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" *Thymoquinone improves methotrexate-induced lung injury in adult female albino rats through antioxidant and anti-inflammatory mechanisms* "

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ABSTRACT:

Background: Methotrexate, folic acid antagonist, is a widely used anticancer drug. Many cancers and inflammatory illnesses are treated with it because of its antiproliferative properties. Despite its therapeutic benefits, it produces toxic effects on various organs. Lung toxicity is a potential side effect of methotrexate. Thymoquinone is an oil extract of nigella sativa plant exhibiting great usage nowadays in medical field and attracting researchers due to its antioxidant, anticancer and anti-inflammatory characteristics. Aim of study: To investigate the potential protective role of thymoquinone against methotrexate-induced lung toxicity. Material and methods: sixteen rats were divided randomly into 4 groups (4 rats per group). GroupI received no treatment. GroupII received single dose methotrexate (20 mg/kg). GroupIII and GroupIV received thymoquinone 5 and 10 mg/kg/day for 11 days respectively. Biochemical parameters were done including Malondialdehyde, total glutathion, total antioxidant capacity, superoxide dismutase, and tumor necrosis factor α. Histopathological examinations with Haematoxylin & Eosin and Masson´s trichome stains were done. Results: In comparison to other groups, GroupII's exhibited significantly lower levels of tGSH, TAC, and SOD and significantly higher levels of MDA and TNFα. Groups III and IV exhibited significantly higher levels of SOD, tGSH, and TAC and significantly lower levels of MDA and TNFα, in comparison to groupII. Group II's hematoxylin and eosin sections revealed peribronchial lymphocytic aggregation, thicker alveolar walls, and congested capillaries. Groups III and IV's lung histology was almost normal. GroupII displayed peribronchial fibrosis shown by Masson's trichome stain, whereas GroupIII and GroupIV displayed minor interstitial tissue fibrosis

Key words: histopathology, oxidative stress, pulmonary toxicity, reactive oxygen species

Introduction:

Methotrexate (MTX), an antineoplastic drug, is a folic acid antagonist. It functions by lowering the formation of dihydrofolate reductase and reducing levels of tetrahydrofolate, producing shortage of both proteins and thymidylate with stoppage of DNA and RNA synthesis and ultimately cell death as a result. **(Funk et al., 2014 and Singh et al., 2019).**Because of its antiproliferative effects, MTX is widely used for treatment of many types of cancers including lung cancer, sarcoma, acute lymphoblastic leukemia, breast cancer, and bladder cancer. It is one of the most prominent broad spectrum immunomodulatory therapies for many illnesses as rheumatoid arthritis, psoriasis, and multiple sclerosis **(Gossec et al., 2020 and Smolen et al., 2023).**

The extreme toxicity of MTX typically limits its therapeutic use. Liver, kidney, brain, and lung injuries are potential side effects of MTX usage demonstrated by many previous studies **(Ohbayashi et al., 2014)**.

Specifically, pulmonary toxicity is a frequent side effect that can require stopping treatment **(Ohbayashi et al., 2010)**. It was estimated that the incidence of lung illness caused by MTX is roughly 7.6% **(Zaki et al., 2021)**. In 1969, the first occurrence of lung toxicity was documented with the administration of high dosages of MTX to children with acute lymphoblastic leukemia **(Clarysse et al., 1969)**.

By Research published in 2014, it was found that MTX therapy -low and high doses- elevates the risk of lung diseases. Pneumonia and interstitial lung diseases were seen in the majority of patients treated with MTX **(Conway et al., 2014)**. **Zisman et al. (2001)** reported immediate pulmonary toxicity, even at modest dosages, during MTX treatment that can develop quickly into a potentially deadly disease.

Keeping a close watch on MTX-induced lung damage, pulmonary fibrosis is the most noticeable symptom **(Fragoulis et al., 2019)**. Pulmonary fibrosis is a degenerative condition in which mesenchymal cells and extracellular matrix (ECM) replace the typical alveolar gap. The exact cause of pulmonary fibrosis is yet unknown. Lung injury is subjected to different substances that cause oxidative damage and inflammation **(Elkhouly et al., 2012 and Holm Nielsen et al., 2019)**.

In addition to fibrosis, there are acute interstitial pneumonitis, interstitial infiltrations, common alveolar damage, and perivascular inflammation **(Ohbayashi et al., 2014 and Dawson et al., 2021)**. Symptoms, like cough, fatigue, high temperature, and dyspnea, may present from a few days to more than a year after the beginning of MTX treatment and for several weeks after MTX stoppage **(Vinay et al., 2022)**.

To identify the mechanism causing MTX-induced lung injury, several mechanisms have been investigated. Oxidative stress is one of these processes. MTX generates lipid peroxidation, diminishes glutathione levels, and tampers with the antioxidant defense enzymes. Reduced antioxidant defenses increase the generation of reactive oxygen species (ROS), which cause interstitial and alveolar fibrosis as well as parenchymal lung damage **(Mohamed et al., 2019 and Althagafy et al., 2023)**. The inflammatory response of MTX is another potential mechanism of MTX-induced lung injury. Interleukin-1beta (IL1β) and tumor necrosis factor alpha (TNF-α), which are indices of an inflammatory reaction, are enhanced by MTX. Furthermore, MTX overdose may result in release of proinflammatory cytokines **(Althagafy et al., 2023)**.

This reveals that treatment with anti-inflammatory and antioxidant drugs may be effective in the prevention of pulmonary toxicity induced by MTX **(Kisaoglu et al., 2013).**

People have looked for natural materials with the ability to cure illnesses since ancient times. Traditional and alternative medicine have made extensive use of medicinal plants, which seem to offer pharmacological benefits. Phytochemicals, which include terpenes, glycosides, alkaloids, saponins, and terpene-like substances, have been linked to the medicinal properties of plants and herbs **(Akhtar et al., 2020)**.

Nigella sativa (NS), which is also known as black seed, is a traditional medicinal herb commonly used in various alternative medicine systems for long time in different cultures. It has been used for treatment of many illnesses such as asthma, hyperglycemia, hypotension, bronchitis and eczema **(Ali and Blunden, 2003 and Entock et al., 2014)**.

The biological activities of Nigella sativa are being studied, and a wide range of activities, including antibacterial, antihypertensive, analgesic, gastroprotective, antidiabetic, anti-inflammatory, immunomodulatory, and anticancer properties, have been reported **(Ahmad et al., 2013)**.

Thymoquinone (TQ), which is an active biological component of NS seed oil, is responsible for the majority of the biological activities that have been documented in NS seeds **(Darakhshan et al., 2015)**. It has been established that TQ possesses a variety of beneficial qualities, including anticonvulsant, antimicrobial, anticancer, anti-histaminic, anti-diabetic, cardioprotective, anti-inflammatory, and antioxidant effects **(Taysi et al., 2022)**.

Kohandel et al. (2021) stated that Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), nuclear factor kappa-light-chain-enhancer of activated B (NF-Kβ), cyclooxygenase-2 (COX2), nuclear factor erythroid 2-related factor 2 (Nrf2), and cytokine

release are some of the molecular pathways through which TQ exerts its anti-inflammatory and antioxidant effects.

Numerous academic publications have documented the antioxidant qualities of TQ in relation to mitigating oxidative stress. TQ stimulates the synthesis of cytoprotective enzymes, which aid in preventing oxidative stress-related cell damage. TQ-based activation of cytoprotective enzymes, including glutathione peroxidase (GPX), and H2O2 scavenges the highly reactive oxygen by upregulating mRNA **(Mahmoud and Abdelrazek, 2019)**. Glutathione production induced by TQ simultaneously inhibits superoxide radical production. Also, it may initiate the redox cycle through participation of various enzymes **(Demir et al., 2020)**.

Many researchers **(Mahmoud and Abdelrazek, 2019)** reported that due to antioxidant properties of TQ, it may shield cells from oxidative stress-related damage. Oxidative stress causes the cell's molecules to be destroyed, which leads to cell damage and death.

Based on these findings, the current study was constructed to evaluate lung damage resulting from a high dose of MTX. We looked into the postulated underlying processes of lung affection and how TQ might modulate this kind of affection through biochemical and histopathological investigations.

2. Materials and methods

2.1. Drugs and chemicals

MTX was purchased from Hikma Specialized Pharmaceuticals (Badr City, Cairo, Egypt) and TQ from Acro’s Organics (Belgium, China).

2.2. Experimental animals

The study was conducted under the permission of the Port Said University Ethical Committee with an approval number (MED (1/3/2023) s.no (78) ANA_ 002).

The study was performed on sixteen female albino rats weighing 150-250 grams. Rats were purchased from the animal house faculty of veterinary Mansoura University. They were housed in environmentally controlled rooms with spacious wire mesh cages at room temperature and good ventilation and kept for at least seven days to acclimatize to their new environment. They received freshwater food ad libitum and rodent pellets, and they were handled only by the researchers throughout all the steps of the experiment.

2.3. Experimental design

The rats were randomly allocated into 4 groups, with 4 in each group. Group I (control) received an intraperitoneal injection of 0.5 ml of saline. Group II (MTX) received an intraperitoneal (IP) injection of a single dose of methotrexate (20 mg/kg) **(Zaki et al., 2021)**. Group III (MTX + TQ1) received the same dose of methotrexate and IP injections of thymoquinone (5 mg/kg/day) for 11 days **(Boskabady et al., 2021).** Group IV (MTX + TQ2) received the same dose of methotrexate and IP injections of thymoquinone (10 mg/kg/day) for 11 days **(Boskabady et al., 2021)**.

2.4. Samples collection

After 11 days of thymoquinone treatment, animals were euthanized with 50 mg/kg thiopental sodium **(Altun et al., 2019)** and sacrificed by cervical dislocation. Both lungs were harvested for biochemical and histological examination.

2.5. Biochemical assessment

Oxidative stress markers were assessed using lung homogenates. The lung homogenate was prepared in a receptor isolation buffer with a tissue grinder, and centrifuged at 12,000×g for 20 min at 2°C. The supernatant was recuperated, kept on ice, filtered, diluted, and mixed with E2-A-FITC reagent, and incubated at room temperature for 30-60 min. The mixture was centrifuged, the bound reagent was retained on the membrane filter, and the free reagent passed through it. The fluorescence associated with the membrane or in the eluate was measured using a microplate reader **(Gagne, 2014)**. After preparation of the lung homogenate, the activities of MDA (malondialdehyde), SOD (superoxide dismutase), TNF-a (tumor necrosis factor alpha), tGSH (total glutathione), and TAC (total antioxidant capacity) were measured.

2.6. Histological assessment

2.6.1. Hematoxylin and Eosin

Sections of lung tissue were prepared as described by Suvarna et al. 2019. Lung tissues were sliced to 3-4 mm thick, fixed in 10% neutral buffered formalin, dehydrated using ethanol in graded concentrations, cleared in xylene, and embedded in paraffin wax. A microtome was used to section the paraffin blocks at (4-5μm) thickness and the sections were dyed with Hematoxylin and Eosin (H&E) stain.

2.6.2. Masson Trichrome

For collagen fiber visualization, lung sections were dyed with Masson Trichrome stain **(Jones et al., 2008)**. Sections were deparaffinized, rehydrated, and washed in distilled water.

They were stained in Weigert's iron hematoxylin for 10 min and washed. Then stained in Biebrich scarlet-acid fuchsin solution for 15 min and washed in distilled water. They were differentiated in Phosphomolybdic-Phosphotungstic acid solution, transferred, without a rinse, to an aniline blue solution, and then rinsed in distilled water. They were differentiated in 1% acetic acid solution, washed in distilled water, dehydrated rapidly through ethyl alcohol, and cleared in xylene.

Lung was evaluated under light microscope with a digital camera (BX53F, Olympus, Tokyo, Japan) for histopathological examination by a pathologist in a blinded manner.

2.7. Statistical analysis

Data were collected, revised, and edited on a computer using the Statistical Program for Social Science version 25 (SPSS 25). Mean ± Standard deviation were used for descriptive data, and the one-way analysis of variance (ANOVA) to evaluate the results statistically. A P value <0.05 was considered statistically significant.

3. Results

3.1. Biochemical results

Levels of MDA showed a significant increase in group II (MTX) compared to other groups. Meanwhile, in groups III and IV (TQ1 and TQ2), the levels decreased significantly compared to group II (MTX) and showed no significant difference with control group (table 1).

The antioxidant capacity of lung tissue was evaluated in this study using SOD, TAC, and GSH parameters. The administration of MTX was found to result in a significant decrease in their levels. Conversely, the administration of TQ demonstrated an improvement in SOD, TAC, and GSH levels. Their values in group II (MTX) significantly decreased compared to other groups, while in groups III and IV (TQ1 & TQ2) they exhibited a significant increase compared with group II (MTX) and a significant decrease compared to control group (table 1).

As shown in table 1, TNFα as an inflammatory marker was increased in group II (MTX), when compared to other groups and decreased significantly in groups III and IV (TQ1 and TQ2) when compared to group II (MTX) but still significantly increased compared to control group.

There is no significant difference in all biochemical parameters between group III (MTX+TQ1) and group IV (MTX+TQ2) (table 1).

3.2. Histopathological results

3.2.1. Haematoxylin and Eosin stain

Lung sections exhibited normal histological structure with normal alveoli and bronchioles in group I (control) (figure 1). In group II (MTX), lung sections revealed deteriorating histological changes as presented by lymphocytic aggregation in the peribronchial tissues and interstitial fibrosis. Alveoli showed thickened walls with narrow lumens and various areas of atelectasis. Blood vessels were congested and dilated (figure 2). Treatment with TQ in groups III and IV (MTX+TQ1 and MTX+TQ2) remarkably alleviates the histopathological changes caused by MTX. Lung sections showed near normal histological appearance with normal alveolar wall thickness and increased alveolar lumen (figures 3, 4).

3.2.2. Masson Trichome stain:

Histological examination of lung sections stained with MT showed no fibrosis around bronchiole nor in alveolar wall in group I (control) (figure 5). Lung sections from group II (MTX) showed marked fibrosis especially around the bronchiole and in the alveolar wall (figure 6). In groups III and IV, lung sections showed mild fibrosis mainly around the bronchioles (figures 7, 8).

TABLE (1): Levels of biochemical parameters in lung tissues among all groups, presented in mean and standard deviation (mean ± SD)

Biochemical parameters (mean ± SD)					
Groups	MDA (nmol/g. tissue)	SOD (U/g. tissue)	TAC (mmol/g. tissue)	GSH (U/g. tissue)	TNF-α (Pg/mg proein)
Group I (Control)	15.0±1.47	197.6 ± 18.6	1.92 ± .15	129.38 ± 5.8	129.6 ± 8.9
Group II (MTX)	38.1 ± 3.29 ***acd**	69.93 ± 8.3 ***acd**	.77 ± .11 ***acd**	51.05 ±5.05 ***acd**	330.58 ±25.47 ***acd**
Group III (MTX+TQ1)	25.3 ± 5.39 ***b**	128.4 ±28.2 ***ab**	1.23 ± .19 ***ab**	90.3 ± 19.4 ***ab**	208.6 ± 44.37 ***ab**
Group IV (MTX+TQ2)	21.9 ± 10.4 ***b**	123.3 ±24.7 ***ab**	1.19 ± .16 ***ab**	85.5 ± 14.6 ***ab**	195.08 ±35.59 ***ab**

ANOVA test

*=The mean difference is significant at the 0.05 level ($p < 0.05$)

a= compared to control b= compared to Group II (MTX)

c= compared to Group III (MTX+TQ1) d= compared to Group IV (MTX+TQ2)

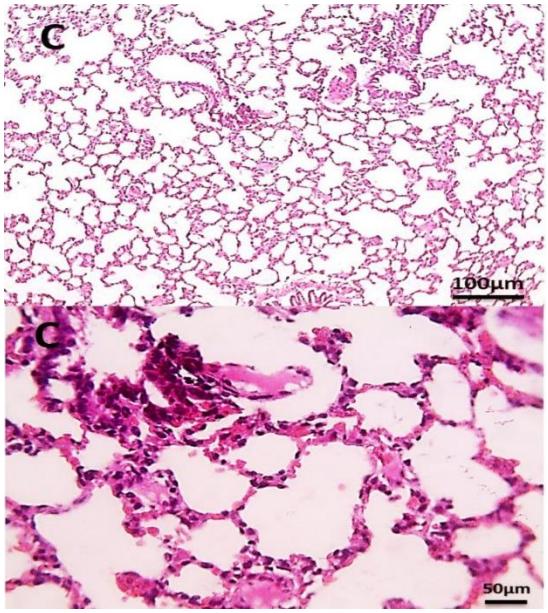

Fig.1. Microscopic pictures of H&E stained lung sections showing normal alveoli and bronchiole in control normal group. Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

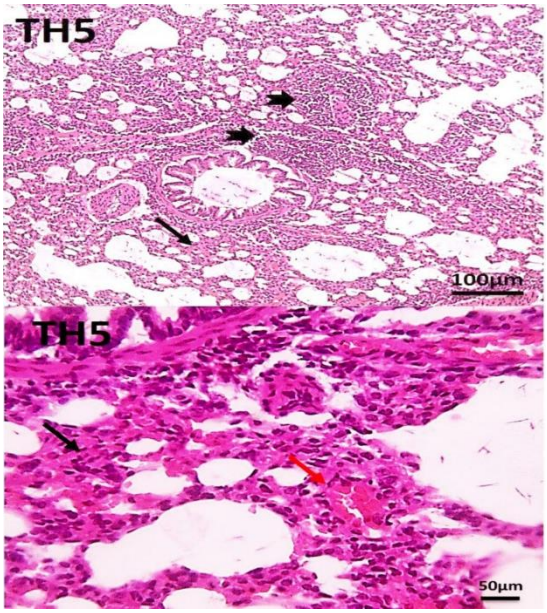

Fig. 3. Microscopic pictures of H&E stained lung sections from TH5 group showing milder lesions than observed in MTX group including: lymphocytic aggregation (thick arrow), interstitial fibrosis (thin black arrow) & congested capillaries (thin red arrow), narrowing of alveolar lumen. Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

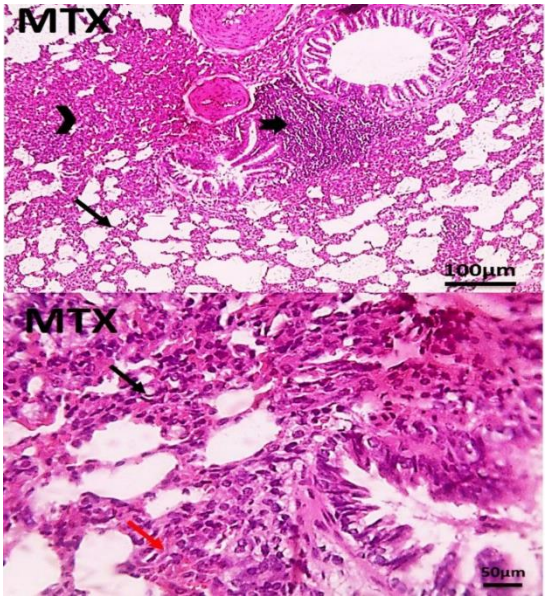

Fig. 2. Microscopic pictures of H&E stained lung sections from MTX group showing peribronchial lymphocytic aggregation (thick arrow), increased alveolar wall thickness due to interstitial fibrosis (thin black arrow) & congested capillaries (thin red arrow), narrowing of alveolar lumen with areas of atelectasis (arrowhead). Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

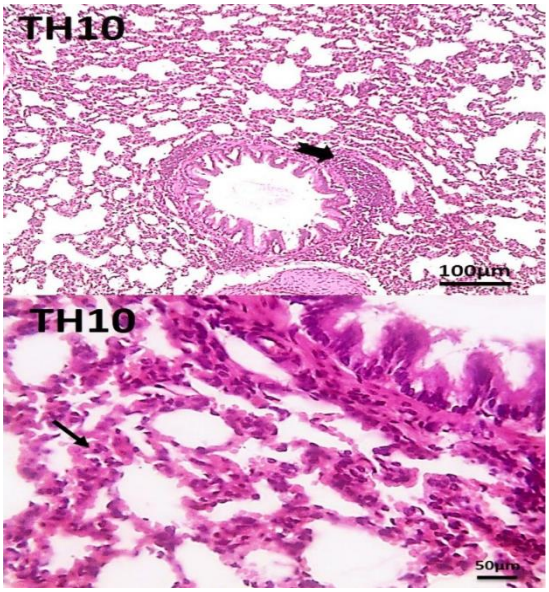

Fig. 4. Microscopic pictures of H&E stained lung sections from TH10 group showing peribronchial lymphocytic aggregation (thick arrow), markedly decreased alveolar wall thickness (thin black arrow) with increased alveolar lumen. Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

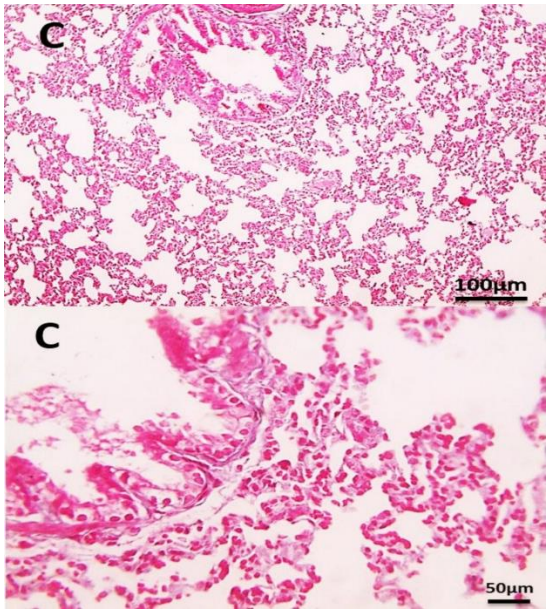

Fig. 5. Microscopic pictures of MT stained lung sections showing no fibrosis around bronchiole and in alveolar wall in control normal group. Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

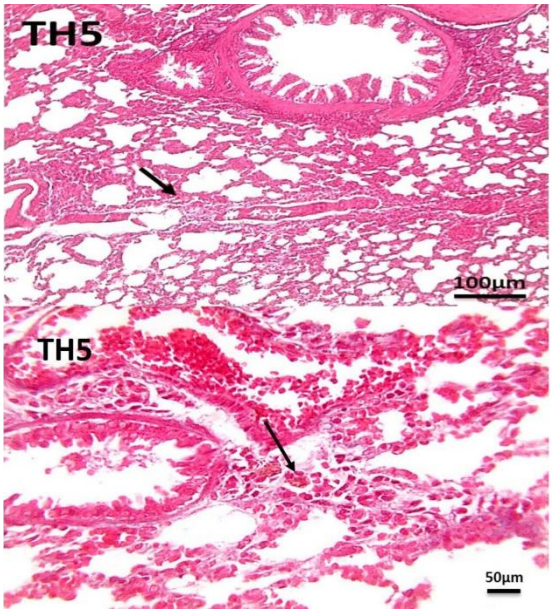

Fig. 6. Microscopic pictures of MT stained lung sections from MTX group showing marked fibrosis around bronchiole and in alveolar wall (thin black arrows). Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

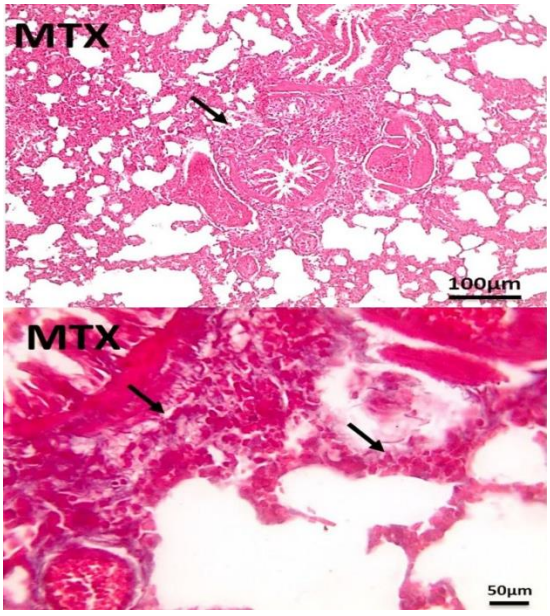

Fig. 7. Microscopic pictures of MT stained lung sections from TH5 group showing mild fibrosis (thin black arrow). Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

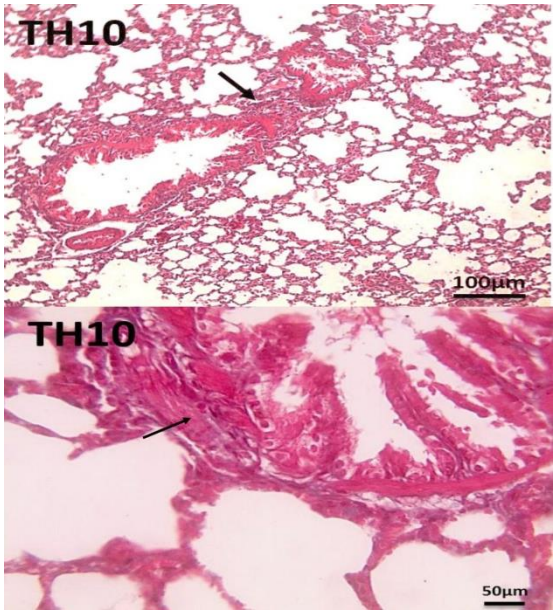

Fig. 8. Microscopic pictures of MT stained lung sections from TH10 group showing mild peribronchial fibrosis (thin black arrow). Low magnification X: 100 bar 100 and high magnification X: 400 bar 50

4. Discussion:

MTX is a common anticancer drug that demonstrates efficacy in treating several illnesses. Because of its extreme toxicity, its therapeutic use is typically restricted. Pulmonary toxicity continues to be a paramount side effect of MTX usage. Although the primary mechanism of MTX toxicity in the lungs remains to be not fully known, several studies reported that oxidative stress (OS) and inflammation are the main causes of lung injury induced by methotrexate **(Althagafy et al., 2023)**. TQ, a natural product, is recently widely used for many diseases due to its potential antioxidant, anti-inflammatory, anticancer, and antiapoptotic effects **(Kohandel et al., 2021)**.

This study was established to evaluate the protective role of TQ against MTX-induced lung injury. In this study, MTX administration caused severe lung tissue injury demonstrated by deteriorating biochemical and histopathological changes.

In the present work, MTX triggered a significant elevation of MDA levels. These results were concurrent with previous studies **(Zaki et al., 2021; Ali et al., 2022 and Demir et al., 2024)**, which stated that MTX administration results in elevated levels of MDA in rats**.**

According to **Shen et al. (2019)**, OS causes damage to nucleic acids, proteins, carbohydrates, lipids, and enzymes as a result of MTX toxicity. Biomolecules that are prone to OS include lipids. **Rafiee et al. (2020)** explained the increased level of MDA due to elevated levels of ROS which induce lipid peroxidation (LPO) and lead to the production of several reactive aldehyde derivatives, including MDA. MDA is generated from the breaking down of extremely reactive lipid hydroperoxides during lipid peroxidation, so it served as a useful indicator of oxidative stress and a suitable gauge for diseases linked to a certain lifestyle **(Sharifi-Rad et al., 2020)**.

Our study revealed low levels of SOD, TAC, and tGSH in the lung homogenate of the MTX group. These findings are similar to studies of **Mammadov et al., 2019; Zaki et al., 2021; and Demir et al., 2024,** as they reported that MTX administration in a single high dose (20 mg/kg) reduces the levels of these biochemical parameters in lung tissue.

SOD, TAC, catalase (CAT), and tGSH are significant biochemical markers that are used to evaluate the antioxidant capacity of tissue. These antioxidant enzymes are vital for responding to stressful conditions and scavenging ROS in different cell compartments **(Jena et al., 2023)**. SOD served as the first line of defense, preventing cellular redox equilibrium and combating free radicals **(Wang et al., 2018)** and has a vital role in cell protection by preventing the conversion of superoxide radicals to hydroxyl radicals **(Ayazoglu Demir et**

al., 2023). Reduction of their levels indicates diminished and deteriorating the antioxidant capacity of the lung tissues **(Zaki et al., 2021).**

Arpag et al. (2018) announced that the major cause of MTX toxicity is OS and explained it as MTX alters the antioxidant defense enzymes, consumes glutathione content, and induces lipid peroxidation. Diminution of the antioxidant defense system results in the production of ROS.

Excessive ROS production causes severe damage to the tissue. ROS react with nitrogen bases of DNA and deoxyribose and form 8-hydroxy-2' deoxyguanosine (8-OHdG). This compound is a main cause of mutations and cancer development. Additionally, ROS induce peroxidation of lipid, resulting in damaged integrity of membrane. Also, elevated levels of ROS reduce the amount of tGSH and cause the intracellular environment to become more oxidant **(Holm Nielsen et al., 2019 and Juan et al., 2021)**.

Oxidative stress is defined as the imbalance between the oxidant and antioxidant systems. The oxidant marker (MDA) was high, while the antioxidant markers (SOD, TAC, and tGSH) were low in MTX-treated rats **(Atug Ozcan et al., 2014)**. Our results indicate that the oxidant-antioxidant balance in the lung tissue of the MTX group declined in favor of the oxidants.

This study demonstrated that a single dose of MTX caused a significant rise of TNFα. This outcome agrees with the findings of **Mammadov et al. (2019)** and **Ali et al. (2022)**, who observed elevated TNF-α in rats treated with MTX during their works.

The MTX-treated group experienced sterile inflammation due to an increase in TNF-α. By releasing chemical mediators, today's neutrophils cause tissue damage and production of ROS. TNF-α is a proinflammatory cytokine that is secreted in greater amounts in response to elevated MTX dosage and IL-1 level. TNF-α plays a pivotal role in the pathophysiology of oxidative stress and elevates ROS **(Aya et al., 2015)**.

Inflammation was often assessed by measuring proinflammatory cytokine levels, such as TNF-α, which was markedly elevated in the lung homogenate of rats treated with MTX. Monocytes and macrophages produce TNF-α. These cells release these cytokines in response to a variety of stimuli, such as activated T cells, immunological complexes, microbial products, and the combined action of other cytokines. They induce leucocytes to migrate from capillaries and gather in areas of damage or infection. The proinflammatory and anti-inflammatory cytokines were in balance in the normal tissues. In MTX-induced lung damage, this equilibrium shifted in favor of the proinflammatory cytokines **(Arpag et al., 2018)**. These results align with the outcomes of our experiment.

Elsawy et al. (2021) provided an explanation for elevated TNF-α, stating that MTX-induced ROS formation causes NF-κB expression to be upregulated. Proinflammatory cytokines

including TNF-α, IL-6, and iNOS enzymes are released when NF-κB, a transcriptional factor, is activated. This worsens the inflammatory response. Furthermore, the release of these proinflammatory cytokines may aid in the infiltration of immune and inflammatory cells, including macrophages and neutrophils, which may worsen oxidative stress and cellular damage by causing the production of ROS.

In TQ treated groups, in the present study, the oxidant-antioxidant balance was apparently restored. It is suggested by decreased level of MDA and increased levels of SOD, TAC, and tGSH in lung tissue. These results agree with **Sakineh et al. (2023)** who reported that using TQ in a dose of 10 mg/kg for 10 days -as an antioxidant- in rats received MTX showed a significant reduction in MDA level and a significant increase in TAC level in lung tissue.

Also, **Alzohairy et al. (2021)** resulted in that thymoquinone can protect the lungs from oxidative damage by increasing the antioxidant enzyme levels while examining the protective role of TQ on rats exposed to benzopyra-induced lung injury.

It has been reported earlier that TQ significantly reduces the level of MDA, increases SOD activity, scavenges free radicals, and maintains the activity of various antioxidant enzymes (**Pourgholamhossein et al., 2016)**.

Our work shows that treatment with TQ (5&10mg/kg) considerably decreased the blood level of TNF-α. **Alzohairy et al. (2021)** reported that TQ reduced the raised TNF-α levels in rats exposed to lung damage.

Umar et al. (2012) observed that during the course of 21 days, oral administration of 5 mg/kg body weight of TQ to Wistar rats resulted in a significant decrease in the levels of various pro-inflammatory mediators, including IL-1β, IL-6, TNFα, and IFNγ.

One of the possible mechanisms of antioxidant activity of TQ was explained by **Kundu et al. (2014)** who stated that the increased expression of heme-oxygenase 1 (HO-1) in human keratinocytes (HaCaT) is responsible for the antioxidant and anti-inflammatory effects of TQ. This is achieved by activating nuclear factor (NF)-erythroid2-(E2)-related factor-2 (Nrf2) through the phosphorylation of protein kinase B (PKB/Akt) and cyclic AMP-activated protein kinase-alpha (AMPKalpha).

The results of our biochemical tests obtained from all study groups are consistent with the histopathological findings. Our histopathological results exhibited destruction of the normal histology of lung tissue in MTX group. H&E stained sections showed massive lymphocytic aggregation mainly around bronchi, thickening of alveolar wall with narrowing of their lumen, interstitial fibrosis and congested blood vessels. These results are in concurrent with **Ahmed et al. (2021)** who evaluate the histological effects of MTX on the lung of albino rats and stated that administration of MTX at 5 mg/kg for 5 days resulted in thickening of interalveolar septa and intracellular inflammatory infiltration.

In agreement with our results, **Zaki et al. (2021)** and **Sakineh et al. (2023**) used MTX in a dose of 20 mg/kg single dose in albino rats and noted cellular inflammatory infiltrations, thickened septa between alveoli, congested blood vessels, and extravasated blood in MTX-treated rats.

These changes were explained by different experimental models proposing that oxidative stress and inflammation play an important role in MTX-induced pulmonary destruction **(Kahraman et al., 2013).**

According to **Sazlar et al. (2012),** cellular infiltrations and blood vessel congestion are caused by the endothelial barrier being destroyed, which interferes with the integrity of the vessels and increases their permeability. This causes an inflammatory response by activating OS-sensitive signaling pathways. The intrabronchial cell debris and the toxic effects of MTX on the bronchiolar lining were closely linked **(Ikeue et al., 2019)**.

Increased inflammatory cytokine production from injured pulmonary cells leads to autoimmune responses and hypersensitivity. Moreover, by drawing mononuclear cells to the area of congestion, red blood cell extravasation might cause inflammation **(Ahmed et al., 2021)**. Our results also provided additional support for these views.

Masson trichome stain of lung section in MTX group in our work revealed marked fibrosis around bronchioles and in the alveolar wall. In accordance with our outcomes, **Zaki et al. (2021)** stated that lung fibrosis was observed in MTX treated group and recorded that the content of collagen fiber in lung tissue was threefold high and explained this as a result of oxidative stress and sterile inflammation caused by MTX.

In addition, **Wanyy, (2011)** informed that OS and inflammatory process play a pivotal role in development of lung fibrosis. Like our study, previous studies showed that a single dose of MTX caused a significant elevation in pro-inflammatory TNFα levels. This mediator with other inflammatory mediators obviously has potent fibroblast-stimulating properties, which boost collagen deposition and ECM protein synthesis **(Varga and Abraham, 2007)**. Reduced antioxidant defense system production leads to the generation of reactive oxygen species (ROS), which cause parenchymal lung damage and interstitial and alveolar fibrosis **(Arpag et al., 2018)**.

In alignment with our study, **Alzohairy et al. (2021)** reported that TQ administration reduces the MTX-induced lung fibrosis, as confirmed by Masson trichome stain. We can explain this reduction in collagen deposition caused by TQ due to its anti-inflammatory and antioxidant activities by lowering the levels of ROS and inflammatory mediators, which are the main causes of the occurrence of fibrosis.

Antioxidants postpone autoxidation by scavenging the species that start peroxidation processes and produce ROS. The balance of the body's natural antioxidant defense system

and regulated ROS production are crucial for preventing pathogenesis. Natural antioxidants are abundant in natural substances and their constituent active chemicals. They impede disease by scavenging ROS **(Nawar, 1996; Alzohairy et al., 2021 and Dragoev, 2024).**

5. Conclusion and Recommendation

In conclusion, our finding provides information that MTX in high dose induced severe lung injury through antioxidant and inflammatory processes. With the use of TQ, the normal structure of lung and normal biochemical analysis were restored with residual affection. TQ exerts its protective effect through its antioxidant and anti-inflammatory activities.

We recommend more researches to declare more benefits of thymoquinone as a natural product and investigate different dosages of it.

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