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Original Article

The Protective Effects of Citral, Silymarin, and Thymoquinone on Methotrexate-Induced Nephrotoxicity

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ABSTRACT

Objectives: Methotrexate (MTX) is an effective chemotherapeutic and immunosuppressive agent; however, its clinical use is limited due to nephrotoxicity associated with oxidative stress. This study aims to evaluate the protective effects of silymarin, citral, and thymoquinone against MTX-induced renal injury in rats.

Methods: Forty-eight adult male Sprague–Dawley rats were randomly assigned into six groups (n=8): healthy control, cosolvent control (DMSO), MTX-only, MTX+citral (30 mg/kg), MTX+silymarin (50 mg/kg), and MTX+thymoquinone (10 mg/kg). Treatments were administered intraperitoneally for 10 consecutive days. Methotrexate (20 mg/kg, i.p.) was administered to all groups except the healthy control. Serum urea and creatinine levels were measured, while renal tissues were evaluated for total antioxidant capacity (TAC), malondialdehyde (MDA), and histopathological alterations.

Results: MTX administration significantly increased serum urea, creatinine, and renal MDA levels while decreasing TAC compared with the healthy control group. Silymarin significantly reduced serum urea and creatinine levels and restored TAC compared with the MTX group ($p<0.05$). Thymoquinone significantly improved TAC and reduced MDA levels ($p<0.05$), although its effects on serum renal biomarkers were not statistically significant. Citral significantly decreased MDA levels ($p<0.01$) but showed limited effects on TAC and renal function markers. Histopathological examination demonstrated vascular and glomerular congestion with hemorrhage in the MTX group, whereas treatment groups exhibited varying degrees of structural preservation, with silymarin showing the greatest improvement.

Conclusion: Oxidative stress plays a major role in MTX-induced nephrotoxicity. Among the tested compounds, silymarin demonstrated the most consistent nephroprotective effects, while thymoquinone and citral provided partial antioxidant and histological protection. These findings support the potential use of natural antioxidants as adjunctive strategies against MTX-induced renal injury.

Keywords: Silymarin, Citral, Nephrotoxicity, Thymoquinone, Methotrexate

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Introduction

Methotrexate (MTX) is a cytotoxic antimetabolite that is routinely used in clinical settings for the management of multiple malignant conditions, including leukemia, osteosarcoma, and lymphoma. It is also prescribed for several chronic inflammatory disorders such as psoriasis, rheumatoid arthritis and inflammatory bowel disease (1). At the molecular level, MTX functions as a folate pathway inhibitor by targeting dihydrofolate reductase, thereby suppressing nucleotide biosynthesis and ultimately impairing DNA replication and cellular proliferation (2).

This antiproliferative mechanism underlies its efficacy against rapidly dividing cancer cells, but it also affects normal tissues. Indeed, MTX's action extends to non-malignant organs (bone marrow, liver, lung, intestine and kidney). Because renal clearance accounts for the elimination of over 90% of methotrexate, the kidney is particularly vulnerable to toxicity, making renal injury a common factor restricting dose escalation. Under acidic conditions within the tubular lumen, methotrexate and its metabolites display limited solubility, leading to intratubular crystallization, precipitation, and subsequent impairment of tubular flow (2, 3). For this reason, high-dose MTX protocols include vigorous hydration and urine alkalinization (pH>7) to prevent crystal formation. Nevertheless, MTX administration often still leads to renal injury leading to acute kidney injury or even renal failure. Renal toxicity following MTX administration is manifested by elevated serum creatinine and urea levels (2, 3), and histologically by tubular damage and glomerular changes (4).

Current evidence indicates that MTX-associated renal injury arises from the interplay of more than one pathogenic process. Two dominant pathways are generally recognized: first, the formation of intratubular crystals due to limited solubility of MTX and its metabolites, leading to crystalline nephropathy; and second, direct damage to tubular epithelium driven by MTX-induced overproduction of reactive oxygen species and other free radicals (5). Reports suggest that MTX also causes renal damage through mitochondrial dysfunction, energy crises, and reduced activity of the antioxidant enzymes (4). In practice these processes overlap: MTX accumulation in proximal tubule cells overwhelms antioxidant defenses, leading to lipid peroxidation and oxidative stress (6). As a result, key mitochondrial functions fail and cells undergo energy crisis and apoptosis. Recent experimental findings support that MTX exposure can suppress the endogenous antioxidant defense system, including glutathione and superoxide dismutase, while simultaneously elevating renal levels of malondialdehyde (MDA), an index of lipid peroxidation, as well as nitric oxide (4). Inflammation also plays a role in renal toxicity following MTX administration. MTX exposure upregulates inflammatory cytokines and mediators in renal tissue, contributing to the injury cascade (7). In this regard, Jalili et al. (2019) reported oxidative damage and tubular injury after MTX treatment, and Khoshnoud et al. (2017) observed significant histological changes (widened Bowman's space, reduced glomerular cellularity) in rat kidneys following MTX administration (8, 9). These results indicate that MTX-induced renal toxicity arises from the concurrent interplay of intrarenal crystal deposition, oxidative imbalance, and inflammatory responses, rather than from a single isolated mechanism.

Given the importance of oxidative stress in MTX toxicity, researchers have increasingly turned to natural antioxidants as potential renal protectants. Antioxidant-rich plant extracts and phytochemicals can scavenge free radicals, boost endogenous defenses, and modulate inflammatory pathways, thereby mitigating drug-induced organ damage (3, 10). For instance, citral, the principal component of *Litsea cubeba* and *Cymbopogon citratus* essential oils, is a potent antioxidant and anti-inflammatory agent (11). Citral has been shown to induce detoxifying enzymes such as glutathione-S-transferase leading to enhancing cellular antioxidant capacity (12). In various models of oxidative injury, citral protects tissues by reducing lipid peroxidation and restoring glutathione levels (13-15). Likewise, silymarin, a bioactive complex of flavonolignans derived from *Silybum marianum*, is widely recognized for its renoprotective properties. Its protective actions are primarily attributed to antioxidant and anti-inflammatory activities, including the maintenance of intracellular glutathione levels and the consequent inhibition of membrane lipid peroxidation (16, 17). It also modulates immune responses and fibrotic signaling (18). In animal studies, silymarin supplementation significantly attenuated drug-induced renal injury: for example, it reduced serum urea and creatinine levels and improved histology in models of antibiotic- or MTX-induced nephrotoxicity (19, 20). Thymoquinone, the chief bioactive of *Nigella sativa* (black seed), exhibits strong antioxidant and anti-inflammatory properties (21). In lung injury models, thymoquinone administration reverses MTX-induced oxidative markers , lowers MDA and nitric oxide levels while restoring glutathione , and improves lung function (10). In a glycerol-induced acute kidney injury model in rats, thymoquinone markedly improved renal function by lowering serum creatinine and blood urea nitrogen levels, attenuating renal MDA accumulation, and enhancing glutathione content (22). Its anti-inflammatory actions through inhibition of the NF-κB/TLR4 signaling further protect renal cells (23).

In light of these data, supplementation with plant-derived antioxidants is a promising strategy to buffer the kidneys against MTX toxicity. Recent reports specifically support that citral, silymarin, and thymoquinone act as tissue protectants (10, 22). These findings provide

a rationale for studying their effects against MTX-induced renal injury. Accordingly, the present study was designed to evaluate the potential renoprotective effects of silymarin, citral, and thymoquinone in the context of MTX-induced renal injury.

Materials and Methods

Ethics statements

The research and all procedures involving laboratory animals were carried out in full compliance with the biological ethics standards outlined by the Research Vice-Chancellor's guidelines (Approval no: 1GCB1M348268), at Shiraz University (Shiraz, Iran).

Experimental Animals and Study Designing

In this study, a total of 48 male Sprague–Dawley rats (approximately one month old; body weight 200 ± 20 g) were used. To ensure their health and adaptation to the environment, rats were monitored for one week in the animal facility of the Veterinary Faculty. During the acclimatization period and throughout the study, the rats had free access to clean water and pellet feed, with the housing temperature maintained at 24±1°C, and a 12-hour light-dark cycle established. The rats were randomly divided into six groups of eight rats each:

Control Group: Received no treatment.

Cosolvent Control Group: Received the vehicle (daily i.p. of 1 ml distilled water + DMSO) for 10 days and MTX (20 mg/kg, i.p.) on the third day of the study.

MTX Group: Administered MTX (20 mg/kg, i.p.) on the third day of the study.

Citral Treatment Group: Administered citral (30 mg/kg, i.p.) for 10 days and MTX (20 mg/kg, i.p.) on the third day of the study.

Silymarin Treatment Group: Administered silymarin (50 mg/kg, i.p.) for 10 days and MTX (20 mg/kg, i.p.) on the third day of the study.

Thymoquinone Treatment Group: Administered thymoquinone (10 mg/kg, i.p.) for 10 days and MTX (20 mg/kg, i.p.) on the third day of the study.

Sampling

At the end of the tenth day, the rats were anesthetized with ketamine (80 mg/kg, Alfasan, Netherlands) and xylazine (10 mg/kg, Alfasan, Netherlands), both administered intraperitoneally. Blood samples were collected for biochemical assessment of renal function, and the rats were euthanized using CO_2 inhalation. Kidney tissues were harvested for histopathological evaluation and the measurement of antioxidant activity.

Biochemical Assessment of Serum Parameters

Collected blood samples were centrifuged at 3000 rpm for 15 min to separate serum. Then, serum levels of creatinine and urea were determined using Pars Azmoon diagnostic kits using an Alpha Classic automated analyzer (Sanjesh Co., Iran).

Measurement of Antioxidant markers in Kidney Tissue

For tissue processing, kidney samples (1 g) were homogenized in 5 mL of phosphate buffer using a mechanical homogenizer. The homogenates were subsequently centrifuged at 3000 rpm for 5–10 min, and the resulting supernatants were collected for biochemical analyses. Total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in kidney tissue homogenates were quantified using commercial assay kits (Zellbio GmbH, Germany) in accordance with the manufacturer's protocols.

Histopathological Evaluation

For histopathological evaluation, kidney tissue samples were fixed in 10% buffered formalin. Standard tissue processing methods were used to prepare histological sections, which were stained using the Hematoxylin-Eosin (H&E) method. The stained tissue sections were examined under a light microscope (Olympus CX23, Japan) to observe histopathological lesions.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism software (version 10.6). Data are presented as mean ± SEM. Differences among groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple-comparison test, with a p value < 0.05 considered statistically significant.

Results

Biochemical Analysis

Methotrexate administration significantly increased serum levels of urea compared with the healthy control group ($p<0.01$). A similar increase was also observed in the cosolvent control group ($p<0.05$ vs. control). Treatment with silymarin significantly reduced serum urea levels compared with the MTX group ($p<0.01$), restoring values to those of the healthy control group. In contrast, citral and thymoquinone did not significantly attenuate the MTX-induced elevation of urea levels ($p>0.05$; Figure 1A).

Serum creatinine level was significantly elevated in both the MTX ($p<0.05$) and cosolvent control groups ($p<0.01$) compared with the healthy control group. Silymarin treatment significantly decreased serum creatinine levels compared with the MTX group ($p<0.05$), restoring creatinine concentrations to near-normal values. Although citral and thymoquinone showed partial reductions in creatinine levels, these effects were not statistically significant ($p>0.05$; Figure 1B).

Antioxidant Activity Assay

Methotrexate administration significantly reduced renal TAC in the MTX group compared with the healthy control group (0.075 vs. 0.155 µM/mg tissue, $p<0.01$). A similar reduction was also observed in the cosolvent

control group (0.085 µM/mg tissue, $p<0.05$ vs. control). Treatment with silymarin and thymoquinone significantly increased TAC levels compared with the MTX group (0.145 and 0.140 vs. 0.075 µM/mg tissue, respectively; $p<0.05$), restoring the antioxidant capacity to near-normal levels. Although citral treatment increased TAC levels to 0.110 µM/mg tissue, this effect was not statistically significant ($p>0.05$; Figure 2A).

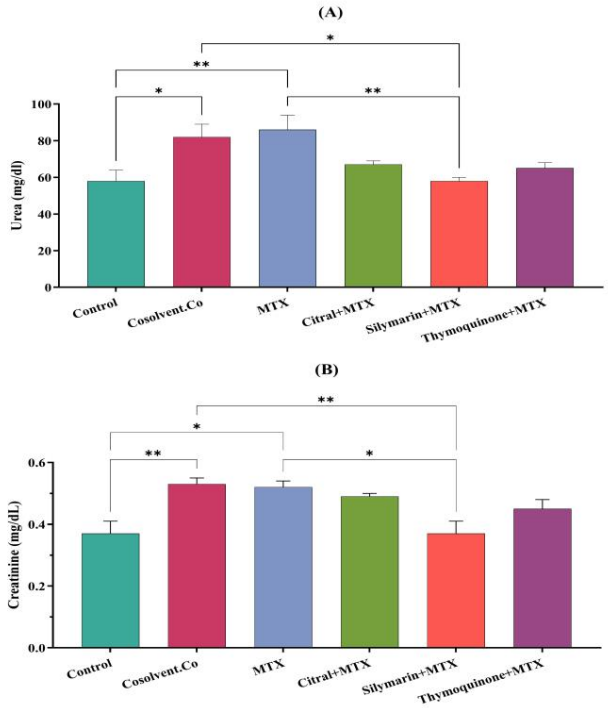


Figure 1. Effects of citral, silymarin, and thymoquinone on serum urea (A) and creatinine (B) levels in MTX-treated rats. Data are presented as Mean ± SEM. Statistical significance between groups is indicated by brackets and asterisks (*p<0.05, **p<0.01). Cosolvent. Co: Cosolvent Control; MTX: Methotrexate

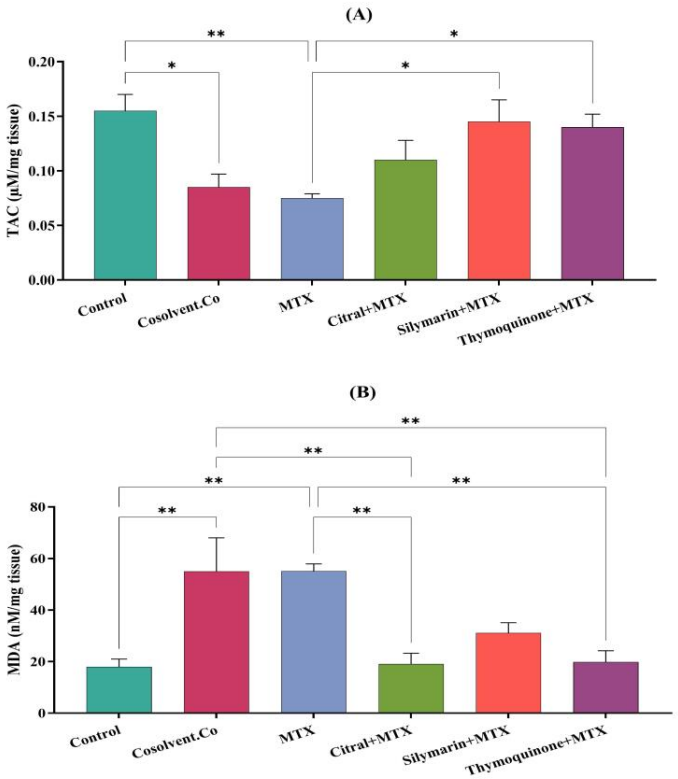


Figure 2. Effects of silymarin, citral, and thymoquinone on renal TAC (A) and MDA (B) levels in MTX-treated rats. Data are presented as Mean ± SEM. Statistical significance between groups is indicated by brackets and asterisks (*p<0.05, **p<0.01). Cosolvent Co: Cosolvent Control; MTX: Methotrexate

Renal MDA levels were markedly elevated in both the MTX (55.06 nM/mg tissue) and cosolvent control groups (55.00 nM/mg tissue) compared with the healthy control group (18.00 nM/mg tissue; $p<0.01$). Treatment with citral and thymoquinone significantly reduced MDA level compared with the MTX group (19.06 and 19.85 vs. 55.06 nM/mg tissue, respectively; $p<0.01$). Silymarin also decreased MDA levels (31.06 nM/mg tissue), but this reduction did not reach statistical significance ($p>0.05$; Figure 2B).

Histopathological Analysis

The histopathological analysis of H&E-stained kidney sections across the experimental groups revealed variable degrees of renal alterations. In the control group (Figure 3A), normal renal histoarchitecture was observed, with intact glomeruli and normal tubular epithelial cells without evidence of pathological lesions. In the MTX group (Figure 3B), renal tissue generally preserved its structural organization; however, marked vascular and glomerular congestion accompanied by hemorrhage was evident, indicating MTX-induced renal injury. No obvious fibrosis, edema, tubular necrosis, or inflammatory cell infiltration was observed. In the drug carrier group (DMSO) (Figure 3C), a focal aggregation of mononuclear inflammatory cells surrounding a blood vessel was detected, suggestive of extramedullary hematopoiesis. In the silymarin-treated group (Figure 3D), renal morphology was largely preserved, with intact glomerular architecture and normal tubular epithelial nuclei. Mild congestion and hemorrhage were still present, together with limited perivascular lymphocytic infiltration suggestive of extramedullary hematopoiesis, indicating partial histological improvement compared with the MTX group. In the citral-treated group (Figure 3E), glomeruli and renal tubules appeared structurally intact without necrosis; however, mild hemorrhage, moderate congestion, and focal inflammatory cell accumulation adjacent to blood vessels were observed, suggesting partial but incomplete protection against MTX-induced damage. Similarly, the thymoquinone-treated group (Figure 3F) exhibited preserved glomerular and tubular architecture with no evidence of necrosis or inflammatory infiltration. Nevertheless, focal cytoplasmic degeneration in some tubular epithelial cells was observed, indicating mild residual cellular injury despite overall histological improvement.

Discussion

Methotrexate, a folate antagonist with immunomodulatory and anti-inflammatory properties, is widely used in treatment of malignancies, psoriasis, and rheumatoid arthritis. It also represents a cornerstone chemotherapeutic agent in paediatric acute lymphoblastic leukemia (ALL) therapy (24). Methotrexate enters cells through active transport mediated by the reduced folate carrier (RFC) and is subsequently converted to polyglutamated derivatives by folylpolyglutamate synthetase. These polyglutamated forms persist intracellularly for prolonged periods and competitively inhibit dihydrofolate reductase (DHFR), thereby reducing tetrahydrofolate availability and impairing DNA and RNA synthesis, particularly in rapidly proliferating cells (25). Despite its therapeutic efficacy, high-dose MTX administration is associated with severe adverse effects, especially nephrotoxicity, necessitating close monitoring and appropriate dose adjustments (26).

Previous studies have demonstrated that MTX administration elevates serum creatinine and Blood Urea Nitrogen (BUN) levels and induces tubular degeneration and oxidative stress in renal tissue (27). Clinically,

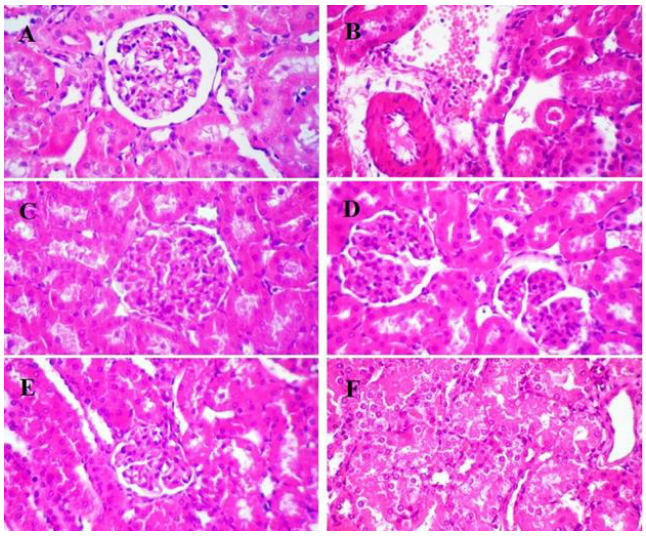


Figure 3. The effects of citral, silymarin, and thymoquinone on histopathological examination of renal tissue. (A) Control group; (B) Cosolvent Control, (C) Methotrexate group, (D) Silymarin treatment group, (E) Citral treatment group, (F) Thymoquinone treatment group (H&E, X400).

MTX-induced nephrotoxicity is frequently associated with acute kidney injury and renal dysfunction (28). Heidari et al. reported that MTX-induced renal injury was accompanied by increased serum creatinine and BUN levels, vascular congestion, interstitial inflammation, mitochondrial dysfunction, depletion of glutathione stores, and reduced antioxidant capacity (4). Consistent with these findings (29, 30), the present study demonstrated that MTX administration significantly increased serum levels of urea, creatinine, and renal MDA while reducing TAC, indicating enhanced oxidative stress. Histopathological examination further confirmed MTX-induced renal injury through vascular and glomerular congestion accompanied by hemorrhage.

Interestingly, the cosolvent control group also exhibited alterations in renal oxidative stress markers and renal function indices compared with the healthy control group. These findings suggest that the vehicle system may have partially contributed to oxidative and biochemical alterations and should therefore be considered when interpreting the protective effects of the tested compounds.

Among the investigated compounds, silymarin exhibited the most consistent nephroprotective effects. Silymarin significantly reduced serum urea and creatinine levels compared with the MTX group and restored these parameters to values comparable with the healthy control group. In addition, silymarin significantly increased TAC levels, indicating restoration of the antioxidant capacity. Although renal MDA levels were reduced following silymarin treatment, the decrease did not reach statistical significance. These findings are in agreement with previous studies demonstrating that silymarin exerts renoprotective effects through antioxidant, anti-inflammatory, and membrane-stabilizing properties that limit oxidative injury and preserve renal cellular integrity (31-33).

Thymoquinone demonstrated partial nephroprotective activity primarily through modulation of oxidative stress markers rather than renal functional indices. In the present study, thymoquinone significantly increased TAC and significantly reduced renal MDA levels compared with the MTX group, suggesting attenuation of lipid peroxidation and oxidative damage. However, its effects on serum urea and creatinine levels did not reach statistical significance. These findings indicate that thymoquinone may alleviate oxidative stress–mediated renal injury without fully restoring renal functional biomarkers under the current experimental conditions. Similar antioxidant and renoprotective properties of thymoquinone have been previously reported in experimental nephrotoxicity models (6, 34, 35).

Citral treatment also demonstrated partial antioxidant activity, particularly through significant reduction of renal MDA levels, suggesting the inhibition of lipid peroxidation. However, citral did not significantly improve TAC or reduce serum renal function markers, indicating comparatively weaker nephroprotective efficacy than silymarin. These findings are consistent with previous studies reporting that citral possesses antioxidant potential but may exert more limited protective effects against severe renal oxidative injury (10, 36).

The observed reduction in TAC and elevation in MDA following MTX administration further support oxidative stress as a major mechanism underlying MTX-induced nephrotoxicity. Increased MDA levels reflect enhanced lipid peroxidation and membrane damage caused by excessive reactive oxygen species generation, whereas TAC reduction indicates the depletion of endogenous antioxidant defence system. The ability of silymarin and thymoquinone to significantly restore TAC levels, together with the marked reduction in MDA levels suggests that attenuation of oxidative stress contributed substantially to the protective effects observed in this study.

Histopathological findings were generally consistent with the biochemical results. Methotrexate-treated rats demonstrated marked vascular and glomerular congestion with hemorrhage, indicating renal tissue injury. In contrast, treatment groups showed preservation of glomerular and tubular architecture with varying degrees of residual congestion and hemorrhage. Silymarin treatment resulted in relatively preserved renal morphology with only mild pathological alterations, supporting its superior nephroprotective efficacy. Thymoquinone also preserved renal architecture and prevented necrotic changes despite mild residual cellular degeneration, whereas citral exhibited only partial histological improvement accompanied by moderate congestion and focal inflammatory cell accumulation. These observations suggest that histological improvement may occur even in the absence of complete normalization of serum renal biomarkers, particularly in the citral and thymoquinone groups.

Conclusions

Methotrexate administration induced significant renal injury characterized by elevated serum urea and creatinine levels, increased lipid peroxidation, reduced antioxidant capacity, and histopathological alterations, confirming the critical role of oxidative stress in MTX-induced nephrotoxicity. Among the tested compounds, silymarin exhibited the most consistent nephroprotective effects, significantly improving renal function markers, restoring antioxidant capacity, and preserving renal histological architecture. Thymoquinone demonstrated partial protective effects primarily through modulation of oxidative stress markers, although its effects on serum renal biomarkers were not statistically significant. Citral also reduced lipid peroxidation and partially improved histopathological alterations; however, its overall nephroprotective efficacy was comparatively limited.

Overall, these findings suggest that natural antioxidant compounds, particularly silymarin, may serve as promising adjunctive agents for reducing MTX-induced renal oxidative injury. Further studies are warranted to clarify their underlying molecular mechanisms and evaluate their potential clinical applicability in patients receiving MTX therapy.

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Conflict of interest

The authors declare no conflicts of interest related to this study.

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