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Protective Impact of Chia Seed Oil on Autophagic and Ferroptotic Cell Death in Methotrexate-Induced Liver and Kidney Damage

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ABSTRACT

Chia seed oil (CSO) contains essential fatty acids as well as various phenolic compounds. Methotrexate (MTX), which targets rapidly dividing cancer cells and immune system cells, is used in various tumor formations but causes undesirable effects on the kidneys, liver, and various organs. In our study, we primarily investigated the effects of chia seed oil on autophagy and ferroptosis in liver and kidney damage. For this purpose, we formed 3 groups with 21 male Balb/c mice. The Control group received nothing except standard feed and water. The MTX group received a single dose of 20 mg/kg methotrexate intraperitoneally on day 1. The MTX-CSO group received a single dose of 20 mg/kg methotrexate intraperitoneally on day 1, along with 4 ml/kg of chia seed oil administered via gavage. Chia seed oil treatment was continued for 7 days. On the 8th day, the mice were euthanized, and liver and kidney tissues were analyzed using histopathological, immunohistochemical, and double immunofluorescence staining methods. In conclusion, the severe histopathological findings observed in the MTX groups were alleviated in the MTX-CSO group. Immunohistochemical and dual immunofluorescence staining results show that while MTX activates autophagy and ferroptosis markers, CSO suppresses them, exhibiting a protective and regulatory effect. Our research is the first original study to investigate the effect of CSO on autophagic and ferroptotic cell death.

Keywords: Autophagy, Chia seed oil, Ferroptosis, Immunofluorescence, Immunohistochemistry.

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Introduction

Chia is a plant classified within the *Lamiaceae* family, and its seeds have been used by various civilizations due to their nutritional properties. Chia seed oil (CSO) contains alpha-linolenic acid and linoleic acid, as well as powerful antioxidants such as phenolic acids, flavonoids, phytosterols, and tocopherols. (Balakrishnan et al., 2025; Shen et al., 2018). Methotrexate (MTX), a folic acid antagonist, is an antineoplastic drug that inhibits DNA synthesis and cell division. MTX causes cytotoxic spread, particularly in the heart and kidneys (Sweilam et al., 2024). MTX, which targets rapidly dividing cancer cells and immune system cells, is used for therapeutic purposes in various cancers and autoimmune diseases. However, it causes undesirable effects in the kidneys, liver, gastrointestinal system, lungs, and various other organs (Marin et al., 2022). Autophagy is a natural, orderly biological degradation process that breaks down unnecessary components of a cell. Beclin-1 and LC3B proteins are involved in the basic mechanism of autophagy. Ferroptosis, on the other hand, is a new form of cell death caused by the accumulation of lipid peroxides, which are toxic to the cell (Park & Chung, 2019). Products obtained using plant-derived antioxidants

have been used for years in the treatment of many diseases such as diabetes, obesity, and circulatory system disorders, and the number of studies in this field continues to increase day by day (Governa et al., 2018). In this experimental study, we investigated the effect of chia seed oil on autophagy and ferroptosis in the liver and kidney. For this purpose, we investigated the effects of chia seed oil on MTX-induced liver and kidney damage using histopathological methods. We also examined the effect of chia seed oil on autophagy and ferroptosis markers such as Beclin-1, LC3B, GPX4, and TFRC using immunohistochemical and dual immunofluorescence methods. In this respect, our study is important as it is the first study to investigate the effect of chia seed oil on autophagy and ferroptosis.

Methods

Animals and Experimental Protocol

Our research was conducted under ethical approval number 2025-1/191, issued by the Adana Veterinary Institute Local Ethics Committee for Experimental Animals on April 25, 2025. The dosage, amount, and method of application of MTX used in our experimental study were determined considering the cytotoxic effects previously reported by Belhan et al. (2019). The dosage, amount, and method of application of chia seed oil were determined by us as 4 ml/kg, based on the protective dose range (2.5–5.0 ml/kg) reported by Ahmed et al. (2021). Sample size was calculated using a power analysis with 8% bias, a 0.05 type I error (α), and type II error (β) (Power = 0.80), resulting in at least 7 animals per group. Twenty-one male Balb/c mice (8–10 weeks old, 20–25 g) were randomly divided into three groups. The CONTROL group received standard feed and water only. The MTX group received a single 20 mg/kg intraperitoneal dose of methotrexate on day 1. The MTX+CSO group received the same MTX dose on day 1, and chia seed oil administration started on day 1 concurrently and continued once daily for seven consecutive days. On day 8, all animals were euthanized, and liver and kidney tissues were collected and fixed for histopathological, immunohistochemical, and double immunofluorescence analyses.

Drugs and Natural Products Used in the Study

The methotrexate used in the study was obtained from a nearby pharmacy by Koçsel İlaç Sanayi ve Ticaret A.Ş. (Istanbul, Turkey). The natural product, chia seed oil, was obtained from the manufacturer, Botaniksan (Isparta, Türkiye).

Histopathological Method

Liver and kidney tissues obtained from necropsied mice were incubated in 10% buffered formaldehyde. After undergoing standard alcohol/xylene follow-up steps, the tissues were subjected to paraffin blocking. Sections averaging 3-5 µm in thickness were placed on slides and stained with hematoxylin-eosin. Following staining, histopathological changes in liver and kidney tissue were evaluated in detail. Histopathological evaluation was performed on hematoxylin–eosin–stained liver and kidney sections by two independent observers blinded to the experimental groups. Three non-overlapping fields per tissue section were examined at ×50 magnification. Tissue injury was scored semi-quantitatively using a 0–4 scale: 0, none; 1, mild; 2, moderate; 3, severe; 4, very severe.

Immunohistochemical Staining Method

Kidney and liver tissues from mice were stained immunohistochemically using Beclin 1 and LC3B primary antibodies. For this purpose, tissue sections taken on 3-5 µm thick polylysine-stained slides were subjected to xylene/alcohol processes and washed with PBS. They were incubated in 3% H_2O_2 for 10 minutes to allow endogenous peroxidase inactivation. To reveal the antigen in the tissues, antigen retrieval was performed by incubation at 500 watts for 2 x 5 minute periods. Afterwards, they were incubated overnight at +4°C with Beclin 1 (BT-LAB brand, Catalog number: BT-MCA0211) and LC3B (SANTA CRUZ brand, Catalog number: SC-271625) primary antibodies at a dilution of 1/200. Anti-Polyvalent, HRP secondary (THERMOFISCHER brand, Catalog number: TP-125-HL) was applied according to the manufacturer's recommendation. DAB chromogen was used in the method. After counterstaining with Mayer’s Hematoxylin, the sample was covered with entellan and examined using a Zeiss brand, Axiocam 305 color camera and Colibri 3 light microscope imaging system. In the study, immunoreactivity severity was assessed using the Zen Blue 3.1 software program. Immunohistochemical staining (Beclin-1, LC3B, GPX4, TFRC) was assessed manually, considering both the percentage of positive area and staining intensity using the same 0–4 scoring scale. Observers were blinded to group allocation to minimize bias.

Dual Immunofluorescence Staining Method

Sections 3-5 µm thick, obtained on polylysine-containing slides, were washed with PBS following xylene/alcohol steps. Endogenous peroxidase inactivation was achieved by incubation in 3% H_2O_2 for 10 minutes. To reveal the antigen in the tissues, the sections were incubated with antigen retrieval at 800 watt current for 2x5 minutes each. After washing with PBS for 2x10 minutes each, the sections were incubated in PBS/Gelatin/Triton x100 (0.25%) solution for 10 minutes. Subsequently, they were blocked with 5% BSA for 60 minutes. Following blocking, they were incubated overnight at +4°C with GPX4 (BT-LAB brand, Catalog no: BT-AP02883) and TFRC (BT-LAB brand, Catalog no: BT-MCA2826) primary antibodies at a dilution ratio of 1/200. The sections, washed again with PBS, were incubated for another 10 minutes in PBS/Gelatin/Triton x100 (0.25%) solution before proceeding to the secondary antibody process. During this process, sections were incubated for 1 hour in 1% BSA with Goat Anti-Mouse FITC (IMMUNORESEARCH, Catalog number: 115-095-003) and Anti-rabbit Alexa Fluor 595 (CELL SIGNALING, Catalog number: 8889S) secondary antibodies mixed at a 1/200 dilution ratio. Sections from tissues washed with PBS were washed in 10 mM $CuSO_4$ /50 mM NH_4Cl solution for 10 minutes. After washing with distilled water, DAPI (4',6-diamidino-2-phenylindole) was added to the sections and examined using a Zeiss Axiocam 305 color camera with fluorescence attachment and a Colibri 3 light microscope imaging system. Based on the examinations, GPX4 immunopositives were displayed in red and TFRC immunopositives in green using the Zen Blue 3.1 software program, and their severity was evaluated accordingly. Dual immunofluorescence staining (Beclin-1/LC3B, GPX4/TFRC) was scored separately for each marker in the same tissue sections at ×50 magnification using the same 0–4 semi-quantitative scale, with observers blinded to group allocation.

Statistical Evaluation

Histopathological, immunohistochemical, and dual immunofluorescence data were analyzed using SPSS 20.0 (IBM SPSS Corp., Armonk, NY, USA). Normality was assessed using the Shapiro–Wilk test. Non-parametric variables were compared using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests with Bonferroni correction for multiple comparisons. Results are presented as median and interquartile range (IQR) or mean ± standard deviation (SD), as appropriate. Exact p-values are reported when possible, and $p < .05$ was considered statistically significant. Graphs were generated using GraphPad Prism 8 (GraphPad Software, USA).

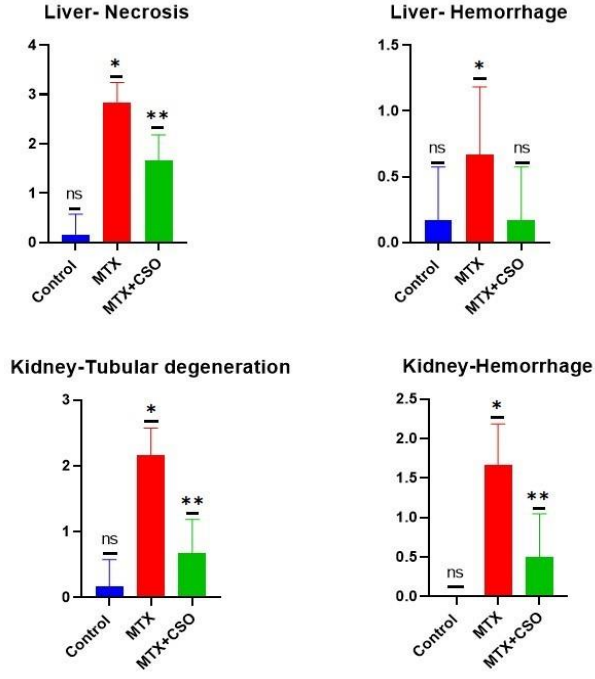
Results

Histopathological Results

Our evaluations revealed statistically significant differences in histopathological findings in the kidneys and liver (Figure 1).

Figure 1.

Statistical analysis of histopathological findings.

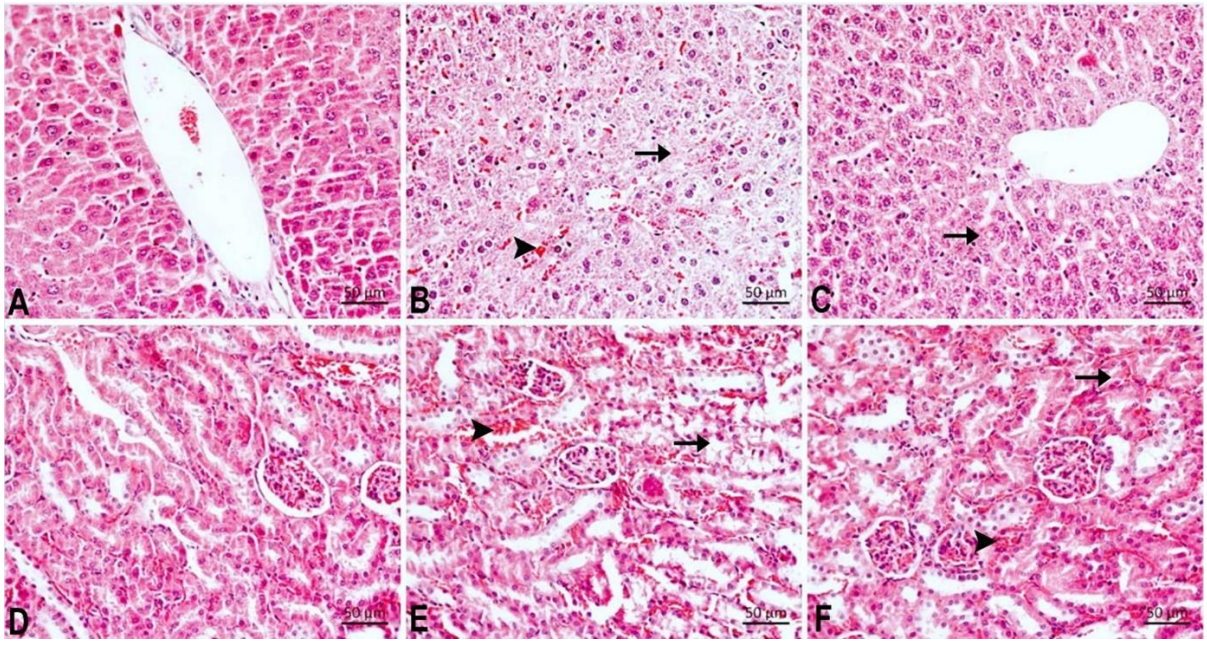


*Note: *p <.05, **p <.01; ns: not significant. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).*

We observed that the control groups maintained their normal histological structure. When examining the other treatment groups, we observed severe hepatocyte necrosis and mild hemorrhage in the livers of the MTX group. In the MTX+CSO group, we observed a moderate reduction in hepatocyte necrosis and the absence of hemorrhage. Our examinations of the kidneys revealed that the moderate degeneration and hemorrhage in the renal tubules observed in the MTX group were reduced to a milder level in the MTX+CSO group (Figure 2).

Figure 2.

Histopathological appearance of liver (A–C) and kidney (D–F).



Note: Liver: (A) Control group, showing normal hepatic architecture; (B) MTX group, showing severe necrosis (→) with moderate hemorrhage (►); (C) MTX+CSO group, showing severe necrosis (→). Kidney: (D) Control group, showing normal renal architecture; (E) MTX group, showing moderate tubular degeneration (→) with intertubular hemorrhage (►); (F) MTX+CSO group, showing mild tubular degeneration (→) with intertubular hemorrhage (►). Hematoxylin and eosin (H&E) staining, 50 µm scale bar. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).

Immunohistochemical Findings

Immunohistochemical analysis revealed significant differences in Beclin 1 and LC3B (Figure 3).

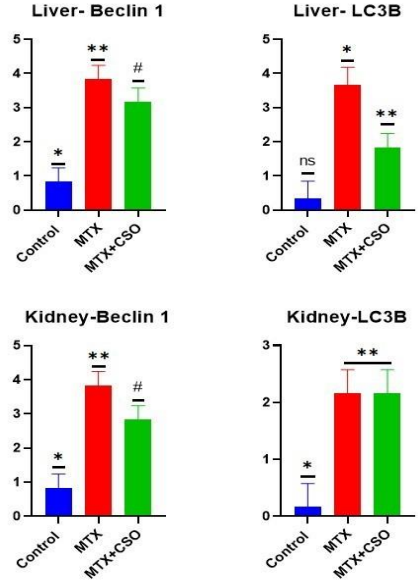
Beclin 1 immunopositivity in the liver and kidney was mild in the control group, very severe in the MTX group, and severe in the MTX+CSO group. LC3B immunopositivity was not observed in the control groups, but was very severe in the liver and moderate in the kidney. When we examined the MTX+CSO group, both markers were moderate (Figure 4-5).

Dual Immunofluorescence Findings

When we evaluated the dual immunofluorescence stainings performed for GPX4 and TRFC purposes, we observed statistically significant differences between the groups (Figure 6).

Figure 3.

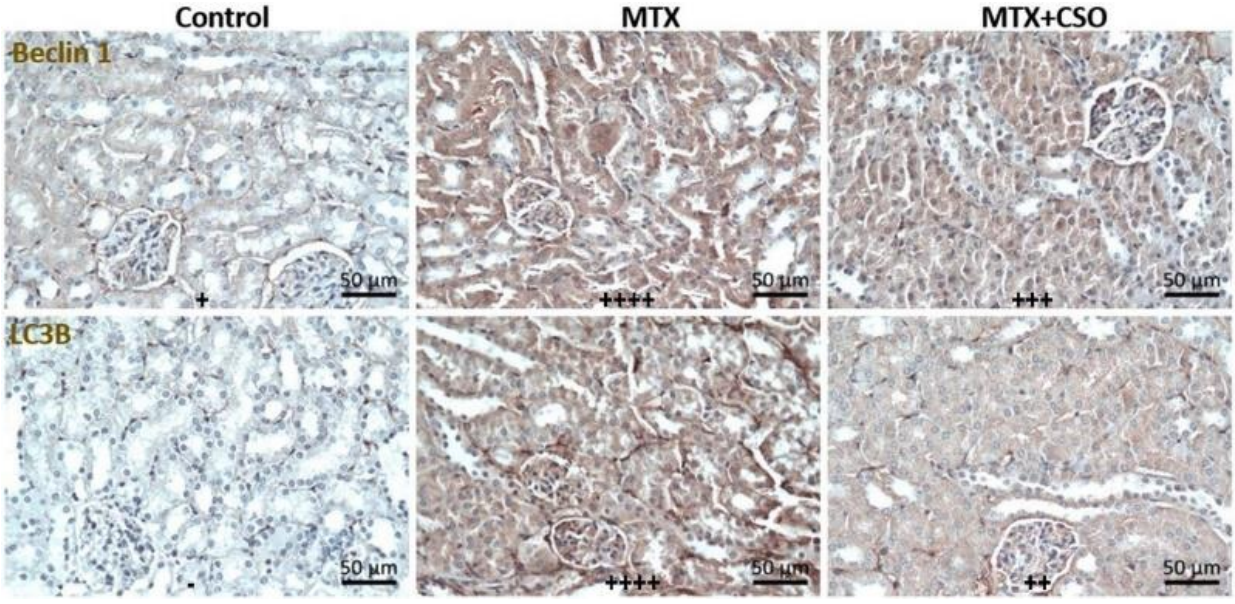
Statistical analysis of immunohistochemical findings.



*Note: *p < .05, **p < .01, #p < .001. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).*

Figure 4.

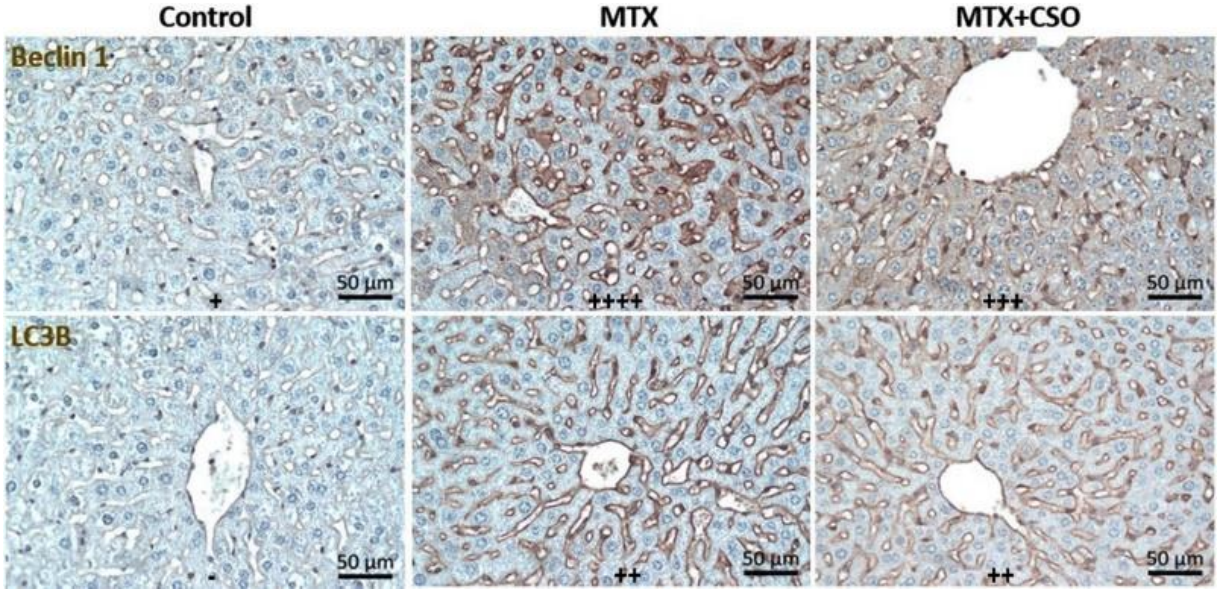
Immunohistochemical staining of liver tissue for Beclin 1 and LC3B.



Note: Control group showing mild Beclin 1 positivity (+) and LC3B immunonegative (-); MTX group showing very severe Beclin 1 (++++) and moderate LC3B positivity (++); MTX+CSO group showing severe Beclin 1 (+++) and moderate LC3B positivity (++). None (-), Mild (+), Moderate (++), Severe (+++), Very Severe (++++). Immunohistochemical staining, 50 µm scale bar. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).

Figure 5.

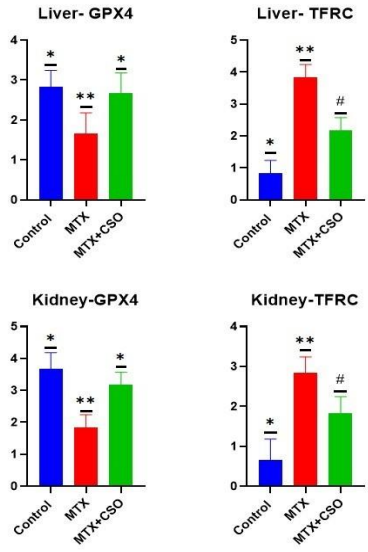
Immunohistochemical staining of kidney tissue for Beclin 1 and LC3B.



Note: Control group showing mild Beclin 1 positivity (+) and LC3B immunonegative (-); MTX group showing very severe Beclin 1 (++++) and LC3B positivity (++); MTX+CSO group showing severe Beclin 1 (+++) and moderate LC3B positivity (++). None (-), Mild (+), Moderate (++), Severe (+++), Very Severe (++++). Immunohistochemical staining, 50 µm scale bar. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).

Figure 6.

Statistical analysis of immunofluorescence findings.



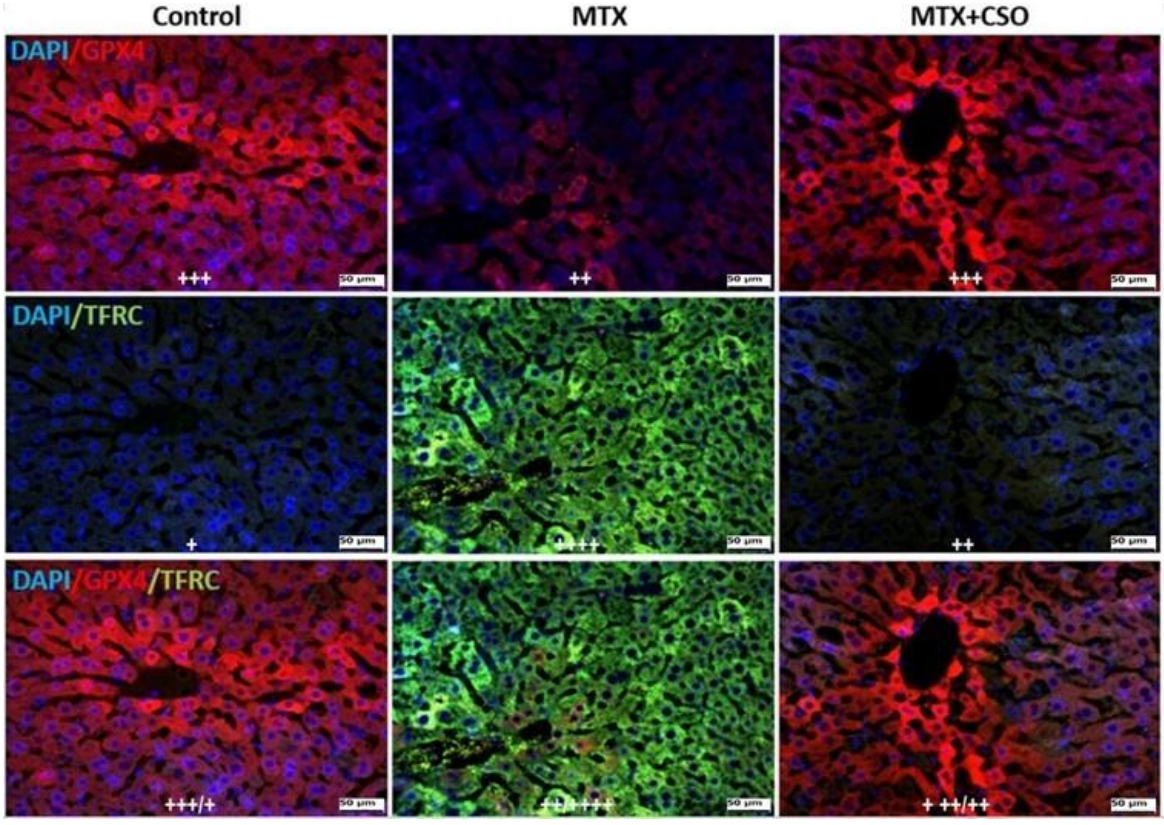
*Note: *p <.05, **p <.01, #p <.001, indicating differences between groups. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).*

In the dual immunofluorescence stainings, we saw an inverse relationship between GPX4 and TRFC rates. In the liver, GPX4 immunopositivity, which was very severe in the Control group, was moderate in the MTX group and severe in the MTX+CSO group. TFRC immunopositivity was mild in the Control group, severe in the MTX group, and moderate in the MTX+CSO group, respectively (Figure 7).

In the dual immunofluorescence stainings we performed on the kidneys, GPX4 immunopositivity was moderate in the MTX group and severe in the Control and MTX+CSO groups. TFRC immunopositivity was mild in the Control group, very severe in the MTX group, and moderate in the MTX+CSO group, respectively (Figure 8).

Figure 7.

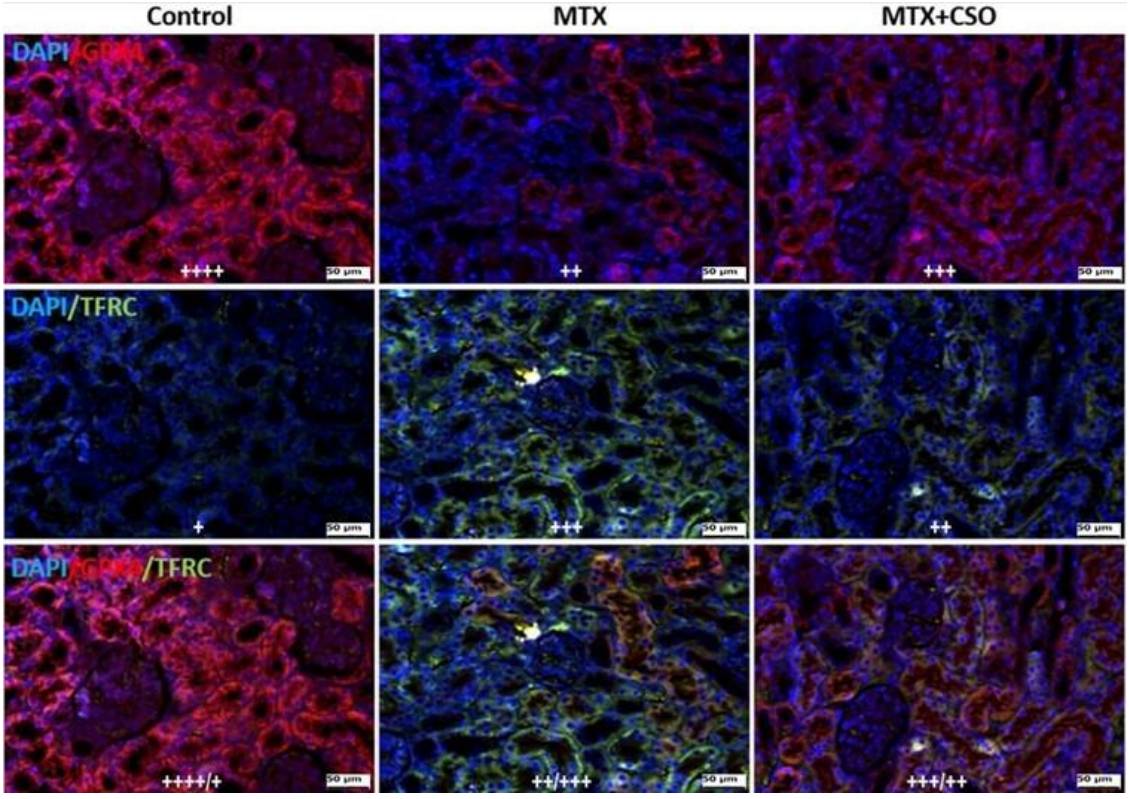
Dual immunofluorescence staining of liver tissue for GPX4 and TFRC.



Note: Control group showing severe GPX4 (+++) and mild TFRC positivity (+); MTX group showing moderate GPX4 (++) and very severe TFRC positivity (++++); MTX+CSO group showing severe GPX4 (+++) and moderate TFRC positivity (++). None (-), Mild (+), Moderate (++), Severe (+++), Very Severe (++++). Dual immunofluorescence staining, 50 µm scale bar. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).

Figure 8.

Dual immunofluorescence staining of kidney tissue for GPX4 and TFRC.



Note: Control group showing very severe GPX4 (++++) and mild TFRC positivity (+); MTX group showing moderate GPX4 (++) and severe TFRC positivity (+++); MTX+CSO group showing severe GPX4 (+++) and moderate TFRC positivity (++). None (-), Mild (+), Moderate (++), Severe (+++), Very Severe (++++). Dual immunofluorescence staining, 50 µm scale bar. (MTX: methotrexate; MTX+CSO: methotrexate + chia seed oil).

Discussion

MTX, an antineoplastic drug with cytotoxic effects on organs, is excreted via the proximal tubules of the kidneys. Therefore, the kidneys are the primary target for MTX (El-Agawy et al., 2022). Numerous studies on experimental animals have shown widespread cytotoxic effects on the liver associated with MTX use, including acute hepatocyte necrosis, liver fibrosis, and fatty liver disease (Ezhilarasan, 2021). Due to the common liver and kidney damage associated with MTX, we focused our study on these organs. Histopathology of the MTX groups showed advanced hepatocyte necrosis and hemorrhage in the livers, while moderate degeneration and hemorrhage occurred in the renal tubules. These histopathological results support the results reported by Sahindokuyucu-Kocasari et al. (2021) in studies on mice. In many studies, plant-based antioxidants have been used therapeutically against MTX and other agents with undesirable effects. Khalifa et al. (2023) reported that CSO improved neural dysfunction in the brains of rats experiencing stress. Chia seeds are a rich source of lipids, proteins, polyphenolic compounds, and omega-3 (ALA, LA) fatty acids (Imran et al. 2016). Olayinka et al. (2016) reported that gallic acid, a component of CSO, has protective properties against MTX-induced liver and kidney damage. Balakrishnan et al. (2025) showed that CSO contains 6 different phenolic acids, including rosmarinic acid and gallic acid. Cetin et al. (2008) reported that grape seed extract, a plant product, protects the liver by suppressing oxidative stress. Zhang et al. (2024) reported that high amounts of omega-3 fatty acids in CSO provide protection against cisplatin-induced kidney damage, a chemotherapeutic agent. In a similar study, Abd Alhusen and Hasan (2023) reported that omega-3-6-9 showed a protective effect in cisplatin-induced kidney damage. Satyam et al. (2025) reported that CSO showed a protective effect in experimental liver damage. Kankılıç et al. (2024) reported that Naringin showed a protective effect via the Beclin-1, LC3B, and LC3A signaling pathways in rat testicular damage. Jia et al. (2024) reported that in an experimental study, it suppressed ferroptosis in the ischemic brain via the TFR1 pathway. In our study, the effect of CSO on MTX-induced liver and kidney damage was investigated using Beclin-1, LC3B, GPX4, and TFRC markers.

The effect of CSO on autophagic and ferroptotic cell death was examined. In this respect, our study has specific features for CSO. In our study, changes in liver and kidney tissues were evaluated histopathologically. In addition, autophagy and ferroptosis markers were evaluated using immunohistochemical and double immunofluorescence staining methods, making a significant contribution to the literature. Our results suggest that MTX+CSO treatment may alleviate the impairment of MTX-induced autophagy and ferroptosis markers. Future, more comprehensive studies with CSO alone would help clarify the direct role of CSO and contribute to a better understanding of its protective mechanisms.

Conclusion

In conclusion, our experimental study using immunohistochemical and dual immunofluorescence analyses suggests that MTX may induce autophagy and modulate ferroptosis markers in liver and kidney tissues. CSO appears to mitigate MTX-induced changes in these pathways, suggesting a potential protective effect on autophagy and ferroptosis in the liver and kidneys. Increases in Beclin-1 and LC3B may indicate autophagy induction, but do not directly confirm autophagic flux; similarly, changes in GPX4 and TFRC may reflect modulation of ferroptosis, but confirmation would require assessment with additional parameters.

Ethics Committee Approval: Our experimental study was conducted subject to the approval of the Adana Veterinary Control Institute Local Ethics Committee for Experimental Animals, dated April 25, 2025 and numbered 2025-1/191.

Author Contributions: Concept – A.G, M.Ö.; Materials Used – A.G, M.S.S, M.Ö, U.Ç.; Data Collection M.S.S, U.Ç.; Critical Review – M.Ö, M.S.S, U.Ç.; Analysis – M.Ö, U.Ç, A.G.; Article Writing – A.G, M.Ö.

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Declaration of Interests: The authors declare that they have no conflicts of interest.

Use of Artificial Intelligence: Artificial intelligence was not used.

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