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Original Article
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Evaluation of the Protective Effect of Citral, Silymarin, and Thymoquinone on Methotrexate-Induced Lung Injury in Rats

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Objectives: Several studies have reported that methotrexate is an anti-cancer and immunosuppressive drug leading to lung injury. Therefore, the present study aimed to investigate the protective effects of silymarin, citral, and thymoquinone on methotrexate-induced pulmonary toxicity.
Methods: Forty-eight rats were divided into six groups, including healthy, Methotrexate, and drug carrier control groups and silymarin, citral, and thymoquinone treatment groups. At the end of the experiment, the studied rats were anesthetized and sacrificed by CO_2. Lung tissue samples were isolated to measure the antioxidant activity and histopathological evaluation.
Results: In the thymoquinone treatment group, the concentration of total antioxidant capacity and Malondialdehyde increased and decreased significantly, respectively, compared to the methotrexate group. The histopathological evaluation of the lung of the methotrexate group showed hemorrhage and congestion, the nodule-like accumulation of mononuclear inflammatory lymphocytes around the blood vessel, a small number of neutrophils around the blood vessel, and the inflammatory cells around the small vessels. However, no significant pathological alterations were observed in the treatment groups, especially the thymoquinone treatment group.
Conclusion: Thymoquinone has the greatest protective effect on methotrexate-induced lung injury, probably due to its antioxidant effect.

Keywords: thymoquinone, pulmonary toxicity, citral, silymarin, methotrexate

INTRODUCTION

Methotrexate is used to treat pediatric hematological malignancies [1] and autoimmune diseases such as rheumatoid arthritis [2]. Clinical and preclinical studies have examined the side effects of methotrexate on the kidney [3], liver [4], heart [5], intestinal [6], and lung [7]. The side effects of methotrexate on the lung include hypersensitivity pneumonitis, interstitial fibrosis, and pulmonary nodules [8]. Hypersensitivity, immune modulation, idiosyncrasy, and direct lung toxicity play key roles in the development of such complications [9]. Oxidative stress and lipid peroxidation are implicated in methotrexate-induced lung injury [10]; methotrexate also causes oxidative damage in the kidney, liver, and testes [11-13]. Although pulmonary complications are critical, few studies have been performed to investigate these complications.

Citral, the most crucial constituent of *Litsea cubeba* and *Cymbopogon citratus* oil [14], has antioxidant effects [15, 16]. Nakamura et al. (2003) [17] reported that the activity of glutathione *S*-transferase (GST) is induced by citral, while Shen et al. (2015) [18] showed that it inhibits lipopolysaccharide (LPS)-induced lung damage. The extract of *Silybum marianum* contains several flavonolignans known collectively as silymarin [19], which has antioxidant, immunomodulatory, and antifibrotic

effects. It also prevents the lipid peroxidation of cell membranes by affecting intracellular glutathione levels [20] and reduces bleomycin-induced lung damage [21].

Thymoquinone, the major component of black seed oil with antioxidant and antiinflammatory effects, can reduce LPS-induced lung damage [22, 23]. Based on these findings, the present study aimed to investigate the protective effects of citral, thymoquinone, and silymarin against methotrexate-induced lung injury.

MATERIALS AND METHODS

Forty-eight male Sprague-Dawley rats with uniform weight were purchased from Shiraz University of Medical Sciences. Acclimation was performed by keeping the rats in the laboratory animal house center of the Faculty of Veterinary Medicine for 2 weeks before the experiment. The rats had free access to water and pelleted food during the acclimation and experimental periods and were kept under 12-hour light/12-hour dark conditions. The animal handling procedures were conducted in conformity with the international guidelines for the care and use of experimental animals and approved by the local Research Ethical Committee at Shiraz University, Iran (Approval No.: 1GCB5M348200). The rats were randomly divided into the following six groups:

A: Healthy control group, without therapeutic intervention

B: Methotrexate group, intraperitoneal injection of 20 mg/kg methotrexate on the third day of the study, (n = 7) [7]

C: Vehicle group, injection of vehicle (dimethyl sulfoxide + water) during all 10 days of the study + 20 mg/kg methotrexate on the third day of the study (n = 7)

D: Citral (Merck, Germany, 96%) treatment group, intraperitoneal injection of 25 mg/kg citral during all 10 days of the study + 20 mg/kg methotrexate on the third day of the study (n = 7)

E: Silymarin (Sigma-Aldrich, Germany, 95%) treatment group, intraperitoneal injection of 50 mg/kg silymarin during all 10 days of the study + 20 mg/kg methotrexate on the third day of the study (n = 7)

F: Thymoquinone (Sigma-Aldrich, Germany, 98%) treatment group, intraperitoneal injection of 10 mg/kg thymoquinone during all 10 days of the study + 20 mg/kg methotrexate (i.p.) on the third day of the study (n = 7)

On the 10th day, the rats were anesthetized with ketamine (80 mg/kg i.p, alfasan) and xylazine (10 mg/kg i.p, alfasan) and then sacrificed by CO_2 inhalation. Lung samples were collected for histopathological and antioxidant activity evaluation.

1. Lung tissue extraction

One gram of heart tissue was transferred to a test tube, 5 mL of phosphate buffer added, and the mixture of tissue and phosphate buffer homogenized using a homogenizer. The resulting suspension was centrifuged (at 2,500 rpm for 5-10 min), and the supernatant was collected and used to measure the desired parameters.

2. Histopathological evaluation

For this purpose, the rats were immediately sacrificed, and their tissue samples were collected as 1 cm × 1 cm × 1 cm blocks and placed in 10% neutral buffered formalin. After 48-hour fixation, different steps of dehydration, clarifying, and preparation of paraffin molds were performed. The tissues were cut using a microtome to obtain 4-5 μm-thick paraffin sections, which were stained with hematoxylin and eosin (H&E) and examined using a light microscope.

3. Measuring the antioxidant activity in the lung

A commercial kit (ZellBio GmbH kit, Germany) was used to determine the total antioxidant capacity (TAC). The formation of the colored product of the chromogenic substrate (tetramethyl benzidine) was monitored using a spectrophotometer (Jenway 6300 Spectrophotometer, UK) at 450 nm and represented as mmol/L. This method could determine TAC with a sensitivity of 0.1 mM (100 μmol/L). The intra- and inter-assay coefficients of variation (CVs) were below 3.4% and 4.2%, respectively. An assay kit purchased from ZellBio GmbH (Germany) was used to measure malondialdehyde (MDA) content (μmol/L; Cat. no. ZB-MDA96A). With this kit, MDA is measured based on its reaction with thiobarbituric acid in an acidic condition and at high temperature. The colored complex was measured colorimetrically at 535 nm. The sensitivity of the assay kit was 0.1 μM (inter-assay CV: 5.8%) for MDA.

4. Data analysis

The obtained data were presented as mean ± SEM. ANOVA and Tukey's post hoc test were used for data analysis. A p-value

< 0.05 was considered statistically significant.

RESULTS

1. Histopathological evaluation of the lung

1) Healthy control group

Histological examination of the lungs of the control group showed normal histological structure and architecture of the lung with sac-like pouches (alveoli). The inter-alveolar septum of the lung was usually thin, except in some areas of the lung tissue. Alveolar septum cells and pneumocytes lining the inner surface of the alveoli were normal. Further, the structure of the bronchi and bronchioles and their epithelium appeared normal and healthy. Except for mild hyperemia, no specific histopathological alterations were observed in the lung tissues of the control group rats (Fig. 1).

2) Methotrexate group

The sections stained with H&E showed severe hemorrhage and congestion in parts of the lung parenchyma tissues of this group. Additionally, an increase in the thickness of the inter-alveolar septum, a nodule-like accumulation of mononuclear inflammatory lymphocytes around the blood vessel and bronchioles and a small number of neutrophils around the blood vessels were observed (Fig. 2). No necrotic tissue, emphysema, alveolar dilation, pulmonary edema, and fibrin secretions were observed.

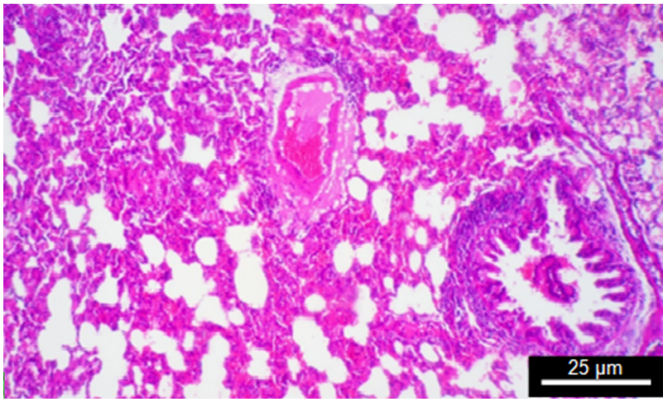

Figure 1. The structure of alveoli and pulmonary bronchioles with normal epithelial tissue and without any specific histopathological lesions in the parenchyma of the lungs of the healthy rats (H&E, 100×).

3) Vehicle group

The histological evaluation of lung parenchyma sections of this group showed an increase in the thickness of the inter-alveolar septum, mild to moderate intra-alveolar hemorrhage, mild vascular congestion, accumulation of lymphoid cells around the bronchioles, and the presence of inflammatory cells around the pulmonary vascular and intravascular aggregates of the small number of neutrophils at its margin (Fig. 3). No necrotic tissue, emphysema, alveolar dilation, interstitial fibrosis, pulmonary edema, and fibrin secretions were observed.

4) Citral group

The sections stained with H&E showed a nodule-like accumulation of mononuclear inflammatory lymphocytes around the blood vessel and bronchioles, a small number of neutrophils

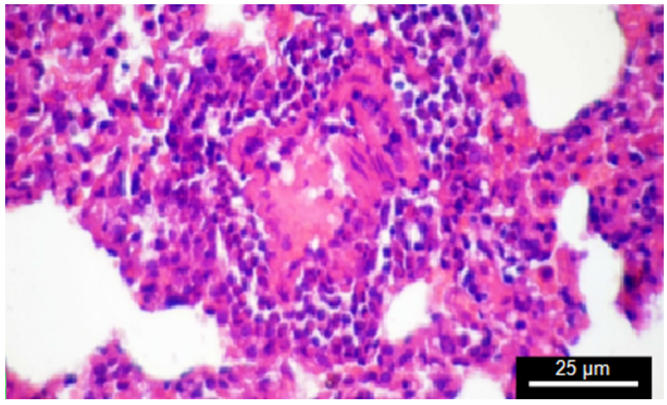

Figure 2. It shows the presence of inflammatory cells, including a large number of lymphocyte cells and a small number of neutrophils around the blood vessels of the lungs of the methotrexate group (H&E, 400×).

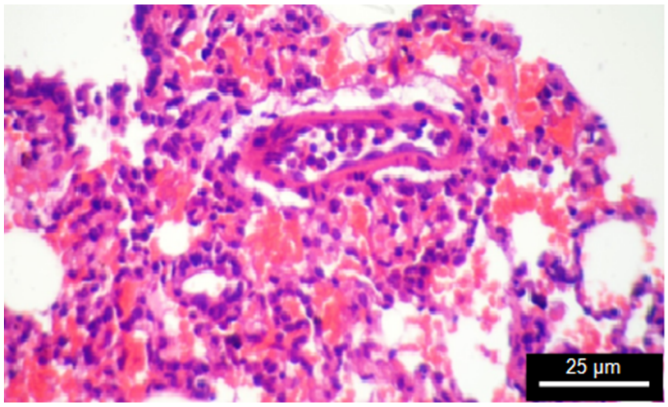

Figure 3. Hemorrhage of the alveoli and the presence of inflammatory cells, especially neutrophils, in the inner margin and around the blood vessels of the lungs of the drug carrier group (H&E, 400×).

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in the parenchyma around the vessel, a slight increase in the thickness of the inter-alveolar septum, congestion, and hemorrhage (Fig. 4). No necrotic tissue, emphysema, alveolar dilation, interstitial fibrosis, pulmonary edema, and fibrin secretions were observed in the citral group.

5) Silymarin group

An increase in the thickness of the inter-alveolar septum, healthy alveoli without any red blood cells, pulmonary edema and fibrin secretions, and inflammatory cells were observed in the lung tissue sections of the silymarin group. There was vascular congestion, lymphocyte inflammatory cells, and a small number of neutrophils around the blood vessels of the lung parenchyma (Fig. 5). No necrotic tissue, emphysema, alveolar dilation, and pulmonary fibrosis were observed.

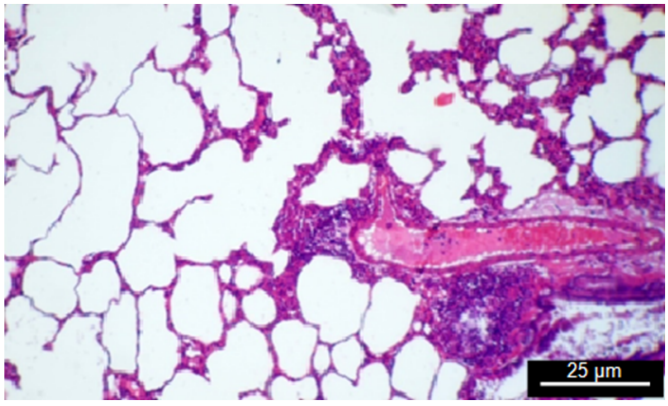

Figure 4. It shows vascular congestion and the presence of inflammatory lymphocyte cells and a small number of neutrophils around the blood vessels of the lungs of the silymarin group (H&E, 100×).

6) Thymoquinone group

The histological evaluation of the lung tissue sections of this group showed an intra-alveolar accumulation of foamy macrophages, mild to moderate intra-alveolar hemorrhage, mild congestion of the pulmonary vessels, the presence of inflammatory cells around the vessels and bronchioles, an increase in the thickness of the inter-alveolar septum, and a small number of neutrophils around the vessels (Fig. 6). No necrotic tissue, emphysema, alveolar dilation, pulmonary edema, and fibrin secretions were observed.

2. Evaluation of antioxidant parameters of lung tissue

Citral, thymoquinone, and silymarin increased the TAC by 51.50%, 63.87%, and 46.65%, respectively, compared to the methotrexate group. This increase was significant in the thymoquinone group ($p < 0.05$, Fig. 7A).

The MDA content in the citral (0.068 ± 0.004 μmole/mg protein), thymoquinone (0.062 ± 0.004 μmole/mg protein), and silymarin (0.062 ± 0.001 μmole/mg protein) treatment groups decreased compared to the methotrexate group (0.077 ± 0.002 μmole/mg protein). This reduction was significant in the silymarin and thymoquinone groups ($p < 0.05$, Fig. 7B).

DISCUSSION

The present study showed that citral, silymarin, and thymoquinone increased TAC and decreased pathological lesions and MDA concentration; these changes were significant in the thymoquinone group. Methotrexate, a chemotherapeutic agent,

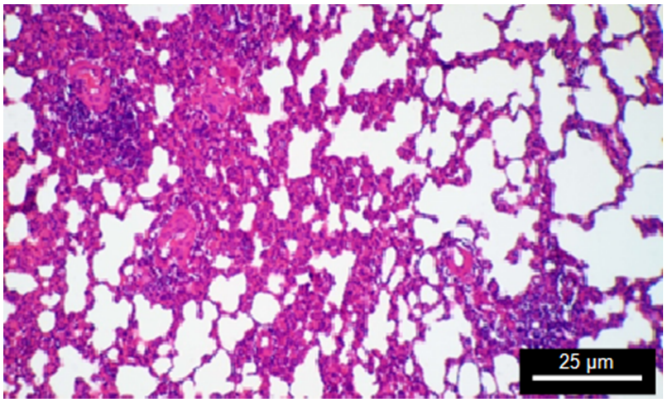

Figure 5. It shows the increase in the thickness of the alveoli wall and the presence of inflammatory cells around the blood vessels and lung parenchyma of the citral group (H&E, 100×).

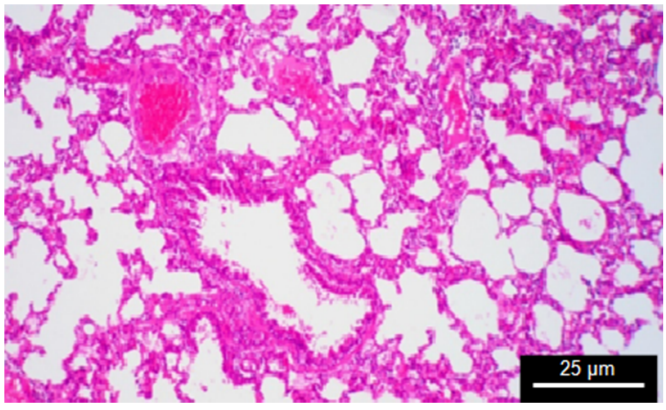

Figure 6. It shows mild congestion of pulmonary vessels and the presence of inflammatory cells around the vessels and bronchioles in the lung parenchyma of the thymoquinone group (H&E, 100×).

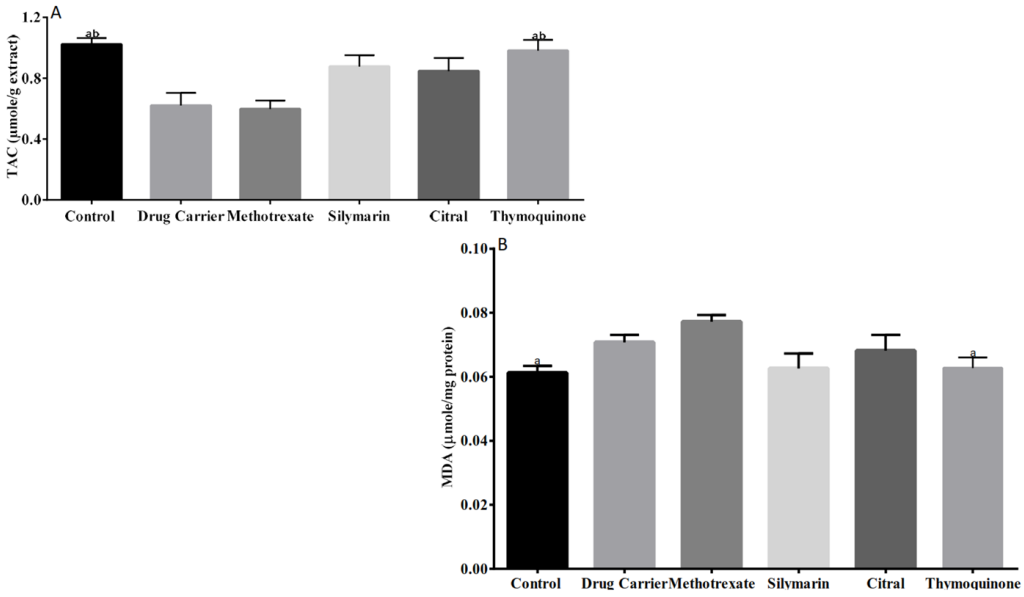

Figure 7. The effects of silymarin, citral, and thymoquinone on methotrexate-induced oxidative stress. A: total antioxidant capacity (TAC) of the lung tissue (a: $p < 0.05$ vs. methotrexate group and b: $p < 0.01$ vs. drug carrier). B: malondialdehyde (MDA) in lung tissue ($p < 0.05$: a vs. methotrexate group). The results were expressed as mean ± S.E.M.

is used to treat cancer and autoimmune diseases and one of its major side effects is pulmonary toxicity [2, 24]. According to the study by Cannon (1997) [25], interstitial pneumonitis, bronchiolitis, and giant cell formation are three key characteristics of methotrexate-induced pneumonitis. A single dose of methotrexate causes considerable lung lesions in rats with thickening of the alveolar wall and an increase in the numbers of lymphocytes and macrophages. Interstitial inflammation in the alveolar space can cause partial or total atelectasis, and there is limited infiltration of eosinophils, hyperplasia of alveolar cells, and dysplasia [25].

Zeller et al. (1984) [26] investigated the effects of high doses of methotrexate on rats' alveolar and peritoneal macrophages and found the number of macrophages in the alveoli and peritoneum to be reduced. Thaniyan et al. (2017) [27] showed that methotrexate causes lymphoproliferative disorders, interstitial fibrosis, and infections in patients with rheumatoid arthritis, with the incidence of these complications increasing remarkably with the increasing duration of methotrexate treatment. According to the study by Chhabra et al. (2012) [8], the histopathological features of methotrexate-induced lung lesions in patients with rheumatoid arthritis include pneumonia, interstitial fibrosis, pulmonary edema, and pulmonary nodules. The lung biopsy also showed the presence of lymphocytes, neutrophils, eosinophilia, type 2 pneumocyte hyperplasia, and interstitial fibrosis [8]. Jakubovic et al. (2013) [28] evaluated methotrexate-induced pulmonary toxicity and reported that methotrexate induces injury to the alveolar epithelial walls. Alveolar injury, pulmonary fibrosis, granulomas, and peribronchial inflammation were observed in the lung biopsy of patients with psoriasis vulgaris who used methotrexate [9]. Methotrexate treatment leads to decreased TAC and increased MDA levels, which may cause lung damage [29, 30]. Ahmed et al. (2021) [31] showed methotrexate-induced lung injury in rats and reported an increase in the thickness of the inter-alveolar wall, infiltration of mononuclear cells, and alveolar collapse. In the present study, methotrexate damaged lung tissue by increasing oxidative stress. Intra-alveolar accumulation of foamy macrophages, slight hemorrhage, congestion, the presence of inflammatory cells around the vessels and the bronchioles, an increase in the thickness of the inter-alveolar septum, and a small number of neutrophils were observed in the lungs of the vehicle group. Very severe hemorrhage and congestion, nodule-like accumulation of mononuclear inflammatory lymphocytes around the vessel, and neutrophils around the vessels were observed in the lungs of the methotrexate group.

Toklu et al. (2008) [32] evaluated the protective role of silymarin against sepsis-induced lung damage and found that silymarin prevents lipid peroxidation and protects membrane integrity through its antioxidant effects. Several studies re-

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ported that the protective role of silymarin might be attributed to its anti-fibrotic, anti-inflammatory, and antioxidant activities [32-34]. Silymarin improves pathological lung injury by reducing MDA concentration, increasing antioxidant activity [35], and reducing microcystin-LR-induced lung damage [36]. In this study, silymarin increased the TAC of the lung tissues of the methotrexate group and its antioxidant activity may have helped reduce methotrexate-induced pathological alterations.

Citral alters the levels of oxidative stress markers and their concentrations, ameliorating oxidative stress. In addition to antioxidant activity, citral demonstrates antiinflammatory activity by inhibiting the production of inflammatory cytokines [37]. It increases the TAC and decreases the concentration of MDA. The result obtained in this study is consistent with the results obtained by Long et al. (2019) [37].

Alzohairy et al. (2021) [38] investigated the protective effect of thymoquinone on rats with benzopyran-induced lung injury and concluded that thymoquinone protects the lungs against oxidative damage by increasing antioxidant enzyme levels. In addition, thymoquinone is essential in maintaining tissue structure by reducing damage to the lung epithelium and the alveolar system [38]. Abdo et al. (2021) [39] showed that thymoquinone could reduce malathion-induced pulmonary toxicity in the form of pulmonary vascular damage and pneumonia. Pourgholamhossein et al. (2016) [40] showed that thymoquinone moderates lung fibrosis induced by the herbicide paraquat. They also reported that thymoquinone significantly reduces the level of MDA, increases superoxide dismutase (SOD) activity, scavenges free radicals, and maintains the activity of various antioxidant enzymes [40]. El-Khouly et al. (2012) [41] demonstrated the protective effect of thymoquinone against bleomycin and toluene-induced lung toxicity. The results of the present study also showed that it protects the lungs against the oxidative damage of methotrexate by increasing and decreasing the TAC and concentration of MDA, respectively. These results are consistent with the results of the previous studies.

CONCLUSION

This study showed that the lung damage caused by methotrexate is related to oxidative stress, and that antioxidant compounds such as silymarin, citral, and thymoquinone could control it. Thymoquinone had the most antioxidant and protective effect compared to silymarin and citral.

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AUTHORS' CONTRIBUTIONS

Noorbakhsh MF., and Ahmadi N. conceived and planned the experiments. Noorbakhsh MF., Ahmadi N., Nazifi S., and Amani Sakineh. Barzan Behdokht carried out the experiments. Noorbakhsh M.F. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

ETHICAL APPROVAL

All experiments involving animal experiments have been received ethical approval from the Institutional Ethics Committee. These tests were performed according to the standard procedures outlined by that committee.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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