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Fisetin ameliorates methotrexate induced liver fibrosis

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Abstract. – OBJECTIVE: Methotrexate (MTX), a widely used chemotherapeutic and immunosuppressive agent, is associated with hepatotoxicity, leading to liver fibrosis and cirrhosis. This study explores the regenerative and reparative effects of fisetin, a flavonoid with known antioxidant and anti-inflammatory properties, on MTX-induced liver fibrosis in a rat model.

MATERIALS AND METHODS: Thirty-six male Wistar albino rats were divided into normal, MTX and saline, and MTX and fisetin. Liver injury was induced in the latter two groups using a single intraperitoneal dose of MTX (20 mg/kg). Fisetin (50 mg/kg/day) or saline was administered intraperitoneally for ten days. After sacrifice, liver tissues were subjected to histopathological evaluation and biochemical analyses, including Transforming Growth Factor-β1 (TGF-beta), sirtuins-1 (SIRT-1), malondialdehyde (MDA), cytokeratin 18, thrombospondin 1, and alanine transaminase (ALT) levels.

RESULTS: MTX administration significantly increased liver injury markers, including TGF-beta, MDA, cytokeratin 18, thrombospondin 1, and ALT, while reducing SIRT-1 levels. Fisetin treatment attenuated these effects, demonstrating its potential therapeutic impact. Histopathological analysis confirmed that fisetin mitigated MTX-induced hepatocyte necrosis, fibrosis, and cellular infiltration.

CONCLUSIONS: This study proves that fisetin administration can alleviate MTX-induced liver damage in rats. The reduction in oxidative stress, inflammation, and apoptosis, along with the histological improvements, suggests fisetin's potential as a therapeutic agent against MTX-induced hepatotoxicity. Further investigations and clinical studies are warranted to validate these findings and assess fisetin's translational potential in human cases of MTX-induced liver damage.

Key Words:
Methotrexate, Liver injury, Fisetin, Fibrosis.

Introduction

Liver fibrosis presents a significant public health challenge, given its increasing prevalence and associated mortality rates[1]. This condition arises from persistent liver damage, leading to the excessive accumulation of extracellular matrix proteins and, consequently, liver dysfunction. Its progression to cirrhosis and hepatocellular carcinoma amplifies the urgency for effective therapeutic strategies. Despite advancements, the complexity of liver fibrosis etiology remains incompletely elucidated, with oxidative stress and inflammation emerging as pivotal initiators and propagators of fibrogenesis[2,3]. Notably, reactive oxygen species (ROS), generated during liver injury induced by various agents, including viruses, medications, and toxins, exacerbate hepatic injury and fibrosis progression[4].

Central to fibrogenesis is the activation of hepatic stellate cells (HSCs), which transdifferentiate into myofibroblasts upon exposure to diverse stimuli[5]. This phenotypic switch prompts excessive extracellular matrix synthesis, fostering fibrosis progression. Oxidative stress exacerbates liver injury through inflammation, apoptosis, and mitochondrial dysfunction[6]. Methotrexate-induced liver injury is characterized by oxidative stress, impaired mitochondrial respiration, and endoplasmic reticulum stress. These factors contribute to the development of liver fibrosis through two distinct pathways[7]. Among the agents implicated in liver fibrosis induction, methotrexate (MTX) stands out due to its widespread use in cancer and autoimmune disease treatment. Approximately half of MTX users develop liver damage, with fibrosis and cirrhosis as potential outcomes[8,9]. The development of liver fibrosis is associated with the accumulation of methotrexate and its metabolites

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in the liver. The levels of methotrexate, 2,4 diamino-N (10)-methylpteroic acid, and methotrexate polyglutamate were significantly higher in the samples from the three patients with the highest increase in fibrosis[10].

The pathogenesis of MTX-induced liver toxicity involves disruption of DNA synthesis, leading to decreased folic acid levels and subsequent hepatocellular injury. Additionally, MTX triggers HSC activation, promoting oxidative stress, inflammation, and apoptosis in hepatocytes. These events collectively contribute to liver damage, as evidenced by elevated liver enzymes and inflammatory markers[11,12].

To discover fisetin's therapeutic potential in liver fibrosis and support our hypothesis, we used some biochemical tests such as cytokeratin 18 (CK18), thrombospondin 1, and sirtuins 1 (SIRT-1). Cytokeratin-18 has the potential to serve as a biomarker for drug-induced liver injury. It can help diagnose the condition early and provide valuable information about the mechanisms of hepatocellular injury[13]. Deficiency of thrombospondin-1 has a significant impact on liver metabolism, which can potentially affect liver injury and the progression of nonalcoholic fatty liver disease[14]. Targeting SIRT-1 could be a novel therapeutic approach to address age-related tissue fibrosis, specifically in liver fibrosis and aging-related tissue damage[15].

In this milieu, exploring natural antioxidants as therapeutic interventions gains significance. Fisetin (3,3′,4′,7-tetrahydroxyflavone), a flavonoid abundant in various fruits and vegetables, exhibits diverse pharmacological properties, including antioxidant, anti-inflammatory, and antifibrotic effects[16,17]. The wood of the purple smoke bush (*Cotinus coggygria*) contains large quantities of these compounds[18]. Similar to other phytochemicals and plant polyphenols, it possesses antioxidative properties[19]. In addition, it has been documented to exhibit antiviral, antibacterial, and anti-inflammatory properties[20]. Experiments conducted on live rats demonstrated that fisetin can increase the levels of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD). This leads to a reduction in the amount of reactive oxygen species (ROS) within the cells, thus explaining, at least partially, the antioxidative effects of fisetin. Notably, in the context of the liver, fisetin's elevated intracellular glutathione levels underscore its potential as a therapeutic agent against oxidative stress-induced liver injury[21]. Moreover, fisetin dramatically lowers blood glucose levels and raises insulin levels in the plasma of diabetic rats by adjusting important enzymes in hepatic and renal tissues[22,23].

Despite promising findings in other liver pathologies, the protective role of fisetin against MTX-induced liver fibrosis remains unexplored. Thus, this study aims to investigate fisetin's preventive and therapeutic potential in a rat model of MTX-induced liver fibrosis. Through biochemical and histopathological analyses, we seek to elucidate fisetin's mechanisms of action and its efficacy in mitigating MTX-induced liver fibrosis.

Materials and Methods

Animals

In this study, 36 male Wistar albino rats weighing 150-200 g and 10-12 weeks old were utilized. The experiments adhered to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA). Ethical approval was obtained from the Animal Ethics Committee of Science University (Istanbul, Turkey Ethical number: 16/01/2023-2823115702). The rats were procured from the Experimental Animal Laboratory at Science University, where they were housed in steel cages under controlled environmental conditions, with a temperature of 22 ± 2°C and a 12-hour light/dark cycle, and provided with unlimited access to food.

Experimental Protocol

The study involved 36 male rats, with 24 rats receiving a single intraperitoneal dose of methotrexate (MTX) at a concentration of 20 mg/kg to induce liver injury. The control group, consisting of 12 rats, received no chemical treatment. The 24 rats administered with MTX were divided into two groups. Group 1 received intraperitoneal administration of fisetin at a dosage of 50 mg/kg/day, while Group 2 received 1 ml/kg/day of 0.9% NaCl intraperitoneally. The treatment duration for all groups was ten days. At the study's conclusion, all animals were euthanized *via* cervical dislocation under anesthesia induced by administering 100 mg/kg of ketamine (Ketasol, Richterpharma AG, Austria) and 50 mg/kg of xylazine (Rompun, Bayer, Germany). Blood samples for biochemical analysis were collected *via* cardiac puncture, and the liver was excised for histopathological and biochemical evaluations.

Histopathological Evaluation

Liver sections, preserved with formalin, were stained with hematoxylin and eosin to a thickness of 4 μm. The Olympus C-5050 digital camera, mounted on the Olympus BX51 (Shinjuku, Tokyo, Japan) microscope, captured images of all sections. Liver histopathological scoring analysis followed the method described by Lobenhofer et al[24], assigning scores ranging from 1 (minimal) to 4 (marked) for parameters including hepatocyte necrosis, fibrosis, and cellular infiltration. The final score was determined by summing the individual grades for these parameters.

Liver Biochemical Analysis

Following decapitation, liver tissues were promptly extracted and stored at -20°C until biochemical analysis. Tissue analysis involved mechanical disruption of liver tissues using a glass homogenizer in a solution comprising five times the volume of phosphate-buffered saline (pH 7.4). The resultant mixture underwent centrifugation at 5,000 x g for 15 minutes, and the liquid portion containing suspended particles was collected. Protein concentration in the liver samples was measured using Bradford's technique with bovine serum albumin as the reference standard[25]. The concentrations of Transforming Growth Factor-β1 (TGF-beta), and SIRT-1 in liver supernatants were quantified using commercially available rat enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's guidelines. Plasma thrombospondin 1 and cytokeratin 18 levels were also measured using commercially available ELISA kits (Sigma-Aldrich, St. Louis, MO, USA).

Determination of Lipid Peroxidation

Malondialdehyde (MDA) levels, as thiobarbituric acid reactive substances (TBARS), were measured to assess lipid peroxidation in tissue and plasma samples[26]. Tissue samples were treated with trichloroacetic acid and TBARS reagent, followed by incubation at 100°C for 60 minutes. After cooling, samples underwent centrifugation at 3,000 rpm for 20 minutes, and the supernatant absorbance was measured at 535 nm. Tissue MDA levels were determined using a standard calibration curve with tetraethoxypropane and expressed as nmol/g protein.

Determination of Plasma Alanine Transaminase (ALT) Levels

Plasma ALT levels were measured using a commercially available ELISA kit (USCN, Life Science Inc, Wuhan, China).

Statistical Analysis

Data analysis was performed using IBM SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics are presented as mean ± standard error of the mean (SEM). Variables with more than two categories were analyzed using the nonparametric Kruskal-Wallis H Test. If significant, the Dunn-Bonferroni test was employed for multiple comparisons. Statistical significance was considered at $p < 0.05$ and $p < 0.001$.

Results

Effect of Fisetin on Liver Injury Markers

Comparative analysis between the MTX and saline group and the MTX and fisetin group unveiled a robust protective effect of fisetin. Fisetin administration resulted in a substantial reduction in TGF-beta (0.81 ± 0.02 pg/g, $p < 0.001$), MDA (55.7 ± 1.6 nmol/g tissue, $p < 0.001$), cytokeratin 18 (0.92 ± 0.3 pg/ml, $p < 0.01$), thrombospondin 1 (2.8 ± 0.1 pg/ml, $p < 0.001$), ALT (28.2 ± 0.7 IU/L, $p < 0.05$), accompanied by a noteworthy increase in SIRT-1 levels (0.81 ± 0.04 pg/g, $p < 0.001$) (Table I).

Biochemical Analysis

The MTX and saline group exhibited marked signs of liver injury, demonstrating significant elevations in key markers compared to the normal group. Notably, TGF-beta (1.29 ± 0.05 pg/g, $p < 0.001$), MDA (63.4 ± 1.1 nmol/g tissue, $p < 0.001$), cytokeratin 18 (2.46 ± 0.3 pg/ml, $p < 0.001$), thrombospondin 1 (5.02 ± 0.4 pg/ml, $p < 0.001$), ALT (48.4 ± 1.7 IU/L, $p < 0.001$), and a notable reduction in SIRT-1 levels (0.56 ± 0.02 pg/g, $p < 0.001$) (Table I). In-depth biochemical analyses provided further insights into the protective role of fisetin. The MTX and saline group demonstrated elevated levels of malondialdehyde (MDA) in both liver tissue and plasma, indicative of heightened oxidative stress (Liver MDA: $p < 0.001$, Plasma MDA: $p < 0.001$). Fisetin administration resulted in a significant reduction in MDA levels, both in liver tissue (MTX + fisetin: 55.7 ± 1.6 nmol/g tissue, $p < 0.001$) and plasma (MTX + fisetin: 60.9 ± 5.5 nM, $p < 0.001$) (Table

Table I. Comparison of normal Methotrexate (MTX) + saline and MTX+ fisetin groups in terms of liver injury findings. Results were presented as mean ± Standard Error of the Mean (SEM).

	Normal	MTX and Saline	MTX and Fisetin
Liver TGF-beta level (pg/g)	0.65 ± 0.01	1.29 ± 0.05**	0.81 ± 0.02$^{\#}$
Liver SIRT-1 level (pg/g)	1.95 ± 0.02	0.56 ± 0.02*	0.81 ± 0.04$^{\#}$
Liver MDA level (nmol/g tissue)	26.3 ± 0.5	63.4 ± 1.1*	55.7 ± 1.6$^{\#}$
Plasma cytokeratin 18 level (pg/ml)	0.52 ± 0.1	2.46 ± 0.3*	0.92 ± 0.3$^{\#\#}$
Plasma Thrombospondin 1 level (pg/ml)	0.81 ± 0.2	5.02 ± 0.4*	2.8 ± 0.1$^{\#}$
Plasma MDA level (nM)	48.1 ± 0.9	122.5 ± 4.6**	60.9 ± 5.5$^{\#\#}$
ALT (IU/L)	15.3 ± 1.3	48.4 ± 1.7*	28.2 ± 0.7$^{\#}$
Hepatocyte necrosis	0.1 ± 0.1	1.6 ± 0.2**	0.8 ± 0.1$^{\#\#}$
Fibrosis	0.1 ± 0.1	1.8 ± 0.1**	0.4 ± 0.1$^{\#\#}$
Cellular infiltration	0.1 ± 0.1	1.5 ± 0.2**	0.6 ± 0.2$^{\#\#}$

Statistical analyses were performed using one-way ANOVA. *$p < 0.01$, **$p < 0.001$ different from normal groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ different from MTX and saline group. Sirtuins 1 (SIRT-1), malondialdehyde (MDA), Transforming Growth Factor-β1 (TGF-beta).

I). Moreover, fisetin treatment led to a substantial decrease in plasma alanine aminotransferase (ALT) levels, reflecting improved liver function (MTX + fisetin: 28.2 ± 0.7 IU/L, $p < 0.05$). This result aligns with previous literature, highlighting Fisetin's potential in ameliorating hepatotoxicity (Table I).

Histopathological Evaluation

Histopathological examination reinforced the biochemical findings, highlighting the remarkable protective effects of fisetin. The MTX and saline group exhibited severe liver damage, characterized by prominent hepatocyte necrosis, fibrosis, and cellular infiltration. Conversely, the MTX and fisetin group displayed a considerable attenuation of these histopathological features, indicating the robust efficacy of fisetin in preventing MTX-induced liver injury (Figure 1). Significant differences emerged among the groups when assessing hepatocyte necrosis, fibrosis, and cellular infiltration. The MTX and saline group exhibited a substantial increase in hepatocyte necrosis, fibrosis, and cellular infiltration compared to the normal group ($p < 0.001$). Notably, fisetin administration in the MTX-induced liver injury model significantly mitigated these histopathological alterations ($p < 0.01$) for hepatocyte necrosis and $p < 0.001$ for fibrosis and cellular infiltration), emphasizing fisetin's potential in preventing and ameliorating the structural damage associated with MTX-induced hepatotoxicity, (Table I and Figure 1). These histological findings complement the biochemical results, comprehensively understanding fisetin's protective effects at both molecular and tissue levels. Further studies are warranted to elucidate the mechanistic pathways underlying these observations and assess the translational potential of fisetin in clinical contexts (Table I and Figure 1).

Discussion

Methotrexate (MTX) is a commonly used drug for cancer and rheumatological disease treatment, albeit with the potential to induce severe liver damage[27]. In this study, we observed that fisetin, when administered after MTX-induced liver injury in rats, mitigated various biomarkers and histopathological manifestations indicative of liver damage.

Transforming Growth Factor-Beta (TGF-β) is a cytokine released during all stages of liver damage, reflecting cellular damage and inflammation. It plays a pivotal role in fibrosis development by stimulating collagen synthesis and hepatic stellate cell transformation into myofibroblasts. Previous studies[28-30] have shown that inhibiting TGF-β signaling can prevent MTX-induced liver injury. Additionally, fisetin has been reported to reduce TGF-β levels in rat models[31]. Consistent with these findings, our study revealed decreased liver TGF-β levels with Fisetin administration following MTX-induced injury.

SIRT-1 mitigates tissue damage by regulating apoptosis, inflammation, and oxidative stress. Studies[32,33] have demonstrated that modulating SIRT-1 activity reduces apoptosis and inflammation, thereby preventing liver damage. Biel et al[33] observed that SIRT-1 protects the liver from

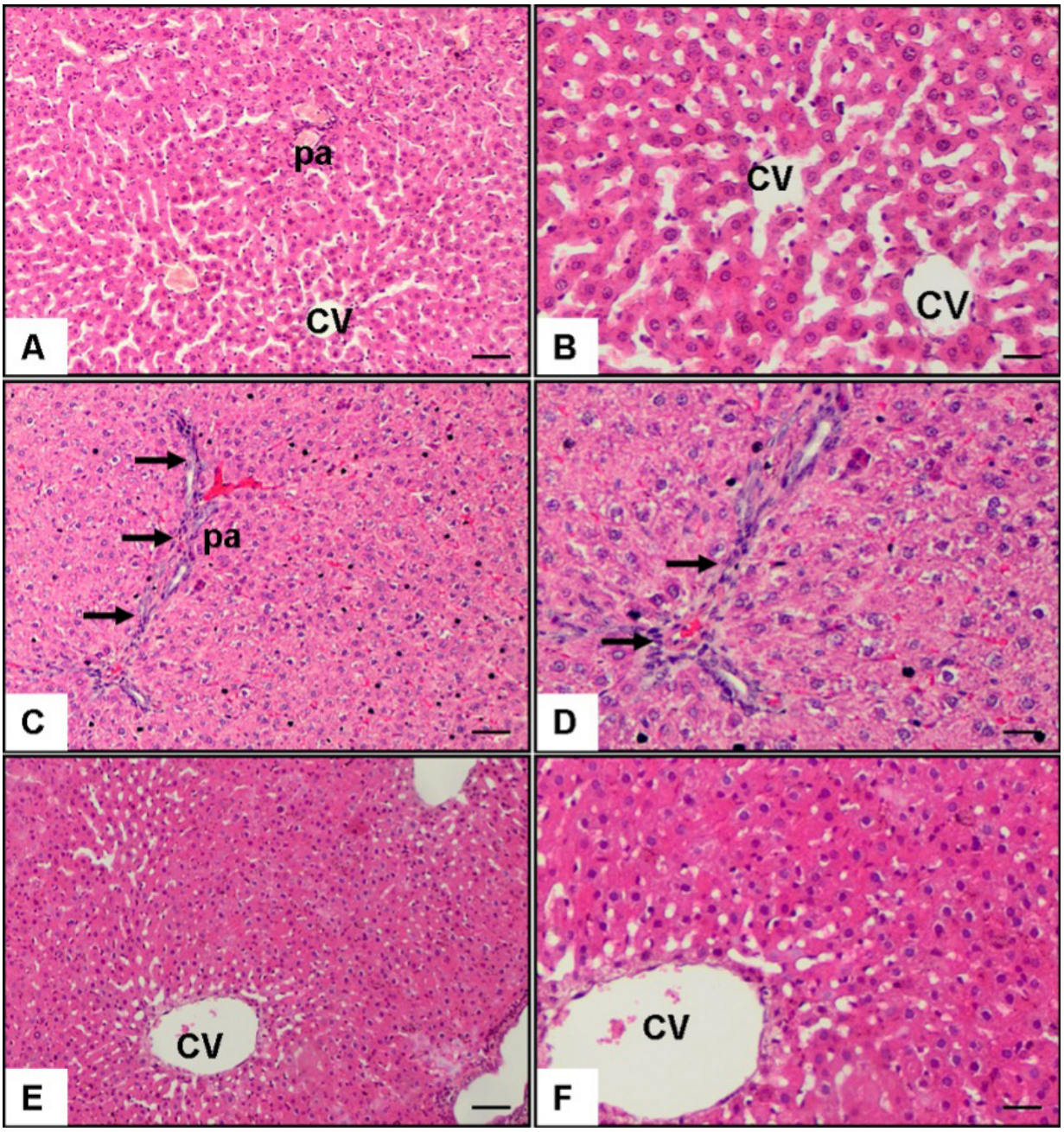

Figure 1. Liver histopathology Hematoxylin and Eosin stain (×10 and ×20 magnification). **A-B**, Normal Group rats have normal liver; central vein (cv) and portal area (pa). **C-D**, MTX and Saline Group rats have Bridging necrosis, fibrosis and cellular infiltration in the portal area (pa) (arrow). **E-F**, MTX+Fisetin Group rats have decreased bridging necrosis fibrosis and cellular infiltration.

ischemic injury by inhibiting faulty autophagy, mitochondrial dysfunction, and hepatocyte death. Fisetin has been shown to increase SIRT-1 expression and inhibit intracellular lipid accumulation[34]. Our study observed increased liver SIRT-1 activity in rats administered MTX, further augmented by fisetin administration. Moreover, fisetin can upregulate SIRT-1 expression and activity, leading to the deacetylation of target proteins involved in cellular stress responses. By activating SIRT-1, fisetin may enhance cellular resilience to oxidative stress, suppress inflammation, and mitigate apoptosis, promoting tissue repair and regeneration.

Malondialdehyde (MDA), a marker of oxidative stress, increases due to elevated free radicals. MTX administration has been shown to increase liver MDA levels in rats[35,36]. Conversely, fisetin administration has been reported to reduce MDA levels[37]. Consistent with these findings, our study revealed elevated liver and plasma MDA levels in MTX-administered rats, which were attenuated by fisetin administration.

Plasma cytokeratin 18 is typically present in liver cells, and its increase indicates hepatocyte damage[38]. In a study of Castéra[39], it is suggested that noninvasive techniques such as guided attenuation parameters and cytokeratin-18 hold the possibility of identifying and measuring hepatic steatosis in patients with nonalcoholic fatty liver disease, which could assist in screening and deciding who requires a liver biopsy. Similarly, in a study by Gonsebbat al[40], they observed that elevated cytokeratin 18 levels in the liver could act as an initial indicator of oxidative stress and harm induced by arsenite, potentially impacting liver function while being exposed. This study demonstrated increased CK-18 levels with MTX administration, suggesting hepatocyte damage, whereas fisetin administration reduced CK-18 levels, indicating its hepatoprotective effect.

A study conducted by El- Youssef et al[41] indicates that TGF-1Beta and thrombospondin-1 likely contribute to liver fibrosis in congenital hepatic fibrosis, possibly originating from hepatic stellate cells. On the other hand, Li et al[42] prove that thrombospondin 1 may be involved in chronic liver diseases like non-alcoholic fatty liver disease, fibrosis of the liver, and hepatocellular carcinoma. Moreover, thrombospondin secretion is upregulated in response to tissue damage and inflammation. Increased plasma TSP-1 levels have been associated with liver damage inhibition[43,44]. In this study, MTX administration increased plasma TSP-1 levels, while fisetin administration decreased them, potentially enhancing liver regeneration.

Of particular interest are our observations regarding the effects of fisetin on CK-18 and TSP-1. CK-18, a marker of hepatocyte damage, exhibited a significant decrease following fisetin administration, indicating the hepatoprotective effects of this compound. This finding aligns with previous studies[44] suggesting a role for fisetin in attenuating cellular apoptosis and inflammation, thereby preserving hepatic integrity.

Plasma alanine aminotransferase levels generally rise in liver diseases, including MTX-induced hepatotoxicity. Fisetin has been shown to reduce elevated ALT levels[45]. Consistent with these findings, our study demonstrated increased ALT levels with MTX administration, mitigated by fisetin administration.

Histopathological findings in MTX-induced hepatotoxicity include necrosis, cell infiltration, and fibrosis[46,47]. Previous studies[48] have shown that fisetin administration prevents liver necrosis in arsenic-induced liver injury. However, research demonstrating fisetin's preventive effects on histopathological manifestations in MTX-induced liver damage still needs to be completed.

Limitations

While this study uncovered the preventive effects of fisetin against liver toxicity in rats, it is important to acknowledge its limitations. The extent of its benefits for humans remains uncertain, as no studies have been conducted on this subject involving human participants.

Conclusions

This study suggests that fisetin administration alleviates MTX-induced liver injury in rats by modulating various biomarkers and histopathological manifestations associated with liver damage. However, further studies are warranted to elucidate the underlying mechanisms and confirm fisetin's therapeutic potential in treating MTX-induced hepatic injury.

Authors' Contributions

Oytun Erbaş, Ejder Saylav Bora, Ahmet Kayalı, Gökhan Yılmaz, and Hüseyin Acar contributed equally during the study and made critical revisions related to the relevant intellectual content of the manuscript.

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Conflict of Interest

No conflict of interest.

Informed Consent

Not applicable as the study did not involve human participants.

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Ethics Approval
Ethical approval Animal Ethics Committee of Demiroğlu Science University Istanbul, Türkiye (Ethical Permission Number: 16/01/2023-2823115702) and reported in compliance with the Animal Research.

Data Availability
The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

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