorrhoids[9], while decoction product of its fresh shoots has been used in the treatment of urinary inflammation, gout and abdominal pain and its tar against diarrhea.[8,10] The tar of *Juniperus drupacea* is obtained through the combustion of its stem. It is applied externally for conditions such as alopecia, eczema, and animal wounds. Internally, it is used to treat cough, cold, urinary tract inflammation, and diarrhea.[8,11] Analysis of the extracts of

Juniperus drupacea fruits suggested that this fruit may be a natural antioxidant supplement for food and beverages.[12] In this study, it was aimed to investigate the curative effects of flavan-3-ol rich extracts from *Juniperus drupacea* fruit by looking at the levels of cytokines, blood gases and lymphocyte in rats with COPD induced by porcine pancreatic elastase (PPE). Some bioactive components found in plants are better dissolved in water, while others are better dissolved in solvents such as methanol. At the same time, methanol is a solvent with a lower density and boiling point than water. With this feature, it penetrates plants faster and allows bioactive components to pass into the solvent faster and in greater amounts. In this study, both water and methanol extracts were used with the idea that they may differ in terms of bioactive components.

The extraction yields of *Juniperus drupacea* fruit used in this study were determined to be 25.35% for the water extract and 27.96% for the methanol extract, with the difference being statistically significant ($p = 0.012$).

EXPERIMENTAL PROCEDURES

Animals

The protocols presented below are based on animal experiments approved by the Ethics Committee of ……University Experimental Medicine Research and Application Center (ethics committee decision no. 2020/52). In this *in vivo* research study, a total of 30 Wistar Albino adult female rats aged 4-5 months and weighing 300-350 g were used. Porcine pancreatic elastase (PPE) (Sigma chemical, St Louis, MO) was used to construct a model of COPD in rats. Single-sex use aims to minimize anatomical and hormonal differences that may arise from gender.

Animal treatment and PPE induction

The rats were randomly selected and divided into four groups. The "Draw method" was preferred and used as the randomization method.[13] Six rats were selected for the control group and eight rats for each of the other groups. No procedure was applied to the negative control group. For the other three groups, a COPD model was created with the PPE. The rats were anesthetized by injecting Ketamine and Xylazine before intubation. After anesthesia, PPE mixture was prepared as 55 U/100 g by mixing with 0.5 mL NaCl and administered to rats intratracheally.[14] The negative control group was received by gavage only water throughout the 21-day treatment period. The flavan-3-ols extracts from *Juniperus drupacea* fruit were started to be given 24 h after the PPE-treatment in the groups. The methanol and water extracts of *Juniperus drupacea* fruits were separately given by gavage to be 250 mg/kg bw/day to rats with PPE-induced COPD throughout 21 days for treatment. The all rats in groups were housed at 21-22 °C without any water and food restrictions, with 12 h of light and 12 h of dark. Food restriction was applied to all rats 12 h before anesthesia procedures. On the 21 day, the rats were anesthetized by injecting Ketamine and Xylazine again before euthanizing the animals. Intracardiac blood was taken in accordance with euthanasia procedures and the sacrification process was performed. Then, various tissues were collected for analysis.

Treatment start date was June 04, 2021, Ethics committee approval date was November 30, 2020. The 250 mg/kg bw/day concentration used for therapeutic purposes in this study was determined considering the procedures in Laouar et al.[15]

Number of animals to be included in the study

The number of animals required for the animal experiment model (4 groups: Group-1, Control group; Group-2, PPE; Group-3, PPE+WE; Group-4, PPE+ME) created to evaluate the effect of extracts (the methanol and water extracts of *Juniperus drupacea* fruit) applied in the COPD

animal model on blood gas, histopathology (emphysema) and inflammatory markers (pH, PaO_2, $PaCO_2$, IL-6, IL-8, TNF-α, Cytotoxic T cells, B cells, NK cells) was determined by the Resource Equality [16-18] method. According to this method, the number of animals to be included in each group for an experimental design consisting of 4 study groups was determined as the maximum level of (20/4) +1=6. Considering the possible animal losses during the experimental phase, the study was planned to be conducted with 8 animals in each experimental arm and 6 animals in the control group.

Plant materials

Specimens of Juniperus drupacea Labill., a member of the Cupressaceae family, were collected from the Sebil forest area in Çamlıyayla, Mersin Province, Turkey, during July. These botanical samples were accurately identified by ..…… from the ....., Faculty of Pharmacy at ……. University (……, ...) and were cataloged under the herbarium record number .....

The extraction procedures

In November 2020, the fruits of *J. drupacea* at optimal maturity were collected from a forest located at an altitude of 1400 meters in the Çamlıyayla district of Mersin. After transportation to the laboratory, the fruits were thoroughly cleaned to remove any contaminants. Once broken, the fruits were ground using a hammer mill (Arzum, model AR1034, Turkey) to produce a fine powder for extraction. For the extraction process, fifteen grams of the ground fruits were separately treated with methanol (150 mL) and distilled water (150 mL) using a Soxhlet apparatus (Electro-mag MX 425, Turkey). The obtained extracts were collected, and then the solvents were removed using a rotary evaporator (SCILOGEX RE-100 pro) under vacuum at 40°C. The extracts were then frozen at -80°C and lyophilized. The final powdered extracts were stored at -18°C for further analysis.

Akbulut and Akbulut[19] analyzed the phenolic compounds of water and methanol extracts of *Juniperus drupacea* fruit used in this study and published them. The HPLC analysis revealed that flavan-3-ols, including catechin, epicatechin, epicatechin gallate, and procyanidin A2, constituted approximately 92% of the total phenolic compounds in the aqueous extract and 85% in the methanol extract.[19]

Histopathological Evaluation

The lung tissues were fixed in 10% buffered formaldehyde for 24 hours for pathological evaluation. For macroscopic sampling, 1x1 cm samples were taken from both lungs of each animal. The pieces were embedded in paraffin and 4-micron sections were taken on the slide with the help of a microtome. The slides stained with hematoxylin-eosin were evaluated under the Olympus BX53 model light microscope for the development of emphysema. The presence and extent of emphysema were evaluated by scanning the entire lung parenchyma at 40x magnification of the microscope. In the evaluation, the ratio of lung parenchyma developing emphysema to normal lung parenchyma was determined as a percentage. Accordingly, the affected area was scored as <25%: score 1, 25-50%: score 2, 50-75%: score 3, >75%: score 4. The mean of emphysema scores of each group were calculated and the results were compared with statistical methods.[20,21]

Blood Gas Analysis (pH, pCO_2, pO_2)

For blood gas analysis, the blood was carefully drawn from the heart with a 2 ml heparin injector under anesthesia in the experimental rats. Blood gas analyses were performed on the day the study was completed, after the rats were anesthetized and the blood was taken from the heart using the blood gas injectors and analyzed without delay by the ABL9 blood gas analyzer

(Dadiometer, Denmark) in the room at that time. pH, arterial oxygen partial pressure (PaO_2), and arterial carbon dioxide partial pressure ($PaCO_2$) values were determined in the analyses.

Cytokine Analyzes (IL-6, IL-8, TNF-α) by Elisa test kit

For the analysis of cytokines, the blood taken from the heart of the rats under anesthesia was placed in purple capped EDTA tubes and transferred to the laboratory without waiting. This blood was then centrifuged, and the plasma was kept at -80º C until analysis. IL-6, IL-8 and TNF-α analyses were carried out by Elabscience Rat IL-6, Bioassay Technology Laboratory Rat IL-8 and Elabscience Rat TNF-α ELISA test kits, and test procedures in them.

Cytotoxic T lymphocyte, B and NK cell analysis by flow cytometry

The surfaces of cells were stained with monoclonal antibodies targeting Anti-Rat CD8a, CD45RA, and CD161a (BD Bioscience, San Jose, CA) to assess the proportions of B cells, cytotoxic T cells, and NK cells through flow cytometry. Three sample tubes were prepared: one control, one containing CD3 (APC) / CD4 (PE) / CD8 (FITC), and another with CD3 (APC) / CD45RA (FITC) / CD161a (PE). A 100 µL aliquot of rat peripheral blood was added to each tube. According to the kit instructions, the appropriate amounts of monoclonal antibodies were added to all tubes except the control. After vortexing, the tubes were incubated for ten minutes at room temperature in dark. Next, two mL of 10X lysing solution, diluted 1:10 with distilled water to lyse red blood cells, was added. The samples were incubated for fifteen minutes in the dark at room temperature. The tubes were centrifuged at 1500 rpm for five minutes to isolate white blood cells, and the supernatant was discarded. After washing the pellet with **two** mL of phosphate-buffered saline (PBS) and centrifuging again, the supernatant was removed. The remaining cell pellet was resuspended in 500 µL of PBS. Finally, the samples were analyzed using the FACS Aria III flow cytometer (BD Bioscience), with data processed using FACS Diva version 6.1.3 software. In the generated dot plots, the cells were categorized as CD3 (+) CD8 (+) cytotoxic T cells, CD45RA (+) B cells, and CD161a (+) NK cells, and their respective percentages were recorded. [22]

Statistical analysis

To statistically evaluate the Histopathological, Flow cytometry, Blood gas and ELISA test results obtained at the end of the study, the results were subjected to Analysis of Variance using the MINITAB release 16.0 (Minitab Inc., PA, USA) program. Duncan's Multiple Range Test was used to see whether the differences between group means were significant. Significance level was accepted as $p<0.05$. To differentiate between the experimental groups such as Control, PPE, PPE+WE, and PPE+ME, methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA), and heatmap clustering analysis were employed. To estimate the effect sizes, the partial eta squared effect size (η^2) was chosen for this study because it allows the calculation of variation for more than one variable.[23] In one-way ANOVA analyses, the partial eta-squared effect size was used to determine the effect size of the difference between groups for results that were significant between groups. Partial eta-squared tells us how large an effect the independent variable(s) have on the dependent variable. For partial eta-squared, if $\eta 2 \geq 0.14$ the effects are large, between 0.06 and 0.14 If it varies between 0.06, the effects are considered moderate, and if $\eta 2 \leq 0.06$, the effects are considered small.[24]

RESULTS

Lung histopathological evaluation

Histopathological tests are the most important indicator of whether COPD occurs in rats after PPE induction. The photomicrographs of general lung histology are presented in Figure 1. The rats without PPE were indicated as the control group (Fig. 1A); the rats with PPE-induced COPD

were indicated as the PPE group (Fig. 1B); the rats with COPD exposed to PPE and treated by gavage with the water extract (250 mg/kg body weight/day) of *J. drupacea* fruit for 21 days were indicated as PPE-WE group (Fig. 1C); the rats with COPD exposed to PPE and treated by gavage with the methanol extract (250 mg/kg body weight/day) of *J. drupacea* fruit for 21 days were indicated as PPE-ME group (Fig.1D).

The emphysema development in the lung tissues of the rats with PPE-induced COPD and treated with the water and the methanol extracts of *J. drupacea* fruit is shown in Figure 1E. The emphysema scores in the histopathological evaluation were determined to be 0.667±0.516, 2.400±0.548, 1.286±0.488, and 1.000±0.000 in the control group, the PPE, PPE+WE, and PPE+ME group rats, respectively. In the method section, detailed information is given about the development of emphysema. In this determination method given, the ratio of the lung parenchyma developing emphysema to the normal lung parenchyma was determined as a percentage. Accordingly, the affected area was scored as <25%: score 1, 25-50%: score 2, 50-75%: score 3, >75%: score 4. According to the results of this evaluation, the most emphysema development was detected in the rats with the PPE-induced COPD (between 50-75%), while the lowest emphysema development was determined in the control group (<25%). Emphysema in the rats of PPE+WE and the PPE+ME group decreased compared to the rats of PPE group. This indicated that both water and methanol extracts of *Juniperus drupacea* fruits were effective in the treatment of COPD. Emphysema in the rats treated with the methanol extracts of *Juniperus drupacea* fruit was lower than that of the rats treated with its water extracts. It has been determined that the methanol extracts of *Juniperus drupacea* fruit are more effective in the regression of emphysema. It seems that the methanol extracts of *Juniperus drupacea* fruit may be more effective than the water extracts in the treatment of COPD.

Arterial blood gas (ABG) analyses in the rat bloods

The pH, $PaCO_2$ and PaO_2 results determined in the rats in our study are given in Table 1. As seen in Table 1, The pH was determined as 7.3950±0.0152, 7.3520±0.0045, 2.3714±0.605, 7.3529±0.340, and 7.3400±0.0185 for the rats of control group, the PPE, the PPE+WE and the PPE+ME, respectively. The distinction in pH levels between the groups was determined significant, statistically (p<0.05). The highest pH was determined in the rats in the Control group, followed by the PPE+WE, the PPE and the PPE+ME groups, respectively. The pH values of the rats in which the COPD model was applied, but treated and not, were found to be lower than the rats that were not treated with COPD and did not receive any treatment. $PaCO_2$ was determined as 45.317±1.903, 51.520±2.960, 49.757±4.170 and 49.350±2.677 mmHg for the Control, the PPE, the PPE+WE and the PPE+ME, respectively (Table 1). While the highest $PaCO_2$ was found in the rats in the PPE group with COPD but no treatment, the lowest $PaCO_2$ was determined in the rats in the Control group. $PaCO_2$ in the rats with COPD treated by the water and methanol extracts of *Juniperus drupacea* fruits decreased with the treatment process compared to the PPE group and were found to be close to the values in the control group rats. It is seen that $PaCO_2$ values are close to each other in the rats in the PPE+WE and the PPE+ME treated with the water and methanol extracts. The PaO_2 determined in the rats in this study was 63.800±11.52, 48.750±5.37, 51.833±6.43 and 50.857±5.18 mmHg for the Control, the PPE, the PPE+WE and the PPE+ME, respectively (Table 1). The highest PaO_2 values were detected in the rats in the Control group, while the lowest PaO_2 were observed in the rats in the PPE group, in which COPD was formed but no treatment was applied. In the rats with COPD, an increase in PaO_2 was observed with the treatment for 21 days by the water and methanol extracts of *Juniperus drupacea* fruits. It was determined that the increase in PaO_2 between these two groups

was higher in the PPE+WE group rats than in the PPE+ME rats and significant statistically (p<0.05).

The effects of flavan-3-ols rich extracts from *J. drupacea* fruit on the cytokine production in the rat bloods

In the present study, IL-8, IL-6, and TNF-α cytokines were also determined in addition to other markers to observe the COPD status created with PPE in the rats and the treatment process with the water and methanol extracts of *Juniperus drupacea* fruits. IL-8, IL-6, and TNF-α were analyzed by the Elisa method. The changes between the groups are also shown in Figure 2. According to these results, IL-6 was determined as 20.633±2.53, 22.563±2.58, 20.753±9.52 and 20.598±2.235 pg/mL for the Control, PPE, PPE+WE and PPE+ME groups rats, respectively. The highest IL-6 was observed in the rats with PPE-induced COPD. It is seen that IL-6 values obtained in the rats of PPE+WE and PPE+ME groups were quite close to the control group. IL-6 values of the rats treated with COPD, which were treated by *Juniperus drupacea* fruit extracts, decreased to the levels of normal rats without COPD. This indicates that the rats responded positively to the treatment process.

When the IL-8 obtained in this study was investigated (Fig. 2), it was observed that a similar situation occurs here with the IL-6. However, the differences between IL-8 were significant statistically (p<0.05). The IL-8 in the control, PPE, PPE+WE and PPE+ME groups rats were determined to be 174.37±3.71, 222.05±12.54, 210.52±23.49, and 169.71±22.12 ng/mL. It was seen that the highest IL-8, as in IL-6, was found in the rats with PPE-induced COPD group. The IL-8 of the control and PPE+ME groups rats were found to be close to each other. It was seen that IL-8 of rats with COPD treated with *Juniperus drupacea* fruit water extract (PPE+WE) was higher than those treated with methanol extract (PPE+ME). This situation give the result that the methanol extract of *Juniperus drupacea* fruit is more effective in terms of decreasing IL-8.

TNF-α for the control, PPE, PPE+WE and PPE+ME group rats were determined to be 255.12±25.4, 417.97±108.6, 267.44±36.1, and 259.57±39.5 pg/mL, respectively. The differences in TNF-α were determined to have strong statistical significance (p<0.05). As with IL-6 and IL8, the highest TNF-α was observed in the rats with PPE-induced COPD group. It was found that the TNF-α of the control group were close to those of the PPE+WE and PPE+ME groups rats. TNF-α of the rats with PPE-induced COPD increased compared to the control group, but the values decreased with the treatment with *Juniperus drupacea* extracts. These results show that both extracts of *Juniperus drupacea* fruit are effective in decreasing TNF-α.

Effects of flavan-3-ols rich extracts from *J. drupacea* fruit on the B, cytotoxic T and NK cells in the rat bloods

The results of B cells, CTLs and NK cells are exhibited in Figure 3. B cells (%) for the Control, PPE, PPE+WE and PPE+ME group rats was detected as 23.940±5.91, 31.980±5.12, 16.800±3.08 and 22.575±4.43, respectively. The differences between the groups in terms of B cell counts was significant statistically (p<0.05). The highest number of B cells was determined in the PPE-induced COPD group rats (31.980), while the lowest was detected in the PPE+WE group rats (16.800). B lymphocyte counts decreased in the rats with PPE-induced COPD treated by the water and methanol extracts.

The number of NK cells was determined as 2.8167±1.910, 1.3200±0.286, 2.3714±0.605 and 2.3714±0.605 for the Control, PPE, PPE+WE and PPE+ME groups rats, respectively. The highest number of NK cells was determined in the rats in the Control group, followed by the PPE+WE, PPE+ME and PPE group rats, respectively. The NK count was the lowest in the rats with PPE-induced COPD.

Cytotoxic T lymphocyte counts were determined as 11.250±4.57, 16.720±4.52, 17.357±2.88 and 19.400±4.45 (%) for the Control, PPE, PPE+WE and PPE+ME groups rats, respectively. The lowest number of Cytotoxic T lymphocyte cells was determined in the Control group without COPD, while the highest number was found in the PPE+ME group rats (Fig. 3). Although there was an increase in CTLs in the rats with PPE-induced COPD compared to the control group rats, no change was observed as a result of 21-day treatment with both water and methanol extracts of *J. drupacea* fruit.

PCA, HCA and Heatmap analyzes regarding inflammation biomarker values

In this study, statistical methods such as Principal Component Analysis (PCA), Hierarchical Clustering Analysis (HCA) and Heatmap with clustering analysis were employed to visually and comprehensively evaluate the analyses from different perspectives. PCA mitigates correlations among numerous variables under investigation by reducing them to linear combinations of fewer components. In our study, it was utilized to visualize differences in inflammation biomarkers such as Il-6, IL-8, TNF-α, B cells, cytotoxic T cells and NK cells. Figure 4 illustrates a scatter plot (labeled as Fig. 2A) where points represent the Control, PPE, PPE+WE and PPE+ME groups, while the vectors represent tested inflammation biomarkers. PC1 explains 61.8% of the variance, and PC2 explains 31.6%, as shown in Figure 4.

The Control, PPE+WE and PPE+ME groups showed close relationships with NK-cells, pH and pO_2 clustering on the negative left side of PC1. In contrast, the PPE group, which exhibit strong association with B lymphocyte, IL-6, IL-8, emphysema, TNF-α, pCO_2 and Cytotoxic T lymphocyte were clustered on the positive right side of PC1 (Fig. 4). In Figure 5, it is observed that the Control, PPE, PPE+WE and PPE+ME groups were divided into 2 clusters based on inflammation biomarkers tested. The first cluster consisted of the Control, PPE+WE and PPE+ME along with IL-6, TNF-α, emphysema, IL-8, pCO_2 and Cytotoxic T lymphocyte. The second cluster consisted of the PPE group along with B cells, inflammation, NK cells, pH and pO_2. This situation indicated that all groups could be clearly differentiated from each other based on the determined inflammation biomarkers tested. It was understood that PC1 explains 61.8% of the variance and PC2 explained 31.6% of the variance, and that all variances could be explained 93.4% by PC1 and PC2 (Table 2). PC1, PC2 and PC3 vectors that contribute to this separation are seen in Table 2.

DISCUSSIONS

In our study, the number of B cells and Cytotoxic T lymphocytes increased, and NK-cells decreased due to inflammation/emphysema in the rats with PPE-induced COPD. At the same time, the levels of IL-6, IL-8 and TNF-α, which were among the cytokines, increased with COPD. As a result of the 21-day treatment of the rats with PPE-induced COPD with the water and methanol extracts of *J. drupacea* fruit, the numbers of B cells and NK cells and the levels of IL-6, IL-8 and TNF-α returned to the levels of control group rats. This current study found that both extracts of *J. drupacea* fruit showed a protective effect against lung inflammation and alveolar deterioration caused by exposure to the PPE in rats. It was estimated that this might be due to the high levels of catechins contained in *J. drupacea* fruit extracts and phenolic acids such as gallic acid, which have been shown to have a positive effect on COPD. The *J. drupacea* fruit extracts are rich in catechins such as catechin, epicatechin, and epicatechin gallate, and also contain significant amounts of protocatechuic and gallic acid.[19, 25, 26]Akbulut and Akbulut[19] stated that among the phenolic compounds, catechins such as catechin, epicatechin, and epicatechin gallate, appears to be the most abundant phenolic compound isolated in all extracts and the total of these catechins constitute approximately 79.7-81.5% of all phenolics in *J.*

drupacea fruit extracts. In some studies, it was stated that catechins were dominant among phenolic compounds in *J. drupacea* fruit extracts[19], and in some studies, it was stated that protocatechuic acid was the most abundant phenolic compound.[25, 26] It is important to note that different distributions and concentrations of phenolic compounds in *J. drupacea* fruit may vary depending on factors such as geographical location, climatic conditions, and growth and harvest stages of the plant.[19]

Sohn et al.[27] reported that the levels of IL-1β and IL-6, known as pro-inflammatory cytokines, were reduced in the lung BAL fluids of the mice treated with the extracts of a mixture of sixteen medicinal plants (Gamijinhae-tang extract) for the acute lung injury. Ahmad[28] observed that epigallocatechin 3-gallate, a catechin group phenolic, suppressed the expression of IL-6 and IL-8 in vitro. In the present study, the findings obtained regarding the changes in the levels of pro-inflammatory cytokines IL-6 and IL-8 in the blood of COPD rats treated with *Juniperus drupacea* fruit extracts were seen to be consistent with the results obtained from the study of Sohn et al.[27] and Ahmad[28].

In light of the existing body of literature, assessment of arterial blood gas (ABG) exchange is a primary measure to determine the damage to the alveolar wall and confirm the efficiency of gas exchange in the lungs, indicating lung injury in rats.[29,30] ABG analysis is a crucial diagnostic tool for assessing the severity of emphysema.[31] Jiang *et al*.[31] stated that at the end of the treatment process of rats with lipopolysaccharide (LPS)-induced COPD with pyrrolidine dithiocarbamate (PDTC) pH and PaO_2, which are blood gas values, decreased compared to the rats in the control group, $PaCO_2$ increased. In the present study, emphysema increased significantly in the blood of rats with COPD due to PPE exposure, and this value decreased again at the end of the 21-day treatment period with the water and methanol extracts of *J.drupacea* fruit. A similar situation was seen in the blood gas analysis results of rats; $PaCO_2$ increased and PaO_2 decreased in the blood gas values of COPD rats exposed to PPE. However, at the end of 21 days of treatment with a dose of 250 mg/kg body weight/day of both extracts of *J. drupacea* fruit, $PaCO_2$ decreased, and PaO_2 increased, approaching the blood gas values in the control group rats. This shows that the treatment of inflammation/emphysema in the rats with *J. drupacea* fruit extracts makes a positive contribution to the PPE-induced COPD (Table 1).

COPD is a progressive respiratory disorder characterized by permanent airflow limitation, inflammation of the airways, and respiratory symptoms such as shortness of breath, chronic cough, and sputum production. COPD primarily includes two main conditions: chronic bronchitis and emphysema.[**27,32**]

COPD is a long-term lung condition where the flow of air in the respiratory system is consistently restricted. This restriction tends to worsen over time and is linked to a heightened, ongoing inflammatory reaction in the air passages and lungs, triggered primarily by harmful substances like cigarette smoke. Inflammation plays a critical role in the pathogenesis and progression of COPD, leading to structural changes in the airways and lung tissue. Chronic inflammation in COPD primarily involves the small airways and lung parenchyma. In response to inhaled irritants like cigarette smoke, there is an influx of inflammatory cells such as neutrophils, macrophages, and lymphocytes into the airways and lung tissue. These cells release various inflammatory mediators, including cytokines, chemokines, proteases, and reactive oxygen species, contributing to tissue damage and inflammation.[**33,34**]

Cytokines like IL-1, IL-6, and TNF-α are raised in COPD patients and contribute to the inflammatory response, tissue destruction, and recruitment of immune cells. Chemokines such as IL-8 play a crucial role in recruiting neutrophils to the airways, contributing to airway

inflammation and obstruction. Matrix metalloproteinases (MMPs) and neutrophil elastase are proteases released by inflammatory cells that lead to tissue destruction and remodeling in COPD. The chronic inflammatory process in COPD also results in oxidative stress due to an imbalance between reactive oxygen species (ROS) and antioxidants. Oxidative stress further exacerbates inflammation and tissue damage in the lungs. Chronic inflammation in COPD triggers structural changes in the airways, including thickening of the airway wall, mucus hypersecretion, and destruction of lung parenchyma (emphysema). This remodeling further exacerbates airflow limitation and respiratory symptoms. Inflammatory responses are amplified during acute exacerbations of COPD, leading to worsened symptoms, increased airway inflammation, and accelerated decline in lung function.[33,34]

IL-8, IL-6, and TNF-α are pro-inflammatory cytokines associated with COPD. These cytokines play a significant role in the inflammatory process and contribute to the pathogenesis and progression of COPD. IL-6 is a pro-inflammatory cytokine that is elevated in COPD patients. It is produced by a variety of cells, including macrophages, T cells, and endothelial cells. Elevated levels of IL-6 are associated with the severity of COPD and its related comorbidities, including muscle wasting and cardiovascular disease.[35] IL-8 is a chemokine that plays a crucial role in recruiting neutrophils to the airways and promoting inflammation. Increased levels of IL-8 are found in COPD patients and are associated with increased airway neutrophilia, exacerbations, and disease severity.[36] TNF-α is a pro-inflammatory cytokine involved in the regulation of immune cells and inflammation. In patients with COPD, TNF-α levels increase depending on the severity of the disease and are associated with airway inflammation, disease progression, and systemic symptoms.[37] Understanding the role of these cytokines is essential for developing targeted therapies and interventions to manage COPD and improve the quality of life for individuals with this chronic respiratory condition.

B-cells, CTLs, NK cells, and their involvement in COPD are important aspects of the immune response and its impact on respiratory health. However, it's important to note that the exact role and mechanisms of these immune cells in COPD may be complex and not fully understood. B-cells play a role in COPD through antibody production and involvement in the inflammatory response. They can produce autoantibodies, such as rheumatoid factor and anti-elastin antibodies, which may contribute to tissue damage in COPD. B-cell activation and antibody production may contribute to chronic inflammation and lung tissue destruction in COPD.[38] CTLs play a crucial role in cell-mediated immune responses, including targeting infected or damaged cells. In COPD, they are involved in targeting and eliminating infected or damaged lung cells, but an excessive CTL response may also contribute to tissue damage and inflammation in the lungs.[39] NK cells are part of the innate immune response and are involved in early defense against viral infections and tumor cells. In COPD, altered NK cell activity has been observed, which may contribute to impaired antiviral defense and increased susceptibility to respiratory infections.[40]

Monitoring specific parameters like pH (acidity), PaO_2 and $PaCO_2$ are crucial in managing and assessing the severity of COPD. The pH of blood is an important indicator of the body's acid-base balance. In COPD, the blood pH can be affected due to a condition called respiratory acidosis, where there is an accumulation of carbon dioxide in the blood.[41] PaO_2 represents the pressure of oxygen dissolved in the blood. In COPD, impaired lung function often leads to decreased PaO_2 levels, resulting in hypoxemia, which can further worsen the symptoms and prognosis of COPD patients.[42] $PaCO_2$ measures the pressure of carbon dioxide dissolved in the blood. In COPD, the retention of carbon dioxide due to impaired lung function can lead to

respiratory acidosis and affect overall blood gas levels.[1] Regular monitoring of these parameters through arterial blood gas (ABG) analysis is critical in managing and adjusting treatment plans for individuals with COPD. It helps healthcare professionals assess the severity of the disease, optimize oxygen therapy, and make appropriate adjustments to ventilation strategies.

The primary approach for treating COPD involves using a combination of medications and non-drug-based strategies. Pharmacological treatments aim to relieve symptoms, improve exercise tolerance, reduce exacerbations, and improve overall quality of life. There are some common classes of medications such as bronchodilators, inhaled corticosteroids (ICS), phosphodiesterase-4 (PDE-4) inhibitors, methylxanthines, antibiotics, oxygen therapy, vaccinations used in the pharmacological treatment of COPD.[43] In the treatment of inflammatory lung diseases, classical drugs are used for specific purposes in combination with different drugs used for several therapeutic purposes, and include inhaled glucocorticosteroids, β2-adrenoceptor agonists, leukotriene receptor antagonists, methylxanthines, theophylline and others.[27,44-46] However, bronchodilators, ICSs and PDE-4 inhibitors, which are the classes of drugs used in this treatment, may cause potential side effects such as tachycardia, tremor, headache, palpitations, increased heart rate, muscle cramps, thrush (oral yeast infection), hoarseness, increased risk of pneumonia, bone density loss with long-term use, nausea, diarrhea, weight loss, depression, and insomnia.[27,47-51] That's why, there is a need to develop treatment methods that are more effective, safer, and have little or no side effects. In our study, we observed that exposure to PPE persuade structural and functional changes that **were** typical of COPD, including airway remodeling, alveolar expansion, emphysema, lung inflammation, and increased numbers of lymphocytes (B-cells, Cytotoxic T lymphocytes, NK cells), the levels of IL-8, IL-6, TNF-α in the rat blood. At the same time, there were changes in the pH, PaO_2 and $PaCO_2$ blood gas values, and as in typical COPD, a decrease in PaO_2 value and an increase in $PaCO_2$ value occurred. On the other hand, during the treatment process with **the** water and methanol extracts of *Juniperus drupacea* **fruits**, the structural and functional changes in the lung caused by the PPE exposure model began to normalize. These results may be considered that both extracts of *J. drupacea* fruit, especially methanol extracts, are useful therapeutic agents in preventing these structural and functional changes related to COPD.

Medicinal plants are used in traditional and complementary medicine for health problems as an alternative to modern methods or in combination with them. Many studies have reported that various extracts of some medicinal plants such as *Nigella sativa* (L.) seeds extracts[52], *Myristica fragrans*, *Cinnamonum cassia*, *Camellia sinensis*, *Curcuma longa* have anti-elastase activity. This is mainly due to the fact that plant extracts are rich in many bioactive compounds, especially phenolic compounds. Green tea (*Camellia sinensis*) is rich in flavonoids such as catechin and epigallocatechin, and these phenolic compounds are stated to be elastase inhibitors.[53]

Bioactive phytochemicals with high antioxidant and anti-inflammatory activities may have a healing effect on impaired lung functions. It has been observed that the use of fruits, vegetables, and plants as rich sources of bioactive phytochemicals can reduce the risk of COPD.[3,54,55] Bioactive compounds found in plants like phenolic compounds, carotenoids, and alkaloids inhibit DNA methylation by preventing oxidative stress and inflammation and thus preventing the progression of COPD.[3,56]

Juniperus drupacea, commonly known as the Syrian juniper, is a species of juniper native to the eastern Mediterranean region, including parts of Turkey, Syria, Lebanon, Israel, and Cyprus.[19] It is often used in traditional medicine for various purposes, including respiratory ailments.[6] The

phenolic compounds present in *Juniperus drupacea* fruits may include various classes of polyphenols, such as flavonoids, phenolic acids, lignans, and tannins. *J. drupacea* fruits include phenolic acids (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid) and flavonoids (catechin, epicatechin, epicatechin gallate, and procyanidin A2).[19,25,26] Yaglioglu and Eser[57] determined four different phenolic compounds in the cones of four different *Juniperus* species, *J. communis*, *J. excelsa*, *J. foetidissima*, and *J. oxycedrus*, and reported that the most abundant phenolic compound was catechin. Akbulut and Akbulut[19] stated that among the phenolic compounds, catechins such as catechin, epicatechin, and epicatechin gallate, appears to be the most abundant phenolic compound isolated in all extracts and the total of these catechins constitute approximately 79.7-81.5% of all phenolics in *J.drupacea* fruit extracts. In some studies, it has been stated that catechins are dominant among phenolic compounds in *J. drupacea* fruit extracts[19], and in some studies, it has been stated that protocatechuic acid is the most abundant phenolic compound.[25,26] It is important to note that different distributions and concentrations of the phenolic compounds in *J. drupacea* fruit may vary depending on factors such as geographical location, climatic conditions, and growth and harvest stages of the plant.[19]
Oxidative stress significantly contributes to the development and progression of COPD. Antioxidants, including those found in medicinal plants, can help combat oxidative stress and potentially mitigate the progression of COPD. (-)-epigallocatechin-3-gallate, a flavanol polyphenolic compound, is indicated to be a potent natural leukocyte elastase inhibitor that can be used to reduce elastase-mediated emphysema. This flavanol is abundant in green tea and exhibits a dose-dependent, non-competitive inhibition of leukocyte elastase at a non-cytotoxic concentration and is effective in neutrophil culture.[**19,58**] In a study, the use of (-)-epigallocatechin-3-gallate showed promise in reducing the severity of acute lung injury caused by lipopolysaccharide in mice. This was evident through several positive outcomes: improved lung injury scores, reduced total cell, neutrophil, and macrophage counts, suppressed myeloperoxidase activity, lower wet-to-dry weight ratio of lung tissues, and a decrease in the release of inflammatory cytokines TNF-α, IL-1β, and IL-6.[**59**] Some flavonoids exert anti-inflammatory effects through blockade of inflammasome such as NF-κB and NLRP3, suppression of production of pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, TNF-α, and IL-17A, down-regulation of chemokines, and reduction of reactive nitrogen species and reactive oxygen species.[**60**] In a study, BAL cellularity, neutrophil recruitment and BAL MCP-1, IL-6 and TNF-α expressions, lung histological parameters, and platelet uptake increased in rats in which lung inflammation was induced by applying the intratracheal elastase model. However, on the 7th day of treatment with pomegranate peel extract (250 mg/kg body weight), it was determined that MCP-1, MMP-2 and IL-6 levels in the animals decreased to the levels of the animals in the control group, and lung TNF-α and MCP-1 expression decreased significantly.[**61**] Gallic acid (GA) is a naturally occurring and abundant phenolic compound in plants that is known to have antioxidant/anti-inflammatory activities. Singla *et al*.[**62**] stated that elastase and IL-6 and TNF-α cytokine levels in cigarette smoke (CS)-induced COPD mice were significantly increased compared to control group mice, but after gallic acid treatment, these factors returned to the control group level.
In our study, treatment with water and methanol extracts of *Juniperus drupacea* fruit significantly decreased the levels of IL-6, IL-8, TNF- α and B cells in the blood and increased the levels of NK cells. In particular, treatment with methanol extracts of the plant was more effective in reducing IL-6, IL-8 and TNF-α levels. This suggests that the anti-inflammatory effect of *Juniperus drupacea* fruit methanol extracts can be attributed to the suppression of

proinflammatory cytokine production in the lung. It is thought that both extracts of *Juniperus drupacea* fruit may provide beneficial clinical effects in the treatment of COPD, but methanol extract may be more effective in this treatment due to its more effect on inflammation biomarkers. Although in present study, the power calculation result showed that the number of animals used was sufficient to convey statistical significance, the sample size of this study was relatively small (n = 6) that might mislead the data interpretation. To overcome such limitations, additional studies are needed to investigate the *Juniperus drupacea* fruit extracts on lung inflammation.

Juniperus drupacea trees are endemic plants and grow quite widely and abundantly in high altitude forests along the Mediterranean coast of Turkey. From ancient times to the present day, the cones (fruits) of this endemic tree have been collected by local people and evaluated in traditional folk medicine to treat respiratory diseases such as asthma.[6,19] At the same time, its fruits are boiled for a long time to obtain a thick syrup called pekmez (molasses) and is considered a delicious food for health.[63] The reason why these fruits, which grow widely in forests in nature, are widely used by the public is that they are easily accessible, sustainable and cheap.

Herbal remedies, although it is among some of the traditional supportive methods used in the treatment of COPD, the fact that such products are natural does not always mean that they are safe. Herbal products contain active ingredients, and these can have both beneficial and harmful effects. The use of extracts from the fruits of *Juniperus drupacea* in the treatment of COPD should be closely monitored in future clinical studies, particularly regarding potential side effects such as allergic reactions, including skin rashes, itching, swelling, or respiratory distress; drug interactions; gastrointestinal issues such as nausea, diarrhea, vomiting, or stomach discomfort; hormonal effects; respiratory impacts; and other related adverse effects. Therefore, possible toxicity and side effects should be kept in mind that herbal treatments may carry risks in terms of individual health status and interactions with other medications used.[64]

The fact that no negative effects have been observed in the use of the fruits of this tree for a long time in terms of human health indicates that the fruits of this plant can be used in the treatment of COPD together with modern drugs. The use of plant extracts in the treatment of COPD should be considered with caution due to the potential for interactions with modern drugs. Herbal products may affect the efficacy and safety of prescription drugs through pharmacokinetic and pharmacodynamic interactions. In this respect, advanced research should be conducted to evaluate the components found in *Juniperus drupacea* fruit extracts from a broader perspective. It is very important to study and reveal the interactions of bioactive components that may be present in the structure of *Juniperus drupacea* fruits with modern drugs used in the treatment of COPD through in vitro and in vivo studies.

Study Limitations

The findings from animal research do not completely represent the conditions in humans. Therefore, more extensive and detailed studies are needed to assess the therapeutic effects of water and methanol extracts of *Juniperus drupacea* fruits on COPD biomarkers.

CONCLUSION

In this study, the therapeutic effect of water and methanol extracts of *J. drupacea* fruit on PPE-induced COPD was investigated. It was tried to determine with inflammatory mediators whether COPD occurs with PPE and whether both extracts of *J. drupacea* fruit are effective in the treatment of COPD. For this purpose, the changes in the histopathological tests (emphysema), arterial blood gas values (pH, PaO_2 and $PaCO_2$), IL-6, IL-8, TNF-α cytokines and B cells,

Cytotoxic T lymphocytes (CTLs), NK cells were observed. In rats with PPE-induced COPD, emphysema increased significantly, PaO_2 decreased, and $PaCO_2$ increased. In addition, with PPE exposure, an increase in B cells and CTLs was observed in the blood of rats, but a decrease in NK cells was observed. At the same time, IL-6, IL-8 and TNF-α levels, which are among the cytokines examined in the current study, increased. This strongly indicates that COPD occurred in PPE-induced mice. It is seen that in rats with COPD treated with water and methanol extracts (250 mg/kg bw/day) of *Juniperus drupacea* fruit for 21 days via gavage, there is a decrease in IL-6, IL-8 and TNF-α cytokines compared to PPE group rats, and these decreases were close to those of the Control group rats without COPD. The methanol extracts of *Juniperus drupacea* fruit were determined to be more effective in terms of the decrease in IL-6, IL-8, and TNF-α cytokines. In terms of blood gas values, it can be thought that the water extract is more effective, but the difference between the values is low, and that both water and methanol extract affect the result at the same level. In addition, with PPE exposure, an increase in B cells and CTLs was observed in the blood of rats, but a decrease in NK cells was observed. According to the results of flow cytometric analysis, the water extracts of *Juniperus drupacea* fruit were more effective on B lymphocytes. In conclusion, treatment of rats with COPD with *J. drupacea* extracts, which have been determined by studies to be rich in bioactive components, shows that it can reduce the negative effects of COPD. This shows that *J. drupacea* fruit extracts can be used in the treatment of COPD, but more scientific research is needed.

MAIN POINTS

- In all rats with PPE-induced COPD, emphysema was observed as a result of histopathological evaluation in the lungs.
- As a result of treatment with water and methanol extracts of *Juniperus drupacea* fruit given to PPE induced COPD rats, emphysema decreased to control sample levels.
- After PPE-induced COPD, IL-6, IL-8 and TNF-α levels in the blood of rats and B cells and NK cells returned to the level of rats without COPD after extract treatment.
- Both extracts of *Juniperus drupacea* fruit responded positively to COPD treatment.

ETHICS

Ethics Committee Approval: The animal study was reviewed and approved by the Ethics Committee of Selçuk University Experimental Medicine Research and Application Center (ethics committee decision no. 2020/52).

Author contributions

DISCLOSURES

Conflict of interests: The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Financial Disclosure: This article is produced from Dr. Hatice Feyza Akbulut's PhD thesis. This work was supported by the Selçuk University Research Foundation [grant number: [20112008]. The project manager is Prof. Dr. Hüsamettin Vatansev and researcher is Dr. H. Feyza Akbulut.

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Table 1. The changes in arterial blood[5] gas results of **the** rats with PPE exposure and *J.drupacea* fruit extracts treatment			
Groups	**pH**	**$PaCO_2$ (mmHg)**[3]	**PaO_2 (mmHg)**[4]

Control	7.3950±0.0152[a]	45.317±1.903[b]	63.800±11.52[a]
PPE	7.3520±0.0045[b]	51.520±2.960[a]	48.750±5.37[b]
PPE+WE	7.3529±0.0340[b]	49.757±4.170[ab]	51.833±6.43[b]
PPE+ME	7.3400±0.0185[b]	49.350±2.677[ab]	50.857±5.18[b]
Effect sizes (η^2)	**0.514**	**0.363**	**0.411**
***p* value**	**0.009**	**0.026**	**0.016**

[1] Values are expressed as "mean ± standard deviation".
[2] There is no statistical difference between the values indicated with the same letter.
[3]PaO_2: arterial oxygen partial pressure, [4]$PaCO_2$: arterial carbon dioxide partial pressure
[5] Blood gas analyses were performed on the day the study was completed, after the rats were anesthetized and blood was taken from the heart using blood gas injectors and analyzed without delay by the ABL9 blood gas analyzer (Dadiometer, Denmark) in the room at that time.

Table 2. PCA results regarding the evaluation of the effects of water and methanol extracts of Juniperus drupacea fruits on inflammation biomarkers in **the** rats applied with a COPD model

Items	**PC1***	**PC2**	**PC3**
Eigenvalue	6.80	3.48	0.72
Variance percentage (%)	61.8	31.6	6.6
Cumulative variance (%)	61.8	93.4	100
Eigenvectors			
IL-6 (pg/mL)	**0.324**	0.286	-0.031
IL-8 (ng/mL)	0.291	0.103	**-0.734**
TNF-α (pg/mL)	**0.331**	0.272	-0.003
B lymphocyte (%)	0.210	**0.386**	**0.503**
NK Cells (%)	**-0.374**	-0.055	-0.234
Cytotoxic T lymphocyte (%)	0.235	**-0.410**	0.233
pH	-0.270	**0.368**	-0.215
pCO_2 (mmHg)	**0.367**	-0.150	-0.086
pO_2 (mmHg)	**-0.341**	0.243	-0.045
Emphysema	**0.365**	0.148	-0.154
Inflammation	-0.065	**0.525**	0.141

*PC1: the first principal component, PC2: the second principal component, PC3: the third principal component

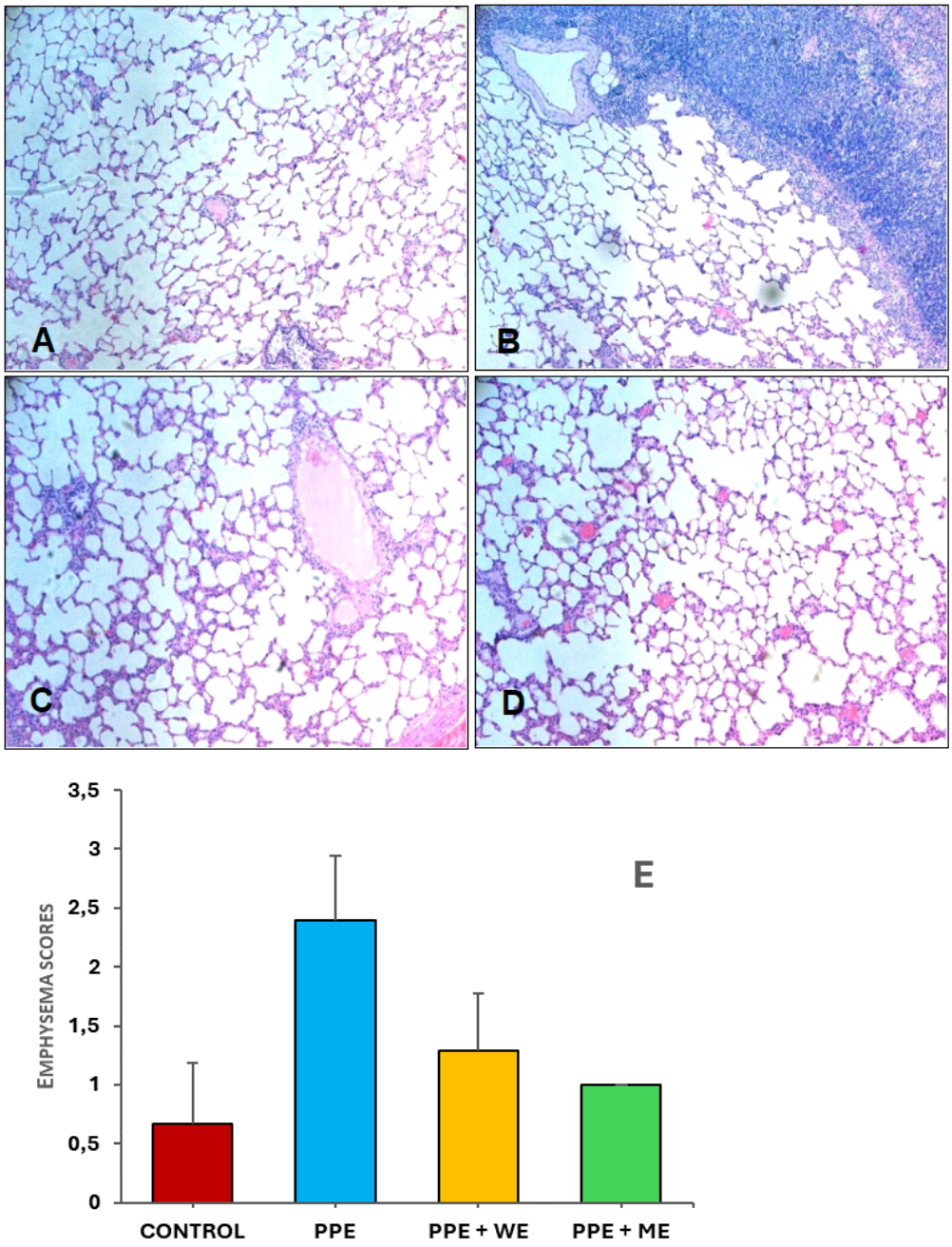

Figure 1. Photomicrographs of general lung histology. (*A*): rats without porcine pancreatic elastase (PPE), (*B*): rats with PPE, (*C*): rats induced COPD with PPE and treated for 21 days by gavage with water extract (WE) of *J. drupacea* fruit, (*D*): rats induced COPD with PPE and treated for 21 days by gavage with methanol extract (ME) of *J. drupacea* fruit, (*E*): Emphysema scores of all rat groups (the effect size (η^2) = 0.698, p = 0.000)

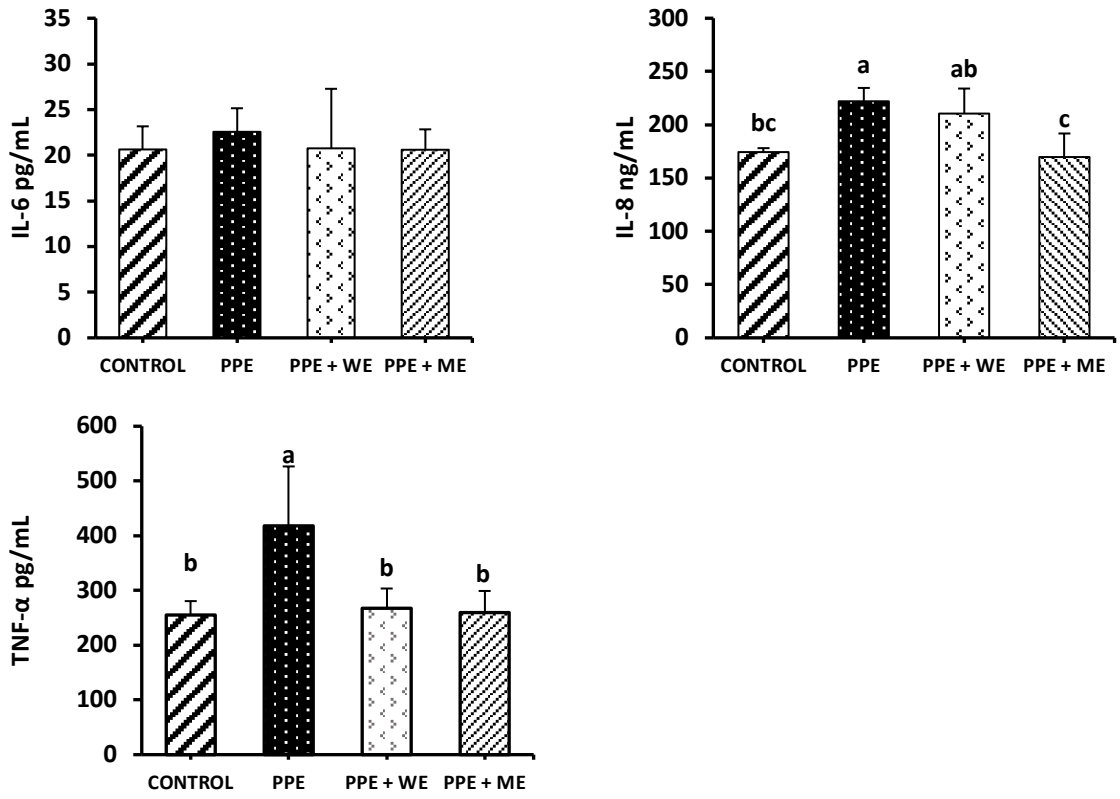

Figure 2. Effect of *Juniperus drupacea* fruit extract on the cytokines in the blood of rats. The levels of IL-8, IL-6 and TNF-α in the rat blood were detected by ELISA. CONTROL: No procedure was applied to the control group, PPE: porcine pancreatic elastase treated, PPE+WE: PPE+Water extract of *J. drupacea* fruit, PPE+ME: PPE+methanol extract of *J. drupacea* fruit, Values are expressed as mean ± standard deviation, there is no statistical difference between the values indicated with the same letter. (p<0.05) (IL-8, the effect size (η^2) = 0.632, p = 0.000; TNF-α, the effect size (η^2) = 0.591, p = 0.000)

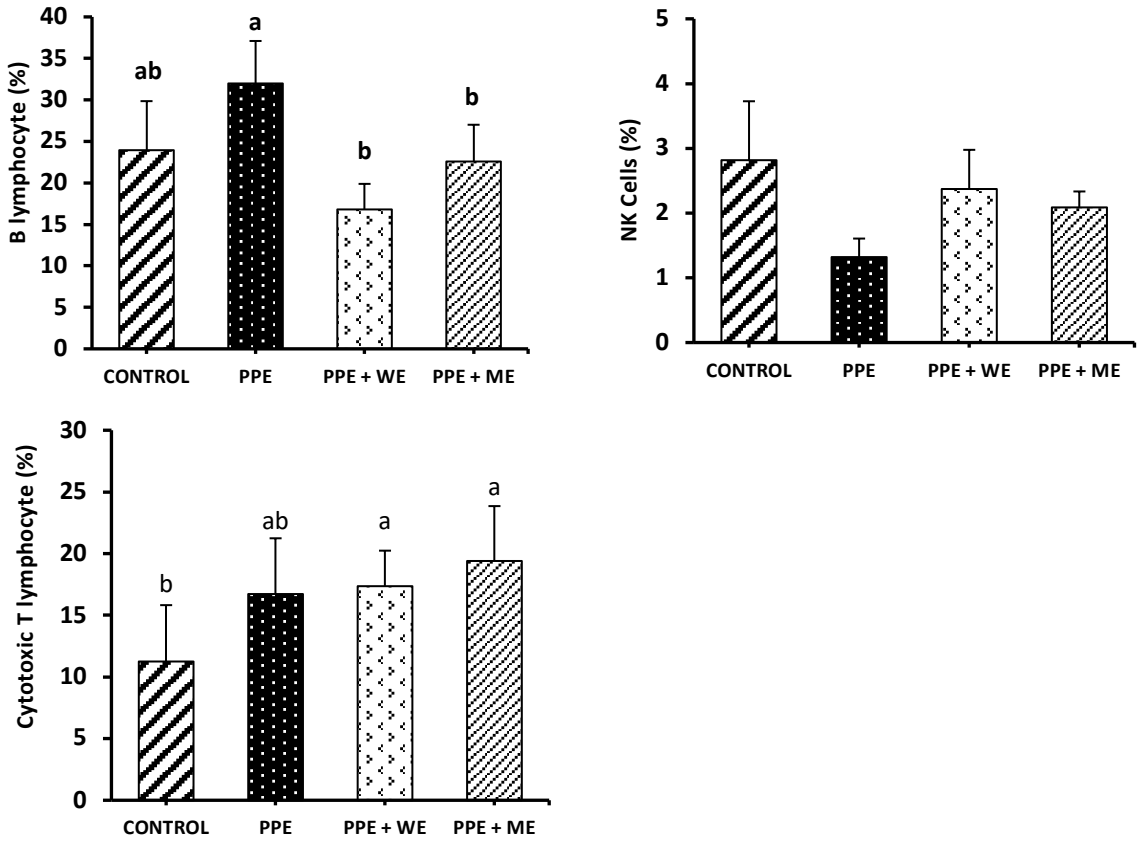

Figure 3. The effect of water and methanol extract treatment of *Juniperus drupacea* fruit on B cells, CTLs, and NK cells in the blood of rats. CONTROL: No procedure was applied to the control group, PPE: porcine pancreatic elastase treated, PPE+WE: PPE+Water extract of *J. drupacea* fruit, PPE+ME: PPE+methanol extract of *J. drupacea* fruit, Values are expressed as mean ± standard deviation, there is no statistical difference between the values indicated with the same letter. (p<0.05) (B cells, the effect size (η^2) = 0.314, p = 0.000; TNF-α, the effect size (η^2) = 0.389, p = 0.032)

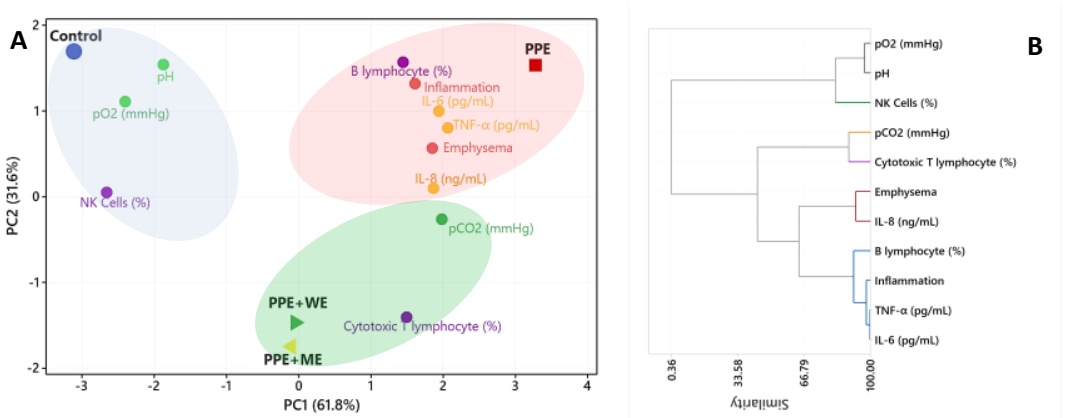

Figure 4. (A) The loading and score plot of PC1 and PC2 describing the changes among the pro-inflammatory cytokines, lymphocytes and blood gas values of the control, PPE, PPE+WE and PPE+ME group rats. (PC1, the first principal component; PC2, the second principal component) and (B) Dendrogram obtained through hierarchical cluster analysis (HCA).

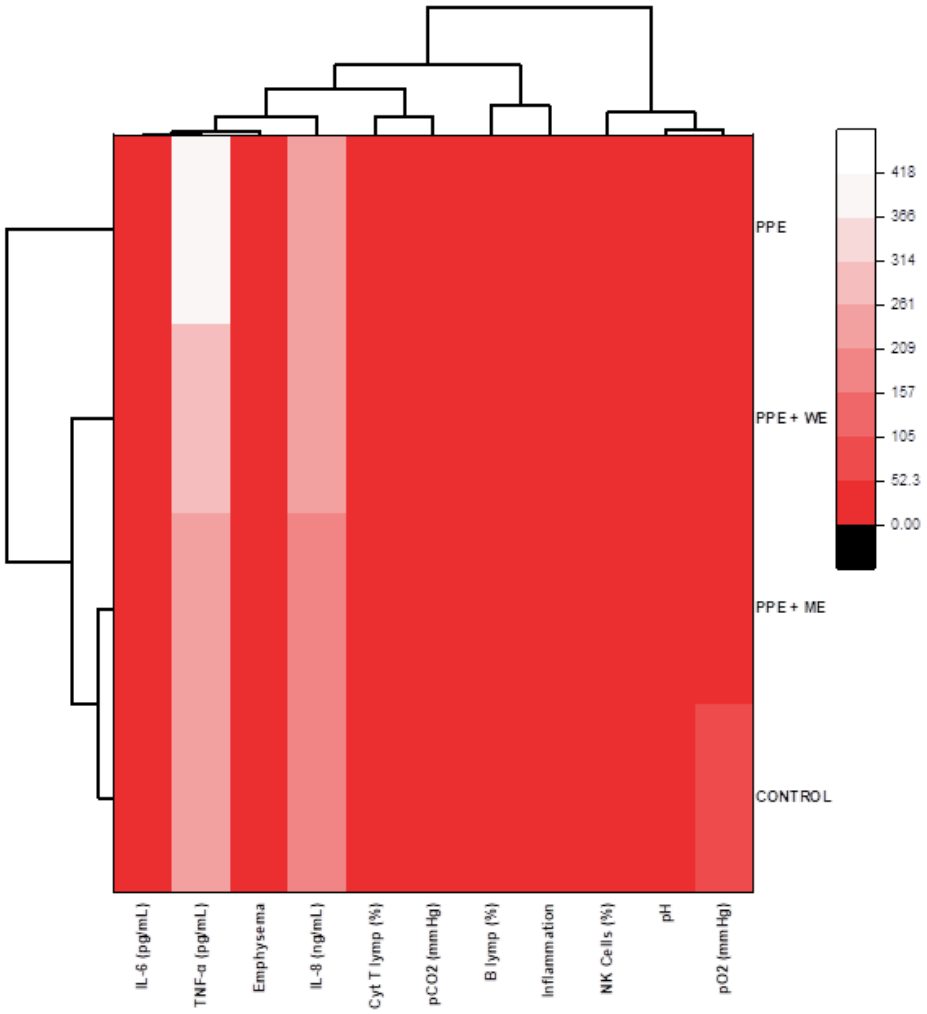

Figure 5. Heatmap obtained through the evaluation of the effects of water and methanol extracts of *Juniperus drupacea* fruits on inflammation biomarkers in rats applied with a COPD model. Heatmap shows the changes and contribution levels of the biomarkers evaluated in the monitoring of the treatment process with the water and methanol extracts of *Juniperus drupacea* fruit to the treatment process of COPD rats.