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Original Article
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Comparative Study of the Protective Effects of Citral, Thymoquinone, and Silymarin on Methotrexate-induced Cardiotoxicity in Rats

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Objectives: Methotrexate (MTX), an immunosuppressant and anti-cancer medication, can harm the heart. The goal of the current investigation was to assess the cardiotoxicity caused by MTX and the potential cardioprotective properties of silymarin, citral, and thymoquinone as antioxidants.
Methods: Forty-eight rats were divided into six groups, which included control, MTX, co-solvent, citral, thymoquinone, and silymarin groups. At the end of the study, the rats were anesthetized (ketamine and xylazine) and killed using CO_2. Their blood samples were collected to measure the enzymatic activities of creatine kinase-myoglobin binding (CK-MB), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH). Also, the heart tissue was sampled to determine the antioxidant capacity and examine the histopathology.
Results: The findings revealed that the activity of CPK, CK-MB, and LDH enzymes significantly reduced in the thymoquinone treatment group compared to the MTX group ($p < 0.05$). On the other hand, total antioxidant capacity was significantly increased in the thymoquinone group compared to the MTX group ($p < 0.05$). The pathological modifications (i.e. severe congestion, edema fluid, the presence of inflammatory cells around the blood vessels, mild to moderate hemorrhaging between cardiac muscle fibers) were seen in the MTX group. The treatment groups, particularly thymoquinone, did not experience any appreciable pathological changes.
Conclusion: The thymoquinone was found to have the strongest protective effect against the heart damage caused by MTX.

Keywords: methotrexate, citral, thymoquinone, silymarin, cardiotoxicity

INTRODUCTION

Methotrexate (MTX), a folate antagonist and anticancer agent, is widely used to treat various types of malignancies, such as lymphoma [1]. In human medicine, MTX is also administered in low doses to manage several autoimmune diseases (e.g., rheumatoid arthritis) [2]. It initially inhibits the enzyme dihydrofolate synthase (DHFS) and subsequently blocks the production of purine and pyrimidine bases required for DNA and RNA synthesis [3]. This medication exerts cytotoxic effects not only on tumor cells but also on vital organs, particularly the heart [4]. Inhibition of glycosylase activity and the antioxidant system in the heart may be related to MTX-induced toxicity [5].

Several medicinal plants with varying degrees of success have been used to prevent MTX-induced cardiotoxicity [6, 7]. Citral, a monoterpene composed of two isomeric acyclic aldehydes known as geranial and neral, is widely used as a flavoring agent in food, cosmetics, and detergents because of its strong lemon flavor and scent [8]. Beyond its sensory properties, citral exhibits antifungal, antibacterial, insecticidal, expectorant, spasmolytic, appetite-stimulating, anti-inflammatory, antioxidant, and mild diuretic effects [9]. Thymoquinone, the primary

constituent of black seed oil [10], possesses antifibrotic, antioxidant, and anti-inflammatory properties and has been shown to decrease the oxidative stress induced by cardiac ischemia/reperfusion injury or drug-induced cardiotoxicity [11]. *Silybum marianum* extract contains several flavonolignans known collectively as silymarin [12]. Recognized as a cardioprotective agent, silymarin exhibits antioxidant and anti-cardiovascular disease effects, thereby offering potential to protect against the oxidative stress induced by atherosclerosis and cardiotoxicity [13].

Based on the above evidence, the present study aimed to evaluate the cardioprotective effects of silymarin, citral, and thymoquinone on MTX-induced oxidative cardiotoxicity in rats.

MATERIALS AND METHODS

1. Animals

The study included 48 male Sprague–Dawley rats, each weighing 200 ± 20 g. These rats were purchased from Shiraz University of Medical Sciences and were given 1 week to acclimate to the new environment of the laboratory animal house at the School of Veterinary Medicine. During this period, they had free access to water and pelleted food. The rats were kept in a controlled environment with a 12-hour light/12-hour dark cycle and a temperature of 25 ± 3℃. To ensure the rats' well-being and minimize the risk of infectious diseases, the researchers cleaned and disinfected the cages every 3 days and changed the bedding regularly. All animal handling procedures followed the international guidelines for the care and use of experimental animals. The study was approved by the local Research Ethical Committee at the University of Shiraz, Iran (1GCB3M348200).

2. Study groups

The rats were randomly divided into 6 groups of 8 as follows: group A (healthy control), healthy rats receiving no intervention; group B (MTX treatment), rats receiving a single intraperitoneal (i.p.) injection of 20 mg/kg MTX on day 3; group C (drug carrier), rats receiving once daily i.p. injections of dimethyl sulfoxide for 10 days, with a single i.p. injection of 20 mg/kg MTX on day 3; and groups D, E, and F (citral, thymoquinone, and silymarin treatment, respectively), rats receiving once daily i.p. injections of 25 mg/kg citral, thymoquinone, and silymarin, respectively, throughout the study, with a single i.p. injection of 20 mg/kg MTX on day 3.

The dose with the highest antioxidant effect was selected based on a review of previous studies that used these three compounds to control oxidative stress [14-17]. In an unpublished study by the same authors of the present study, various i.p. doses of citral were examined, with 25 mg/kg showing antioxidant effects without significant complications. During this study, the biochemical profile of the animals was evaluated.

On day 10, the rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and their blood samples were collected for biochemical analysis. Then, they were euthanized with carbon dioxide, and their heart samples were obtained for histopathological analysis and antioxidant activity measurement.

3. Serum biochemical measurements

Blood samples were allowed to clot and then centrifuged at 750 g for 15 minutes to separate the serum. Serum biochemical parameters, including creatine kinase-myoglobin binding (CK-MB), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH), were measured using standard methods and commercial kits (Pars Azmoon Co., Tehran, Iran) on a biochemical autoanalyzer (Alpha Classic AT++, Sanjesh, Iran).

4. Heart tissue extraction

One gram of heart tissue was weighed and placed in a test tube. Next, 5 mL of phosphate buffer was added to the heart tissue, and the mixture was thoroughly homogenized using a homogenizer. Afterward, the solution was centrifuged at 2,500 rpm for 5 to 10 minutes, and the resulting supernatant was collected for subsequent parameter measurements.

5. Cardiac total antioxidant capacity (TAC) and malondialdehyde (MDA) measurements

A commercial kit (ZellBio GmbH kit, Germany) was used to determine TAC levels. The color product of the chromogenic substrate (tetramethylbenzidine) appeared in the end. The color difference was measured at 450 nm using a spectrophotometer (Jenway 6300 Spectrophotometer, UK) and reported as mmol/L, with a sensitivity of 0.1 mM (100-μmol/L) TAC and intra- and inter-assay coefficients of variation (CVs) of less than 3.4% and

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4.2%, respectively. MDA measurement was conducted using an assay kit (Cat. no. ZB-MDA96A, ZellBio GmbH, Germany), where MDA reacted with thiobarbituric acid under acidic conditions and high temperatures to form a color complex, which was quantified at 535 nm. The assay kit had a sensitivity of 0.1 μM MDA and an inter-assay CV of 5.8%.

6. Histopathological evaluation

The rats were promptly sacrificed, and their heart tissue samples (1 cm × 1 cm × 1 cm) were collected and fixed in 10% neutral buffered formalin for 48 hours. After dehydration, clarification, and paraffinization, a microtome was used to prepare 4- or 5-μm-thick paraffin sections, which were stained with hematoxylin and eosin and subsequently examined under a light microscope.

7. Data analysis

Data are presented as mean ± standard error of the mean. Analysis of variance, followed by Tukey's post hoc test, was used for statistical comparisons. A p value less than 0.05 was considered statistically significant.

RESULTS

1. Serum biochemical parameters of heart function

1) CPK

The CPK level in group B was 560.00 ± 102.70 U/L and did not change with drug carrier administration. However, treatment with silymarin, citral, and thymoquinone reduced CPK levels to 323.30 ± 42.72 U/L, 405.90 ± 67.41 U/L, and 271.20 ± 30.89 U/L, respectively, with the observed reduction in CPK following thymoquinone administration being significant ($p < 0.05$; Fig. 1).

2) CK-MB

After i.p. injection of silymarin, citral, and thymoquinone, CK-MB levels decreased to 861.60 ± 192.90 U/L, 783 ± 153.00 U/L, and 460.00 ± 32.86 U/L, respectively, representing decreases of 16.51%, 24.13%, and 55.43%, respectively, compared with the MTX group (1,032 ± 113.90 U/L). The reduction was significant in the thymoquinone treatment group ($p < 0.05$; Fig. 2).

3) LDH

The LDH level in the MTX group was 1303.00 ± 189.00 U/L. The administration of silymarin, citral, and thymoquinone reduced LDH levels to 897.40 ± 113.60 U/L, 759.30 ± 163.20 U/L, and 642.60 ± 123.40 U/L, respectively. A maximum decrease of 50.68% was observed in the thymoquinone treatment group (Fig. 3).

2. Cardiac histopathology

1) Group A

Normal heart histology was observed (Fig. 4A).

2) Group B

Mild to moderate myocardial fiber hemorrhage, slight edema, severe congestion, and minimal perivascular inflammatory cell infiltration were observed (Fig. 4B). No histopathological lesions (e.g., necrosis, degeneration, and fibrosis) were present.

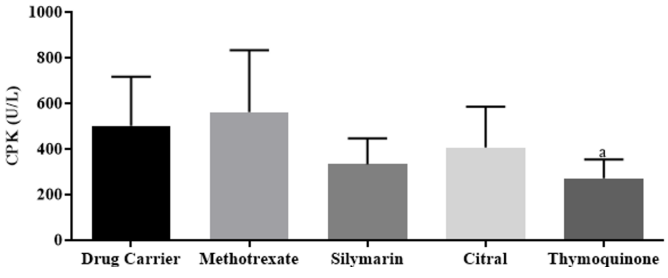

Figure 1. The effect of silymarin, citral and thymoquinone on serum creatine phosphokinase (CPK) concentration in rats under influences of Methotrexate (n = 8). ANOVA statistical test and Tukey's post hoc test are performed and the results are presented as mean ± SEM ($^{a}p < 0.05$ compared to Methotrexate group).

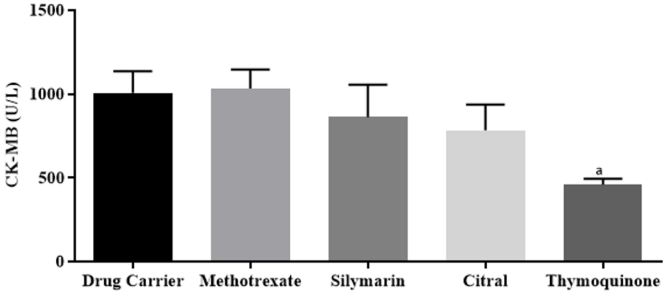

Figure 2. The effect of silymarin, citral and thymoquinone on serum creatine kinase (CK-MB) concentration in rats under influences of Methotrexate (n = 8). ANOVA statistical test and Tukey's post hoc test are performed and the results are presented as mean ± SEM ($^{a}p < 0.05$ compared to methotrexate group).

3) Group C

Except for very mild hemorrhage and congestion, no specific histopathological lesions were observed in the epicardium, myocardium, or endocardium (Fig. 4C). The heart structure was histologically normal and similar to that in the control group.

4) Group D

Focal hemorrhages and mild congestion were observed, with no specific pathological lesions such as necrosis, myocardial fiber degeneration, or inflammatory cell infiltration (Fig. 4D).

5) Group E

Pathological lesions such as necrosis, myocardial fiber degeneration, and inflammatory cell infiltration were not present. The heart structure appeared normal and similar to that in the control group (Fig. 4E). Only congestion and mild hemorrhage were observed.

6) Group F

No pathological lesions were observed, except for congestion and mild hemorrhage (Fig. 4F).

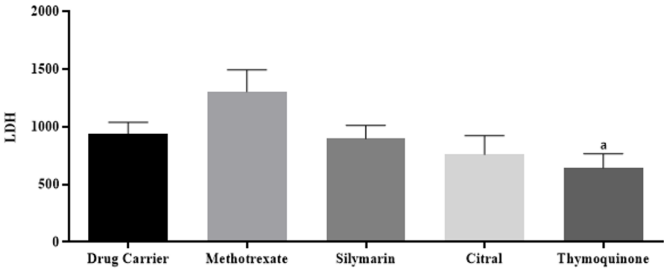

Figure 3. The effect of silymarin, citral and thymoquinone on serum concentration of lactate dehydrogenase (LDH) in rats under influences of Methotrexate (n = 8). ANOVA statistical test and Tukey's post hoc test are performed and the results are presented as mean ± SEM ($^{a}p < 0.05$ compared to methotrexate group).

3. Antioxidant parameters of heart tissue

1) TAC

Silymarin, citral, and thymoquinone increased TAC by 33%, 18.11%, and 59.30%, respectively, compared with the MTX group. However, the increase was significant only for the thymoquinone group ($p < 0.05$; Fig. 5).

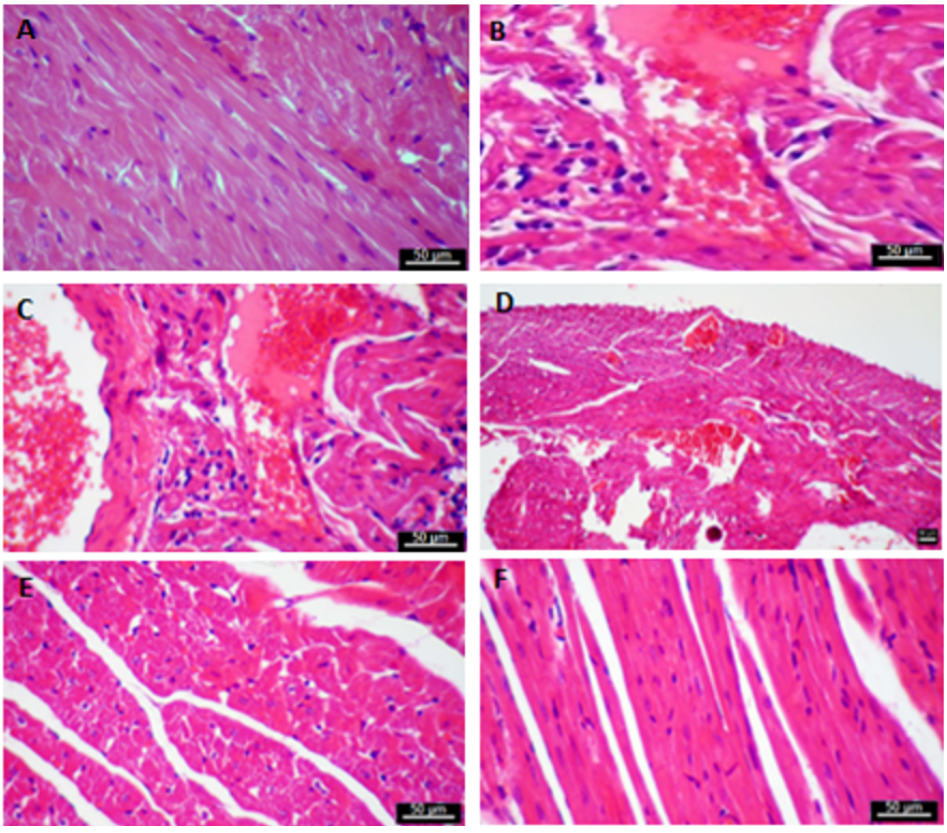

Figure 4. Histopathological examination of myocardia. (A) Normal structure and architecture of myocardia in healthy rats. (B) Severe vascular hemorrhage, congestion, edema fluid and mild presence of inflammatory cells in the heart tissue of methotrexate group rats. (C) Histological structure of cardiac muscle filaments or cells and their cytoplasm in the heart tissue of rats in the drug carrier group (H&E, 400×). (D) Vascular congestion and focal hemorrhages in the myocardial muscle fibers of heart tissue of citral group rats (H&E, 100×). (E) Cardiac muscle fibers without any pathological lesions in rats receiving silymarin (H&E, 400×). (F) Cardiac striated muscle fibers without any pathological lesions in thymoquinone group rats (H&E, 400×).

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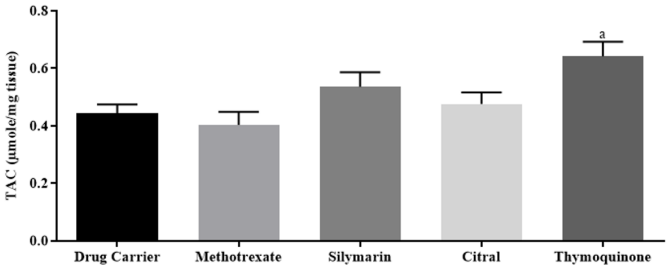

Figure 5. The effect of silymarin, citral and thymoquinone on cardiac tissue concentration of total antioxidant capacity (TAC) in rats under influences of Methotrexate (n = 6). The results of statistical test (ANOVA) and Tukey's post hoc test are shown as mean ± SEM ($^{a}p < 0.05$ compared to methotrexate and drug carrier group).

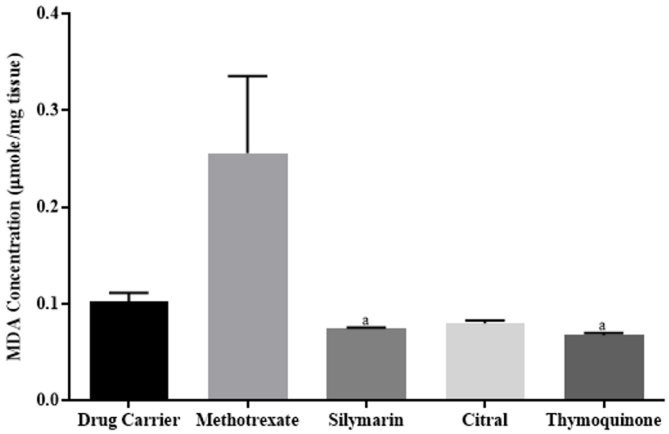

Figure 6. The effect of silymarin, citral and thymoquinone on cardiac tissue concentration of total antioxidant capacity (MDA) in rats under influences of Methotrexate (n = 6). The results of statistical test (ANOVA) and Tukey's post hoc test are shown as mean ± SEM ($^{a}p < 0.05$ compared to methotrexate group).

2) MDA

The concentration of MDA declined in the silymarin (0.074 ± 0.001 mmol/mg protein), citral (0.079 ± 0.00 mmol/mg protein), and thymoquinone (0.067 ± 0.002 mmol/mg protein) groups compared with the MTX group (0.255 ± 0.07 mmol/mg protein), with the reductions being significant for the silymarin and thymoquinone groups ($p < 0.01$; Fig. 6).

DISCUSSION

In this study, MTX injection decreased TAC and increased MDA, leading to elevated CPK and CK-MB levels due to oxidative stress. Conversely, treatment with citral, thymoquinone, and silymarin resulted in increased TAC and decreased MDA levels. In other words, these compounds were able to improve biochemical makers of heart function (i.e., CPK and CK-MB) by inhibiting MTX-induced oxidative stress.

MTX is broadly used to treat various types of malignancies, including lymphoma and breast cancer [1]. In lower doses, MTX is also used to manage several autoimmune diseases, such as rheumatoid arthritis and psoriasis [2]. This medication first inhibits DHFS enzymatic action and then blocks the production of purine and pyrimidine bases essential for DNA and RNA synthesis [3]. Mahmoud et al. [6] reported MTX-induced cardiac toxicity (as evidenced by histopathological and clinical biochemistry examinations), which was accompanied by decreased antioxidant activity. They showed that MTX led to an increase in cardiac enzymes and caused histopathological changes by reducing TAC. In the current study, MTX was found to induce oxidative stress in the heart. This was evidenced by decreased TAC and increased MDA levels. Consequently, heart damage occurred, as indicated by elevated levels of CPK and CK-MB.

Several studies have highlighted the cardioprotective and antioxidant effects of thymoquinone [18-23]. Danaei et al. [18] demonstrated that oral (p.o.) administration of thymoquinone at 10 mg/kg/day increased the activity of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPX) in heart tissue while decreasing the levels of CK-MB, triose-phosphate isomerase, and lipid peroxidation in diazinon-induced cardiotoxicity. El-sheikh et al. [19] reported that thymoquinone (10 mg/kg/day, p.o.) reduced MTX-induced intestinal lesions and improved oxidative stress and inflammation. Nagi and Mansour [20] showed the ability of thymoquinone (10 mg/kg/day, p.o.) to reduce LDH and CPK levels in doxorubicin-induced cardiotoxicity, along with its antioxidant and free radical scavenging activities. Shirmard et al. [21] found that thymoquinone (0.5 mg/kg, i.p.) reduced the levels of troponin and MDA to normal in trastuzumab-induced cardiotoxicity while increasing the level of glutathione (GSH) and preserving normal heart tissue structure. In another study, thymoquinone (10 and 20 mg/kg, p.o.) decreased CK-MB, LDH, and aspartate transaminase (AST) and increased CAT, SOD, GPX, GSH reductase, and GST in rats suffering from doxorubicin-induced cardiotoxicity [22]. Hafez et al. [23] reported that thymoquinone preserved cardiomyocyte structure by reducing reactive oxygen species (ROS) and increasing GSH in clozapine-induced cardiotoxicity. In our study, i.p. injection of 10 mg/kg thymoquinone for 10 days led to a significant increase in TAC and significant decreases in MDA, CPK, CK-MB,

and LDH levels compared with those observed in the MTX group, indicating the preventive effects of thymoquinone.

Cengiz [24] demonstrated that i.p. injection of 50 mg/kg silymarin reduced adriamycin-induced cardiotoxicity by decreasing the levels of lipid peroxides, serum LDH, and serum CPK and increasing the level of GSH in heart tissue. Histopathological evaluation revealed that silymarin mitigated adriamycin-induced lesions. Avci et al. [25] reported that silymarin (100 mg/kg/day, p.o.) attenuated cyclophosphamide-induced cardiotoxicity by normalizing both biochemical parameters of heart function and antioxidant markers in heart tissue. In a study conducted in 2023, it was found that silymarin could protect the heart from damage caused by doxorubicin [26]. According to a study by Aktas and Ozgocmen [27], silymarin (100 mg/kg/day, p.o.) decreased LDH, CK-MB, alanine transaminase, and AST levels in rats receiving valproic acid, and normalized the decreased and increased levels of GSH and MDA, respectively. In another study, silymarin (25, 50, and 100 mg/kg/day, i.p.) was found to normalize the levels of GSH, SOD, and CAT in rats treated with acrolein [28]. Pretreatment with silymarin not only reduced MDA, troponin, and creatinine levels already elevated by acrolein, but also mitigated cardiac tissue pathological changes. Rahimi et al. [29] reported that silymarin reduced diabetes-induced heart failure by downregulating the gene expression of urotensin II and its receptor gene expression while modulating oxidative stress. They also confirmed that silymarin reduced the levels of CK-MB, LDH, and oxidative stress markers (MDA and NO). In the current study, i.p. injection of 50 mg/kg silymarin increased antioxidant capacity compared with the MTX group while decreasing MDA, CPK, CK-MB, and LDH levels as well as mitigating pathological changes.

Phillips et al. [30] demonstrated that citral was rapidly excreted through urine. It was also shown to protect human endothelial cells against hydrogen peroxide-induced oxidative stress and, when used as pretreatment, increase ferric reducing antioxidant power [31]. Citral also reduced myeloperoxidase activity and nitric oxide production in acetaminophen-induced liver toxicity. In our study, i.p. injection of 25 mg/kg citral for 10 days increased cardiac TAC and decreased MDA, LDH, CPK, and CK-MB levels compared with the MTX group. Yardımcı et al. [32] reported that both thymoquinone and silymarin reduced the oxidative stress induced by aortic ischemia-reperfusion in the heart. They also found thymoquinone more effective than silymarin in improving TAC. Our results corroborate this finding, showing that the effect of thymoquinone on improving TAC was greater than that of silymarin and citral. In the present study, thymoquinone also reduced MTX-induced heart failure more effectively than did silymarin and citral, demonstrating the highest antioxidant effect and leading to a greater decrease in MDA production and a greater increase in TAC.

While our study revealed differences in the improvement of heart function parameters and oxidative stress among the treatment groups, these differences were not statistically significant. Nevertheless, this lack of significance may change with an increase in the number of samples. Additionally, future studies could investigate ROS levels and *Nrf2* gene expression to further elucidate the role of oxidative stress in MTX toxicity.

CONCLUSION

This study confirmed that silymarin, citral, and thymoquinone prevent MTX-induced cardiotoxicity through their antioxidant effects. Thymoquinone had the highest cardioprotective and antioxidant effects compared with silymarin and citral.

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ETHICAL APPROVAL

All animal handling procedures followed international guidelines for the care and use of experimental animals. The study was approved by the local Research Ethical Committee at the University of Shiraz, Iran (Approval No.: 1GCB3M348200).

CONFLICTS OF INTEREST

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

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