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Comparative evaluation of citral, silymarin, and thymoquinone in methotrexate-induced neurotoxicity: biochemical and histopathological evidence in rats

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Abstract

Methotrexate (MTX) is an antineoplastic and immunosuppressive agent whose clinical use is limited by neurotoxicity, largely mediated through oxidative stress. Natural phytochemicals with antioxidant properties have emerged as promising candidates for preventing MTX-induced brain injury. Although the neuroprotective effects of silymarin, citral, and thymoquinone have been reported individually, their comparative efficacy against MTX-induced neurotoxicity has not been directly evaluated under identical experimental conditions. This study compared the neuroprotective effects of these phytochemicals in rats with MTX-induced neurotoxicity. Forty-two male Sprague-Dawley rats were randomly assigned to six groups (n = 7): healthy control, MTX, vehicle+MTX, MTX+silymarin, MTX+citral, and MTX+thymoquinone. MTX (20 mg/kg, intraperitoneally) was administered on day 3, while silymarin (50 mg/kg), citral (25 mg/kg), and thymoquinone (10 mg/kg) were injected intraperitoneally once daily for 10 days. Cerebral cortex total antioxidant capacity (TAC), malondialdehyde (MDA), and histopathological alterations were evaluated. MTX significantly reduced TAC and increased MDA levels compared to the healthy control group. Thymoquinone was the only treatment that significantly restored TAC compared to the MTX group ($p < 0.01$), whereas all three phytochemicals significantly reduced MDA levels ($p < 0.05$). Histopathological examination demonstrated marked neuronal degeneration, cytoplasmic microvacuolation, and darkly stained nuclei in MTX-treated rats, whereas all treatment groups exhibited improved neuronal morphology and reduced tissue injury. Overall, silymarin, citral, and thymoquinone attenuated MTX-induced neurotoxicity by reducing oxidative damage and improving histopathological alterations. Thymoquinone demonstrated the most favorable antioxidant profile by significantly restoring TAC, while all three compounds showed comparable efficacy in reducing lipid peroxidation and improving histopathological alterations.

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Introduction

Methotrexate (MTX) is a folate antagonist widely used in the treatment of various malignancies and autoimmune diseases because of its proven therapeutic efficacy. However, its clinical application is frequently limited by dose-dependent toxicities affecting multiple organs, particularly the central nervous system. As an antifolate agent, MTX inhibits DNA synthesis and cell proliferation. While high doses are primarily administered for the treatment of solid tumors and hematological malignancies, lower doses are commonly prescribed for chronic inflammatory disorders such as rheumatoid arthritis. Despite its therapeutic benefits, prolonged treatment or high-dose administration may result in severe adverse effects, including peripheral and central nervous system toxicity (Su *et al*., 2017; Cottin *et al.,* 2021; Hamed *et al.,* 2022; Xu *et al.,* 2022).

Although the precise mechanisms underlying MTX-induced neurotoxicity have not been fully elucidated, accumulating evidence indicates that oxidative stress plays a pivotal role in its pathogenesis (Aslankoc *et al.,* 2022). Excessive generation of reactive oxygen species (ROS), depletion of endogenous antioxidant defenses, and subsequent lipid peroxidation collectively contribute to neuronal dysfunction and structural damage in brain tissue. Accordingly, considerable attention has been directed toward the use of antioxidant and anti-inflammatory agents as adjunctive strategies to alleviate MTX-induced neurotoxicity. Nevertheless, no intervention has consistently demonstrated complete protection against these adverse effects (Aslankoc *et al.,* 2022).

Among naturally occurring antioxidants, citral, silymarin, and thymoquinone have attracted considerable interest because of their potent antioxidant and neuroprotective properties. Citral, the principal constituent of the essential oils of *Litsea cubeba* and *Cymbopogon citratus* (Idrees *et al.,* 2019; Faraji *et al.,* 2025), exhibits strong antioxidant activity (Bakhtiari *et al.,* 2021; Khatinasab *et al.,* 2025) and has been reported to enhance glutathione S-transferase activity, thereby strengthening endogenous antioxidant defense mechanisms (Nakamura *et al.,* 2003; Perez *et al.,* 2010; Li *et al.,* 2018). Silymarin, a flavonolignan complex isolated from *Silybum marianum*, possesses well-documented antioxidant, anti-inflammatory, immunomodulatory, and antifibrotic properties (Abenavoli *et al.,* 2018). Its neuroprotective activity is largely attributed to its ability to preserve intracellular glutathione levels and inhibit lipid peroxidation, thereby protecting cellular membranes against oxidative injury (Akhtar *et al.,* 2023). Furthermore, silymarin has been shown to attenuate doxorubicin-induced neurotoxicity in experimental models (Yardım *et al.,* 2021).

Similarly, thymoquinone, the major bioactive constituent of *Nigella sativa* (black seed), exhibits potent antioxidant and anti-inflammatory activities and has demonstrated protective effects against experimentally induced neuronal injury, including aluminum chloride-induced neurotoxicity (Mohamed *et al.,* 2021; Abbas *et al.,* 2022).

Although each of these phytochemicals has individually demonstrated antioxidant and neuroprotective effects in different experimental models, their relative neuroprotective efficacy against methotrexate-induced neurotoxicity has not been directly compared in a single experimental model. Therefore, this study was designed to comparatively evaluate the neuroprotective efficacy of silymarin, citral, and thymoquinone in a rat model of MTX-induced neurotoxicity through biochemical assessment of oxidative stress and histopathological examination. We hypothesized that all three compounds would attenuate MTX-induced brain injury, with differences in antioxidant capacity resulting in varying degrees of neuroprotection.

Materials and Methods

Animals

Forty-two male Sprague–Dawley rats with comparable body weights were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Before the initiation of the experiment, the animals were acclimatized for two weeks in the Laboratory Animal Center of the Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran. Throughout the acclimatization and experimental periods, the rats had free access to standard pelleted chow and water and were maintained under a controlled 12/12 hours light/dark cycle. All procedures involving animal handling adhered to international guidelines for

the care and use of laboratory animals and received approval from the Shiraz University Research Ethical Committee (Approval No.: 1GCB5M348200).

Experimental protocol

Following the acclimatization period, the rats were randomly allocated into six experimental groups (n = 7 per group). The experimental design, including the dosing regimen for MTX, vehicle, and the investigated phytochemicals, was adopted from the previously published experimental protocol of Sakineh *et al.,* (2023). Vehicle or antioxidant treatments were administered intraperitoneally (i.p.) once daily for 10 consecutive days. MTX (20 mg/kg, i.p.) was administered as a single injection on Day 3 to all experimental groups except the healthy control (Sakineh *et al.,* 2023).

Group I (Healthy control): received no treatment.

Group II (MTX): received a single i.p. injection of MTX (20 mg/kg) on Day 3.

Group III (Vehicle): received the vehicle (dimethyl sulfoxide (DMSO) and water, i.p.) once daily for 10 days and MTX (20 mg/kg, i.p.) on Day 3.

Group IV (Citral): received citral (25 mg/kg, i.p.; Merck, Germany, 96%) once daily for 10 days and MTX (20 mg/kg, i.p.) on Day 3.

Group V (Silymarin): received silymarin (50 mg/kg, i.p.; Sigma-Aldrich, Germany, 95%) once daily for 10 days and MTX (20 mg/kg, i.p.) on Day 3.

Group VI (Thymoquinone): received thymoquinone (10 mg/kg, i.p.; Sigma-Aldrich, Germany, 98%) once daily for 10 days and MTX (20 mg/kg, i.p.) on Day 3.

On Day 10, all animals were anesthetized with ketamine (80 mg/kg, i.p.; Alfasan, The Netherlands) and xylazine (10 mg/kg, i.p.; Alfasan, The Netherlands). Following euthanasia by CO_2 inhalation, brain tissues were immediately collected from the cerebral cortex for biochemical and histopathological analyses.

Preparation of cerebral cortex homogenate

Cerebral cortex samples were weighed, and 1 g of tissue was homogenized in 5 ml of phosphate buffer using a tissue homogenizer until a uniform homogenate was obtained. The homogenate was centrifuged at 2500 rpm for 10 minutes, and the resulting supernatant was collected and stored for subsequent biochemical analyses.

Assessment of oxidative stress biomarkers

Cerebral cortex homogenates were used to determine total antioxidant capacity (TAC) and malondialdehyde (MDA) levels using commercially available assay kits (ZellBio GmbH, Germany) according to the manufacturer's instructions.

TAC was determined using a colorimetric assay based on the formation of a colored product from the chromogenic substrate tetramethylbenzidine (TMB). Absorbance was measured at 450 nm using a Jenway 6300 spectrophotometer (Jenway, UK), and TAC values were expressed as mmol/l. The assay had a sensitivity of 0.1 mM (100 µmol/l), with intra- and inter-assay coefficients of variation below 3.4% and 4.2%, respectively.

MDA concentration was measured using a commercially available assay kit (Cat. No. ZB-MDA96A, ZellBio GmbH, Germany). The assay was based on the reaction of MDA with thiobarbituric acid (TBA) under acidic conditions and high temperature, producing a colored complex that was quantified colorimetrically at 535 nm. The assay sensitivity was 0.1 µM, with an inter-assay coefficient of variation of 5.8%.

Histopathological evaluation

Immediately after tissue collection, brain samples were fixed in 10% neutral-buffered formalin for 48 hours. Following routine histological processing, including dehydration, clearing, and paraffin embedding, tissue blocks were sectioned at 4–5 µm thickness using a rotary microtome. Two representative sections from each animal were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX23, Japan).

Histopathological evaluation was performed by an experienced veterinary pathologist in a blinded manner, with the examiner blinded to the treatment allocation of the tissue sections throughout the microscopic examination.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10.1 (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± standard error of the mean (SEM). Data were assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using Bartlett's test prior to statistical analysis. Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Investigation of antioxidant parameters in cerebral cortex tissue

TAC

MTX administration significantly reduced cerebral cortex total antioxidant capacity compared to the healthy control group ($p = 0.0037$). No significant difference was observed between the MTX and vehicle groups ($p > 0.9999$), indicating that the vehicle had no detectable effect on TAC.

Treatment with silymarin and citral increased TAC compared to the MTX group; however, these increases did not reach statistical significance ($p = 0.1903$ and $p = 0.1122$, respectively). In contrast, thymoquinone treatment significantly restored TAC compared to the MTX group ($p = 0.0073$). Furthermore, TAC values in all treatment groups were not significantly different from those of the healthy control group (Fig. 1).

MDA

MTX administration significantly increased cerebral cortex MDA levels compared to the healthy control group ($p = 0.0004$), indicating enhanced lipid peroxidation. No significant difference was observed between the MTX and vehicle groups ($p = 0.9914$).

Treatment with silymarin, citral, and thymoquinone significantly reduced MDA levels compared to the MTX group ($p = 0.0002$, $p = 0.0050$, and $p < 0.0001$, respectively). Moreover, MDA concentrations in all treatment groups were comparable to those of the healthy control group, with no statistically significant differences observed among them (Fig. 2).

Investigation of cerebral cortex histopathology

Histopathological examination of H&E-stained cerebral cortex sections from the healthy control group revealed normal tissue architecture with well-preserved neurons and no detectable histopathological alterations (Fig. 3A). In the vehicle-treated group, degenerative changes were observed in several regions of the cerebral cortex. These alterations were characterized by the presence of darkly stained (pyknotic) nuclei in affected neurons, indicating neuronal degeneration (Fig. 3B). Similarly, cerebral cortex sections obtained from the MTX-treated group demonstrated marked histopathological alterations. Degenerative changes were evident in neuronal cells and were accompanied by cytoplasmic microvacuolation and numerous darkly stained nuclei. These findings indicated more extensive neuronal injury than that observed in the healthy control group (Fig. 3C). Administration of silymarin noticeably attenuated the histopathological alterations induced by MTX. Cerebral cortex sections from this group exhibited fewer degenerative neurons, while dense cytoplasmic granules were observed in some neuronal cells. Overall, the tissue architecture appeared better preserved than that of the MTX-treated group (Fig. 3D). In the citral-treated group, the severity of neuronal degeneration was also reduced. Cerebral cortex sections showed fewer darkly stained nuclei and a lower number of cytoplasmic granules compared to the MTX group, indicating partial preservation of the histological architecture (Fig. 3E). The thymoquinone-treated group exhibited the greatest preservation of cerebral cortex histological architecture among the treatment groups. Neurons maintained a morphology closely resembling that of the healthy control group, and characteristic degenerative features, including darkly stained nuclei and cytoplasmic microvacuolation, were largely absent (Fig. 3F).

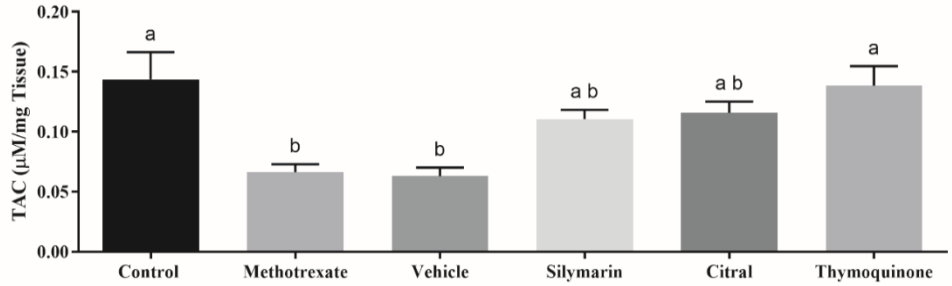


Fig. 1. Cerebral cortex total antioxidant capacity (TAC) levels in the healthy control, MTX, MTX + vehicle (DMSO), MTX + silymarin (50 mg/kg), MTX + citral (25 mg/kg), and MTX + thymoquinone (10 mg/kg) groups. Data were presented as mean ± standard error of the mean (SEM) (n = 7 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Groups with different lowercase letters differ significantly ($p < 0.05$).

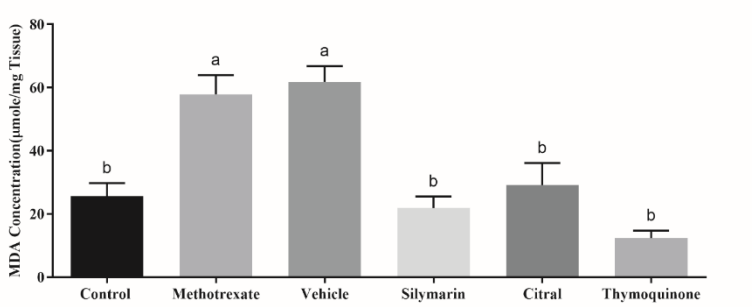


Fig. 2. Cerebral cortex malondialdehyde (MDA) levels in the healthy control, MTX, MTX + vehicle (DMSO), MTX + silymarin (50 mg/kg), MTX + citral (25 mg/kg), and MTX + thymoquinone (10 mg/kg) groups. Data were presented as mean ± standard error of the mean (SEM) (n = 7 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Groups with different lowercase letters differ significantly ($p < 0.05$).

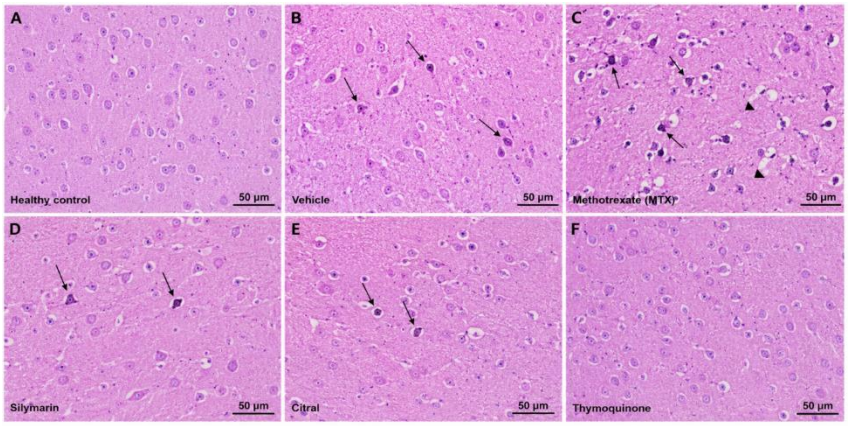


Fig. 3. Representative photomicrographs of hematoxylin and eosin (H&E)-stained cerebral cortex sections from rats in the (A) healthy control, (B) vehicle, (C) MTX (MTX), (D) silymarin, (E) citral, and (F) thymoquinone groups. (A) The healthy control group exhibited normal cortical architecture with well-preserved neurons. (B) The vehicle group showed neuronal degeneration characterized by scattered pyknotic nuclei. (C) The MTX group demonstrated marked neuronal degeneration, cytoplasmic microvacuolation, and numerous pyknotic nuclei. (D) Silymarin treatment attenuated MTX-induced histopathological alterations, with fewer degenerative neurons than in the MTX group. (E) Citral treatment reduced neuronal degeneration and partially preserved cortical architecture. (F) Thymoquinone treatment exhibited the greatest preservation of neuronal morphology, closely resembling that of the healthy control group. Arrows indicate pyknotic (darkly stained) nuclei, whereas arrowheads indicate cytoplasmic microvacuolation. Original magnification, × 400. Scale bar = 50 µm.

Discussion

MTX remains one of the most effective antineoplastic and immunosuppressive agents; however, its clinical application is frequently limited by dose-dependent toxicities affecting multiple organs, including the central nervous system. Increasing evidence indicates that oxidative stress represents a central mechanism underlying MTX-induced neurotoxicity, whereby excessive generation of reactive oxygen species (ROS), depletion of endogenous antioxidant defenses, mitochondrial dysfunction, and activation of inflammatory signaling pathways collectively contribute to neuronal degeneration and functional impairment (Vezmar *et al.,* 2003; Watanabe *et al.,* 2018; Ayalon *et al*., 2019; Ahmed *et al.,* 2021; Kougkas *et al.,* 2022; Aslankoc *et al.,* 2022; Behairy *et al.,* 2024). Consistent with these observations, the present study demonstrated that MTX administration significantly decreased cerebral cortex total antioxidant capacity while markedly increasing lipid peroxidation, as reflected by elevated MDA levels. These biochemical alterations were accompanied by prominent histopathological changes, including neuronal degeneration, cytoplasmic microvacuolation, and darkly stained nuclei, confirming the establishment of oxidative brain injury in this experimental model.

The neurotoxicity of MTX is multifactorial and extends beyond simple ROS overproduction. Experimental studies have demonstrated that MTX disrupts folate metabolism, leading to impaired nucleotide synthesis, mitochondrial dysfunction, excessive homocysteine accumulation, excitotoxicity, and activation of apoptotic cascades (Vezmar *et al*., 2003; Ahmed *et al.,* 2021). Moreover, oxidative stress induced by MTX further amplifies neuroinflammation through activation of redox-sensitive transcription factors, particularly nuclear factor-kappa B (NF-κB), resulting in increased production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, which ultimately aggravate neuronal injury (Behairy *et al.,* 2024). These mechanisms explain why oxidative stress biomarkers and histopathological alterations generally occur simultaneously following MTX administration, as observed in the present study.

One of the principal findings of the present study was the differential effect of the investigated phytochemicals on cerebral cortex antioxidant capacity. Although all three compounds increased TAC compared to the MTX group, only thymoquinone restored TAC to a statistically significant extent. In contrast, the increases observed following silymarin and citral administration did not reach statistical significance. This finding suggests that while all three compounds possess antioxidant activity, thymoquinone exhibited a greater ability to reinforce the overall endogenous antioxidant defense system under the present experimental conditions.

The superior effect of thymoquinone on TAC may be attributed to its pleiotropic antioxidant mechanisms. Unlike conventional free radical scavengers, thymoquinone activates endogenous cytoprotective pathways, particularly the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway. Activation of Nrf2 promotes transcription of numerous antioxidant enzymes, including heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and enzymes involved in glutathione biosynthesis, thereby enhancing the overall antioxidant capacity of tissues rather than merely neutralizing existing ROS (Aslani *et al*., 2024; Jomova *et al.,* 2024).

Our findings are also in agreement with previous experimental studies investigating MTX-induced organ toxicity. Similar improvements in TAC following thymoquinone administration have been reported in experimental models of MTX-induced pulmonary and cardiac injury, in which thymoquinone consistently produced greater restoration of antioxidant status than citral or silymarin (Sakineh *et al.,* 2023; Behdokht *et al.,* 2024).

Lipid peroxidation represents one of the earliest biochemical consequences of oxidative stress and is widely assessed by measuring tissue MDA concentrations. In the present study, MTX administration significantly increased cerebral cortex MDA levels, confirming enhanced oxidative degradation of membrane lipids. Treatment with silymarin, citral, and thymoquinone significantly reduced MDA concentrations compared to the MTX group, indicating that all three compounds effectively attenuated lipid peroxidation.

Interestingly, although thymoquinone produced the lowest mean MDA concentration among the treatment groups, no statistically significant differences were observed between thymoquinone, silymarin, and citral. Therefore, the present findings indicated that all three

phytochemicals were comparably effective in limiting MTX-induced lipid peroxidation, despite the superior effect of thymoquinone on total antioxidant capacity. This distinction is important because restoration of TAC reflects enhancement of endogenous antioxidant defenses, whereas reduction of MDA primarily indicates inhibition of oxidative membrane damage.

The histopathological observations further substantiated the biochemical findings. Cerebral cortex sections obtained from MTX-treated rats exhibited neuronal degeneration characterized by darkly stained nuclei and cytoplasmic microvacuolation, whereas these alterations were markedly attenuated following treatment with silymarin, citral, or thymoquinone. Importantly, the thymoquinone-treated group displayed the greatest preservation of neuronal architecture, with cerebral cortex morphology closely resembling that of the healthy control group. Although histopathological assessment in the present study was qualitative, the morphological improvements observed in all treatment groups were consistent with the reduction in lipid peroxidation and restoration of antioxidant capacity. These findings collectively support the concept that attenuation of oxidative stress contributes directly to preservation of neuronal integrity following MTX exposure.

The neuroprotective effect of silymarin observed in the present study is biologically plausible considering its well-established antioxidant and anti-inflammatory activities. Silymarin has been shown to stabilize cellular membranes, preserve intracellular glutathione, suppress lipid peroxidation, and inhibit activation of NF-κB, thereby reducing oxidative stress-induced neuronal apoptosis (Kandemir *et al.,* 2017; Abenavoli *et al.,* 2018; Yardım *et al.,* 2021; Akhtar *et al.,* 2023). In addition to its direct free radical scavenging activity, recent evidence indicates that silymarin modulates multiple intracellular signaling pathways involved in neuronal survival, including Nrf2, PI3K/Akt, MAPK, and mitochondrial apoptotic pathways. Through these mechanisms, silymarin enhances endogenous antioxidant enzyme activity while simultaneously suppressing inflammatory cytokine production and mitochondrial dysfunction, both of which are major contributors to neurodegeneration (Ashique *et al.,* 2024; Sayyad *et al.,* 2025).

Despite these well-recognized neuroprotective properties, silymarin did not significantly restore TAC in our experimental model, although it significantly reduced MDA levels. This apparent discrepancy may indicate that the antioxidant action of silymarin under the present experimental conditions was sufficient to suppress lipid peroxidation but insufficient to fully restore the overall antioxidant reserve measured by TAC. Since TAC reflects the combined activity of both enzymatic and non-enzymatic antioxidant systems, partial recovery of individual antioxidant pathways may not necessarily translate into a statistically significant increase in total antioxidant capacity.

Citral also demonstrated protective activity against MTX-induced oxidative injury. Although fewer studies have investigated its neuroprotective efficacy compared to silymarin and thymoquinone, citral has consistently been reported to possess potent antioxidant and anti-inflammatory properties. Experimental evidence suggests that citral enhances glutathione S-transferase activity, promotes cellular detoxification, suppresses ROS generation, and reduces oxidative membrane damage (Nakamura *et al.,* 2003; Perez *et al.,* 2010; Li *et al.,* 2018; Idrees *et al.,* 2019; Bakhtiari *et al.,* 2021). These mechanisms likely explain the significant reduction in cerebral cortex MDA observed in the present study. Nevertheless, similar to silymarin, citral failed to significantly increase TAC, suggesting that its primary protective action may involve inhibition of lipid peroxidation rather than broad restoration of endogenous antioxidant capacity.

Among the three investigated phytochemicals, thymoquinone exhibited the most favorable biochemical profile. In addition to significantly reducing MDA concentrations, it was the only treatment capable of significantly restoring TAC following MTX administration. Numerous experimental studies have demonstrated that thymoquinone exerts its antioxidant activity not only through direct scavenging of reactive oxygen species but also by activating endogenous cytoprotective pathways, particularly the Nrf2/ARE signaling cascade. Activation of Nrf2 induces transcription of several antioxidant enzymes, including HO-1, SOD, CAT, GPx, and enzymes involved in glutathione synthesis, thereby enhancing the overall antioxidant capacity of neural tissues. Simultaneously, thymoquinone suppresses NF-κB-mediated inflammatory signaling and attenuates mitochondrial dysfunction, ultimately reducing neuronal apoptosis and oxidative injury. These complementary mechanisms may explain why

thymoquinone produced the most pronounced restoration of TAC in the present study (Sayyad *et al.,* 2025).

Interestingly, although thymoquinone exhibited the lowest mean MDA concentration and the greatest qualitative preservation of cerebral cortex histology, direct statistical comparisons among the three treatment groups revealed no significant differences in MDA levels. Therefore, the present findings indicated that all three phytochemicals effectively reduced MTX-induced lipid peroxidation, whereas thymoquinone demonstrated additional benefits by significantly restoring total antioxidant capacity. This distinction highlights the complexity of antioxidant defense mechanisms, emphasizing that improvement of individual oxidative stress biomarkers does not necessarily occur uniformly across different antioxidant pathways.

The present study has several limitations that should be acknowledged. First, oxidative stress was evaluated using only TAC and MDA, whereas measurement of individual antioxidant enzymes such as SOD, CAT, glutathione peroxidase, and reduced glutathione would have provided a more comprehensive characterization of redox homeostasis. Second, inflammatory cytokines and apoptosis-related markers, including TNF-α, IL-1β, IL-6, Bax, Bcl-2, and caspase-3, were not assessed, limiting mechanistic interpretation of the observed neuroprotective effects. Third, histopathological evaluation was qualitative and was not supported by a standardized histological scoring system or immunohistochemical analysis. Finally, behavioral and cognitive assessments were not performed; therefore, the functional relevance of the observed biochemical and histological improvements remains to be established. Future studies integrating molecular, histopathological, and behavioral analyses will be essential for clarifying the precise mechanisms through which these phytochemicals attenuate MTX-induced neurotoxicity.

The present study demonstrated that MTX-induced neurotoxicity was closely associated with oxidative stress, as evidenced by reduced TAC, increased lipid peroxidation, and histopathological alterations in cerebral cortex tissue. Treatment with silymarin, citral, and thymoquinone attenuated MTX-induced oxidative damage, as reflected by significant reductions in cerebral cortex MDA levels and improved histological architecture. Notably, thymoquinone was the only compound that significantly restored total antioxidant capacity, suggesting a greater ability to reinforce endogenous antioxidant defenses under the present experimental conditions.

Collectively, these findings support the neuroprotective potential of silymarin, citral, and thymoquinone against MTX-induced brain injury. Overall, all three phytochemicals attenuated MTX-induced neurotoxicity by reducing oxidative damage and improving histopathological alterations. Thymoquinone demonstrated the most favorable antioxidant profile by significantly restoring TAC, whereas all three compounds showed comparable efficacy in reducing lipid peroxidation and improving histopathological alterations. Although the precise molecular mechanisms remain to be elucidated, the observed biochemical and histopathological improvements suggest that modulation of oxidative stress may represent a key mechanism underlying their protective effects. Future studies should incorporate molecular analyses of antioxidant and inflammatory signaling pathways, quantitative histopathological scoring, and behavioral assessments to further clarify the therapeutic potential of these compounds and facilitate their translation into clinical applications.

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Conflict of Interest

The authors declare that they have no competing interests.

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