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Carvacrol and Pomegranate Extract in Treating Methotrexate-Induced Lung Oxidative Injury in Rats

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Data Interpretation D
Manuscript Preparation E
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Background: This study was designed to evaluate the effects of carvacrol (CRV) and pomegranate extract (PE) on methotrexate (MTX)-induced lung injury in rats.

Material/Methods: A total of 32 male rats were subdivided into 4 groups: control (group I), MTX treated (group II), MTX+CRV treated (group III), and MTX+PE treated (group IV). A single dose of 73 mg/kg CRV was administered intraperitoneally to rats in group III on Day 1 of the investigation. To group IV, a dose of 225 mg/kg of PE was administered via orogastric gavage once daily over 7 days. A single dose of 20 mg/kg of MTX was given intraperitoneally to groups II, III, and IV on Day 2. The total duration of experiment was 8 days. Malondialdehyde (MDA), total oxidant status (TOS), total antioxidant capacity (TAC), and oxidative stress index (OSI) were measured from rat lung tissues and cardiac blood samples.

Results: Serum and lung specimen analyses demonstrated that MDA, TOS, and OSI levels were significantly greater in group II relative to controls. Conversely, the TAC level was significantly reduced in group II when compared to the control group. Pre-administering either CRV or PE was associated with decreased MDA, TOS, and OSI levels and increased TAC levels compared to rats treated with MTX alone. Histopathological examination revealed that lung injury was less severe in group III and IV relative to group II.

Conclusions: MTX treatment results in rat lung oxidative damage that is partially counteracted by pretreatment with either CRV or PE.

MeSH Keywords: Methotrexate • Oxidative Stress • Punicaceae

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Background

Despite advances in medicine, more than 150 drugs have been reported to induce pulmonary toxicity since 1972 and the list of medications that cause pulmonary toxicity continues to grow as new drugs are developed [1]. Methotrexate (MTX), a folic acid analogue, is commonly used over a wide range of doses for various therapeutic applications [2,3]. Methotrexate's antiproliferative, anti-inflammatory, and immune system modulation effects limit cancer growth and prevent leukocyte proliferation in inflammatory diseases [4]. Besides the many beneficial uses of MTX, this medication may cause adverse effects in several organ systems. Severe methotrexate toxicity may affect the lungs, liver, and bone marrow [2,5]. Methotrexate-induced pulmonary toxicity was first reported in 1969 after children with acute lymphocytic leukemia were administered high doses of this drug [6].

The precise mechanism by which MTX causes pulmonary injury is not fully understood. Direct methotrexate toxicity to the lungs was observed in a mouse model that demonstrated alveolar epithelial injury, apoptosis, and fibrosis [7]. Pulmonary toxicity most often occurs after long-term methotrexate use, but such adverse effects have also been observed following high doses [8,9]. MTX produces free oxygen radicals that enhance lipid peroxidation. Specifically, these free radicals lead to mitochondrial functional impairment [10]. Increased malondialdehyde (MDA) production and decreased glutathione levels in the blood, liver, and kidneys are all associated with MTX administration. [11]. In summary, the mechanisms of MTX pulmonary toxicity remain unclear. An optimal therapy for MTX-induced lung toxicity has not yet been developed and prospective therapy trials have yet to be performed.

Traditional medicinal treatments continue to be popular worldwide as herbal therapies compete with modern technological advances in diagnosis and treatment. Many patients seek alternative and complementary medical treatments to derive some therapeutic benefit from products made from all natural ingredients. Carvacrol (5-isopropyl-2-methylphenol) is a phenolic monoterpene constituent of essential oils produced by numerous aromatic plants and spices [12,13]. The oil from *Origanum vulgare* has the highest concentrations (80%) of carvacrol and carvacrol is thought to be the most biologically active form of this compound [14]. Medicinal plants containing carvacrol have been used in folk medicine for many years [15]. Carvacrol has been found to have antimicrobial, antithrombotic, anticarcinogenic, and antioxidant properties [12,16,17]. Essential oils rich in carvacrol possess strong antioxidant properties equivalent to those of ascorbic acid, butyl hydroxytoluene (BHT), and vitamin E [18]. Carvacrol also increases the activity of endogenous antioxidants [19]. *Punica granatum Linn*. from the family Punicaceae, commonly known as the pomegranate, is a medicinal fruit native to the Himalayas in northern India to the Middle East and Turkey [20]. Pomegranate leaf extract contains antiparasitic, antimicrobial, and antioxidant properties [21,22].

In an effort to develop treatment modalities that reduce lung toxicity following MTX use, we investigated whether antioxidants inhibit MTX-mediated pulmonary injury. Specifically, we studied whether CRV and PE exert protective effects against MTX-induced oxidative lung injury in a rat model. Biochemical analyses and histological examinations were performed to determine the morphologic changes in rat lung tissues after exposure. To the best of our knowledge, this is the first study that has investigated the protective effects of CRV and PE against MTX-induced pulmonary toxicity.

Material and Methods

Animal model and experimental design

The entire experimental protocol was reviewed by the Dicle University Animal Ethics Committee and all experiments were carried out in accordance with the Animal Welfare Act and the Dicle University Animal Ethics Committee Guide for the Care and Use of Laboratory Animals. A total of 32 male 3-month-old Wistar albino rats with a mean body weight of 225±30 g were supplied by the animal laboratory of Dicle University. All animals were housed in standard conditions at an ambient temperature of 25±2°C with a 12-hour light-dark cycle. The rats were kept in cages (8 rats to each cage) and were treated in compliance with the National Institutes of Health guidelines. All experimental procedures were performed in compliance with the animal use regulations of Dicle University Experimental Research Center, Diyarbakır, Turkey. Compound doses were calculated based on animal weight. Orogastric drug administration was performed via a gavage needle. No animals died during the study.

The rats were randomly allocated into 4 groups containing a total of 8 animals each. Group I was composed of control rats that did not receive any treatment, but were housed in identical conditions as the other experimental groups. Group II contained rats administered a single 20 mg/kg dose of intraperitoneal MTX (Medac, GmbH, Theaterstrasse 6, D-22880 Wedel/Germany) on Day 2 of the study. No other medications were administered during the remaining 7 days. Group III consisted of rats treated with both MTX and CAR. On Day 1, the animals received a 73 mg/kg dose of CAR administered via an intraperitoneal injection according to the methods recommended by Canbek et al. [23]. On Day 2, group III rats received a single dose of 20 mg/kg MTX intraperitoneally. Group IV rats were given a 225 mg/kg dose of PE containing 40% ellagic acid via orogastric gavage once daily for 7 days. On Day 2, a single dose of 20 mg/kg MTX was administered by intraperitoneal injection. The total duration of the experiment was 8 days, and on the 8th day all rats were sacrificed by decapitation.

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Plant extract

Carvacrol extract

Steam-distilled essential oils of *Origanum onites L,.* collected from West Anatolia, were isolated as described by Canbek et al. so to extract carvacrol, also called 2-methyl-5-(1-methylethyl) phenol [23]. For the isolation, fractional distillation was performed using a lab-size glass fractional distillation unit containing a column packed with S/S Knit Mesh packing material (2.8×1.35 m). Reflux ratio was set at 10/1 to 20/1 and the medium pressure was 8–10mm Hg. Carvacrol-rich fractions were bulked to obtain CAR with 99% purity (gas chromatography and mass spectrometer analyses) [23].

Pomegranate extract

Pomegranate extract was obtained from GNC Herbal Plus® Standardized Pomegranate Extract Capsules (Pittsburgh, USA) (each capsule contains 250 mg PE and 100 mg Ellagic acid) [24].

Surgical procedures

On Day 8, all animals were anesthetized with an intramuscular injection of 50 mg/kg of ketamine HCl (Ketalar; Parke-Davis, Karachi, Pakistan). Afterwards, the animals received a peritoneal injection of 10 mg/kg of xylazine. Upon achieving surgical anesthesia, the rats were sacrificed via decapitation. Immediately following decapitation, 5 mL of blood was obtained from rat cardiac cavities. Then the lungs were immediately excised from all rats and were longitudinally sectioned into 2 equivalently sized portions. One section was retained for biochemical analysis and the other section was fixed in 10% formalin for subsequent histological examination.

Biochemical methods

Blood samples were collected in tubes without anticoagulants. Blood specimens were centrifuged at 4.000 rpm at 4°C for 10 minutes and then the serum was stored at –80°C until biochemical analyses were performed. Lung tissues were rinsed with ice-cold saline and immediately stored at –80°C. Lung specimens were weighed and then homogenized in a 50-mM phosphate buffered saline (PBS) at pH 7.0. Homogenized lung tissues were then centrifuged at 10.000 rpm at 4°C over 15 minutes to isolate the supernatant for subsequent analysis. Serum and homogenized lung tissue MDA concentrations were determined using an assay (Northwest Life Science Specialties, LLC, Vancouver, Canada) based on the reaction of MDA with thiobarbituric acid (TBA). This reaction results in the production of a MDA-TBA_{2a} compound that has a measurable absorbance at 532 nm [25]. Total antioxidant capacity (TAC) and total oxidant status (TOS) were measured via colorimetric methods as described by Erel from rat serum and lung tissue [26]. Total antioxidant capacity units are µmol Trolox equivalents per gram of tissue, and TOS units are µmol H_2O_2 equivalents per gram of tissue. The oxidative stress index (OSI) was calculated according to the following formula [27]:

$$OSI = \frac{TOS}{TAC}$$

Histological methods

Following excision, the lung tissues were fixed in a 10% formalin solution for 48 hours. Paraffin blocks were sliced in 5-mm-thick sections and were stained with hematoxylin and eosin. Sections were examined via light microscopy (Nikon Eclipse 50i, Japan) and photographed to identify morphological alterations. A blinded pathologist examined all lung tissue specimens. Interstitial edema, alveolar epithelial injury, polymorphonuclear leukocytes (PNL), and chronic inflammatory cell infiltrates (lymphocytes and plasma cells) were determined for each lung specimen at 200× to 400× magnification. Each finding was semi-qualitatively scored on a scale ranging from 0 to 3: (0) absent, (1) weak, (2) moderate, and (3) intense.

Statistical analysis

All data are expressed as the mean ± standard deviation (±SD). Variation from a normal distribution was evaluated using the Kruskal-Wallis analysis of variance test followed by the non-parametric Mann-Whitney U-test. Pearson's chi-squared test was utilized to compare lung injury scores between rat lung histological specimens. A *p-value* less than 0.05 was statistically significant. All data were analyzed with SPSS statistical software package version 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Serum biochemical analysis

TOS (p=0.001), OSI (p=0.001), and MDA (p=0.002) levels were significantly increased in rats treated with MTX alone when compared with controls. However, a significant decrease in TAC (p=0.002) was observed in MTX-treated rats in comparison to control rats. Administering oral PE was associated with significantly decreased TOS (p=0.009), OSI (p=0.009), and MDA (p=0.021) levels and increased TAC (p=0.005) levels relative to animals that received MTX alone. Pre-administering carvacrol was associated with significantly decreased TOS (p=0.003), OSI (p=0.002), and MDA (p=0.005) levels and increased TAC (p=0.016) levels when compared to animals that were treated only with MTX. All biochemical analysis results from rat sera are shown in Table 1.

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Table 1. Serum oxidant and antioxidant parameters by treatment group (means ±SD).

Groups	TAC (mmol trolox equiv./L)	TOS (mmol H_2O_2 equiv./L)	OSI (arbitrary unit)	MDA (μM)
Control (1)	1.67±0.08	11.40±3.38	6.89±2.30	2.47±0.55
MTX (2)	1.34±0.15	35.57±26.45	26.60±19.01	3.55±0.34
MTX + CRV (3)	1.55±0.05	12.28±6.21	7.90±3.94	2.37±0.69
MTX + PE (4)	1.65±0.16	16.18±5.95	9.98±4.33	2.47±0.85
p value between groups				
1–2	0.002	0.001	0.001	0.002
2–3	0.016	0.003	0.002	0.005
2–4	0.005	0.009	0.009	0.021

TOS – total oxidant status; TAC – total antioxidant capacity; OSI – oxidative stress index; MDA – malondialdehyde; MTX – methotrexate; CRV – carvacrol; PE – pomegranate extract.

Table 2. Lung tissue oxidant and antioxidant parameters in different groups (means ±SD).

Groups	TAC (μmol trolox equiv./g tissue)	TOS (μmol H_2O_2 equiv./g tissue)	OSI (arbitrary unit)	MDA (μmol/g tissue)
Control (1)	5.58±0.91	246.91±26.28	44.73±4.75	46.61±17.68
MTX (2)	4.13±0.70	425.44±175.34	107.02±47.38	67.37±27.21
MTX + CRV (3)	5.28±0.96	280.72±110.84	54.17±23.86	54.36±15.72
MTX + PE (4)	4.98±0.42	288.09±52.96.	58.40±14.06	55.83±13.34
p value between groups				
1–2	**0.006**	**0.046**	**0.012**	**0.021**
2–3	**0.036**	0.074	**0.036**	**0.016**
2–4	**0.012**	0.115	**0.021**	**0.046**

TOS – total oxidant status; TAC – total antioxidant capacity; OSI – oxidative stress index; MDA – malondialdehyde; MTX – methotrexate; CRV – carvacrol; PE – pomegranate extract.

Lung tissue biochemical analysis

In the MTX only treatment group, MDA (p=0.021), TOS (p=0.046), and OSI (p=0.012) levels in lung tissues were significantly increased relative to the control group. Pre-administering CRV before MTX treatment was associated with significantly decreased MDA (p=0.016) and OSI (p=0.036) levels in homogenized lung tissues in comparison to animals treated with MTX alone. TOS levels were lower in rats that received both CRV and MTX compared to the MTX-treated group, but the difference was not statistically significant (p=0.074). Pre-administering PE to the rats via orogastric gavage was associated with significantly decreased MDA (p=0.046) and OSI (p=0.021) levels in homogenized lung samples when compared to rats treated with MTX alone. TOS levels were lower in rats that received PE before MTX treatment, but this difference was not statistically significant (p=0.115). In addition, TAC levels were decreased in rats treated with MTX alone relative to controls (p=0.006). Administering CRV prior to MTX significantly suppressed the effects of MTX-induced lung injury (p=0.036). Lung tissue TAC (p=0.012) levels were significantly increased in rats that received PE before MTX treatment when compared to rats that were treated with MTX alone. All results from rat lung tissue biochemical analysis are summarized in Table 2. Figures 1–4 contain bar graphs of lung tissue oxidative markers.

Lung tissue histological analysis

On examination, lung tissues from control rats appeared healthy (Figure 5). Interstitial edema, alveolar epithelial injury, PNLs, and chronic inflammatory cell infiltrates (arrowheads) were observed in rats treated with MTX alone (Figure 6). Interstitial

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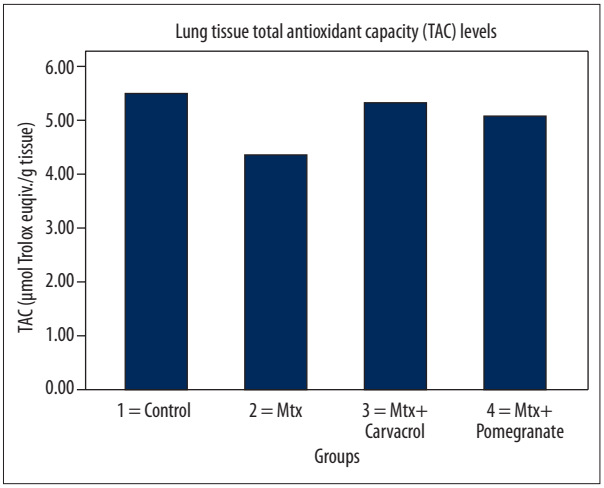

Figure 1. The mean lung tissue Total Antioxidant Capacity (TAC) levels, MTX: methotrexate.

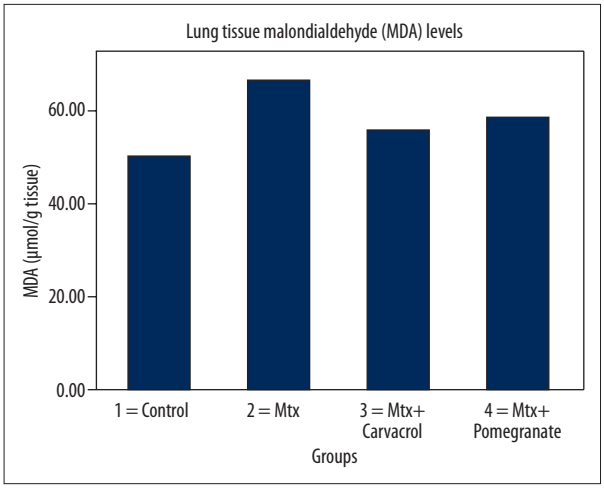

Figure 3. The mean lung tissue Malondialdehyde (MDA) levels, MTX: methotrexate.

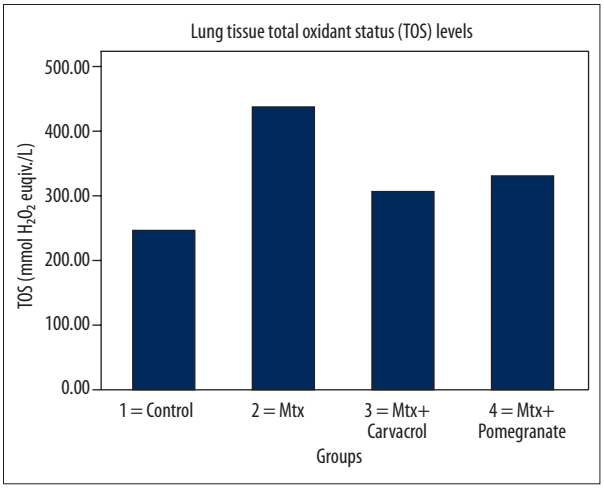

Figure 2. The mean lung tissue Total Oxidant Status (TOS) levels, MTX: methotrexate.

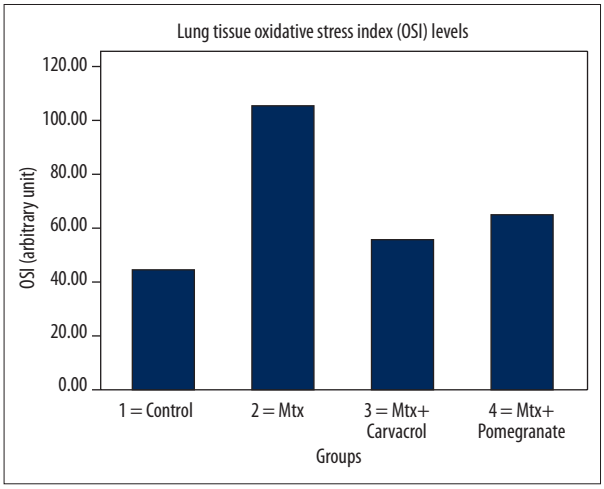

Figure 4. The mean lung tissue Oxidative Stress Index (OSI) levels, MTX: methotrexate.

edema, alveolar epithelial injury, PNLs, and chronic inflammatory cell infiltrates were less severe in lung tissues harvested from animals that received CRV treatment prior to MTX administration when compared to rats treated with MTX alone, and these differences were statistically significant (p=0.001, p=0.003, p=0.02, and p=0.02, respectively) (Figure 7). In rats pretreated with PE before MTX exposure, interstitial edema, alveolar epithelial injury, PNLs, and chronic inflammatory cell infiltrates were observed at decreased levels that approached statistical significance when compared to rats treated with MTX alone (p=0.03, p=0.03, p=0.04, and p=0.03, respectively) (Figure 8).

Discussion

Our findings demonstrate that pretreating rats with CRV and PE may be useful in preventing MTX-induced lung toxicity by attenuating oxidative stress and inflammation. In particular, administering CRV prophylactically might have protective effects on MTX-induced oxidative damage to rat lung tissues. Methotrexate is prescribed for the treatment of a wide range of conditions, including cancer, rheumatoid arthritis, psoriasis, uveitis, asthma, granulomatosis with polyangiitis, sarcoidosis, primary biliary cirrhosis, and inflammatory bowel disease [28,29]. Life-threatening toxicity may result following both high-dose and low-dose MTX therapy [28,30]. Over the past 3 decades there have been numerous reports of MTX-induced pulmonary toxicity, mainly acute interstitial pneumonitis. It is estimated that acute pulmonary toxicity develops in 1–8% of patients that receive methotrexate treatment for rheumatologic conditions, but several reports suggest that the incidence is as high as 33% [9].

The pathophysiology of methotrexate-associated pulmonary toxicity is not fully understood. Oxidative stress significantly

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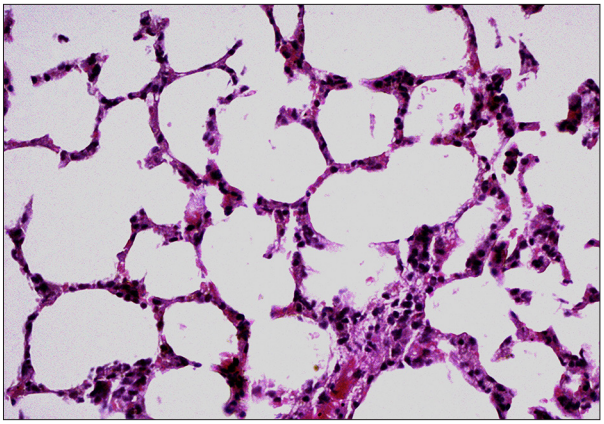

Figure 5. Normal lung tissue architecture in control rats.

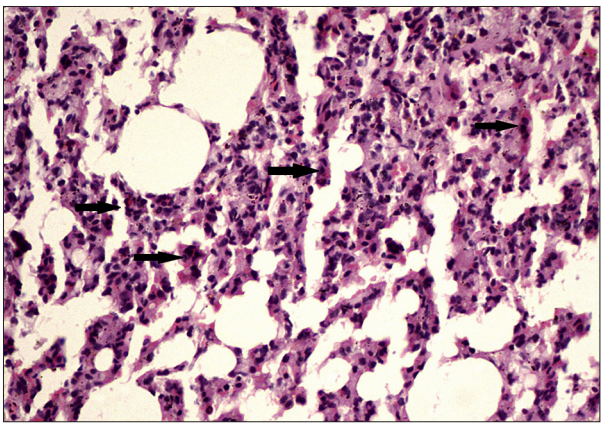

Figure 6. Alveolar epithelial damage, edema, and polymorphonuclear leukocyte infiltration (arrowheads) and chronic inflammatory cell infiltration in lung tissue from rat(s) treated with methotrexate alone.

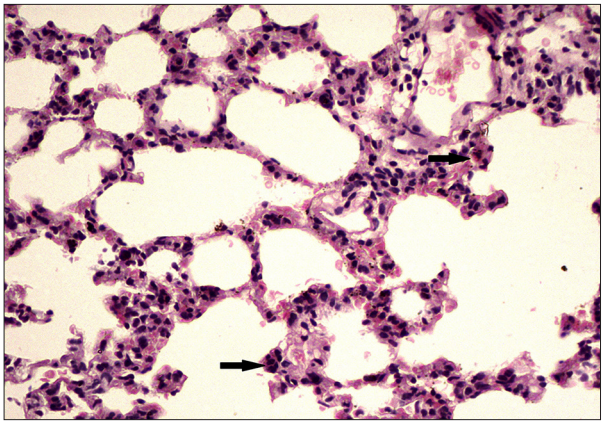

Figure 7. Significant reduction in alveolar epithelial damage, edema, polymorphonuclear leukocyte infiltration (arrowheads) and chronic inflammatory cell infiltration in rats pretreated with carvacrol.

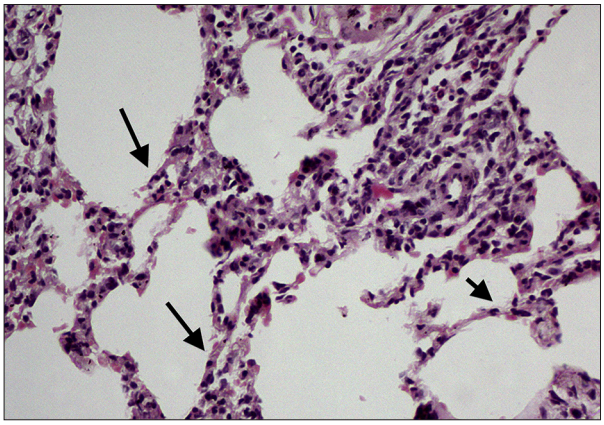

Figure 8. Reduction in alveolar epithelial damage, edema, polymorphonuclear leukocyte infiltration, and chronic inflammatory cell infiltration in lung tissues from rats pretreated with pomegranate extract.

contributes to the development and progression of various diseases, but antioxidants play a key role in protecting against oxidative damage [31]. MTX has anti-inflammatory and immunosuppressive properties because it facilitates the production of reactive oxygen species (ROS) [32]. ROS that result from MTX treatment enhance its effectiveness as a medication, as well as its toxicity [32,33]. Experimental studies have demonstrated that there is an increased production of oxygen free radicals following MTX treatment, and these free radicals might lead to mitochondrial impairment [10]. MTX was demonstrated to stimulate neutrophils and increase the amount of hydrogen peroxide, leading to the release of free radicals that trigger cell damage [32]. MTX-induced toxicity activates an inflammatory response and significantly increases the production of pro-inflammatory cytokines [34]. MDA is a highly biologically active oxidative degradation product from membrane unsaturated fatty acids. As such, MDA serves as a reliable biomarker of lipid peroxidation [34]. MTX administration results in increased MDA levels in the blood, liver, and kidneys [35,36].

In this study, MTX significantly increased MDA levels in rat serum and lung tissue. Pretreatment with CRV or PE significantly ameliorated MTX-induced lipid peroxidation, as decreased MDA levels were observed serum and lung tissue. Increased lipid peroxidation may be induced by the direct or indirect effects of elevated ROS following MTX-induced injury. Our results suggest that MTX injury may result from oxidative damage, and that both intraperitoneal CRV and PE delivered via orogastric gavage exert a protective effect against MTX-induced oxidative lung damage.

A diet rich in phytochemicals and antioxidants helps prevent certain diseases [37]. Natural compounds and derivatives that are rich in antioxidants reduce the adverse effects resulting from the toxicity of many chemicals [38]. Searching for and exploiting natural antioxidants, especially those that originate from plants, has greatly increased in recent years, as certain synthetic antioxidants are suspected to be carcinogenic [39]. Oregano water,

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a hydrosol of oregano, has been used as a Turkish folk remedy to enhance liver health. *In vitro* studies revealed that carvacrol possesses several therapeutic characteristics, including anti-inflammatory, antithrombotic, and anti-carcinogenic properties, in both lung and breast tissues [40]. The findings of this study support that CRV is useful in preventing MTX-induced lung injury.

In traditional medicine over the centuries, pomegranate has been regarded as a food with many beneficial and healing properties. Researchers have investigated the pharmacological mechanisms responsible for the medicinal properties of PE [41]. Previous studies demonstrated that PE has strong antioxidant activity [42]. Our findings also support that PE has antioxidant properties that might be effective against pulmonary oxidative damage caused by MTX use. Treating MTX-induced pulmonary toxicity requires immediately discontinuing MTX and administering high-dose methylprednisolone (1 mg/kg/day) and providing supportive care [43]. Hence, the potential for developing a more conservative therapy against pulmonary toxicity triggered by MTX therapy is exciting. Our findings suggest, for the first time, that CRV and PE are protective against MTX-induced lung damage. Specifically, the oxidative damage caused by MTX is suppressed by CRV and PE in rat serum and lung tissues. These results are also supported by histopathological observations.

Conclusions

A single dose of 20 mg/kg MTX is sufficient to trigger oxidative stress in rats, leading to MTX-induced lung injury. Carvacrol and PE reduces ROS and may enhance the body's intrinsic antioxidant system. Modulating oxidative stress with CRV and PE may be effective against lung toxicity caused by MTX therapy. However, further research is necessary to understand the exact mechanisms by which CRV and PE help prevent lung damage. To the best of our knowledge, this is the first study reporting that CRV and PE are beneficial in protecting against MTX-induced lung toxicity, possibly by scavenging free radicals and boosting the body's endogenous antioxidant system to help restore normal lung histology.

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Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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