

Modulation of Interleukin-6 and Transforming Growth Factor - β Signaling Pathways by Saffron and Silymarin in Methotrexate-Induced Hepatotoxicity

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ABSTRACT

This study examined whether saffron (*Crocus sativus*) and silymarin (*Silybum marianum*), used alone or together, can protect the liver against methotrexate (MTX)-induced acute injury in rats, with a focus on interleukin-6 (IL-6) and transforming growth factor- β (TGF- β) signaling. Ethanol extracts of saffron and silymarin were standardized by GC-MS, and thirty adult male rats were randomly assigned to five groups (n = 6): control, MTX alone (20 mg/kg, intraperitoneally, on day 7), saffron + MTX (100 mg/kg/day orally for 14 days + MTX), silymarin + MTX (100 mg/kg/day orally for 14 days + MTX), and saffron + silymarin + MTX. We measured serum liver enzymes (ALT, AST, ALP, LDH), inflammatory markers (CRP, IL-6, TGF- β) and oxidative stress indices (MDA, GPX1), and examined liver tissue with H&E and immunohistochemistry for IL-6 and TGF- β . MTX caused liver damage, with significant rises in liver enzymes, CRP, IL-6, TGF- β and MDA, and reductions in albumin, total protein and GPX1 (p < 0.05). Histology showed inflammatory cell infiltration, necrosis and strong IL-6 and TGF- β staining in hepatocytes. Saffron or silymarin treatment improved these changes, partially normalizing biochemical markers, restoring GPX1, lowering IL-6 and TGF- β and reducing inflammatory and necrotic lesions. The combined saffron and silymarin regimen produced the greatest protection. These findings suggest that saffron and silymarin, especially in combination, can limit MTX-induced acute hepatotoxicity by reducing oxidative stress and downregulating IL-6 and TGF- β signaling, helping to preserve liver structure and function and supporting their potential use as natural adjuncts to lessen drug-induced liver injury.

Keywords: Acute liver damage, methotrexate, saffron, Silymarin, gene expression

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INTRODUCTION

The liver plays a central role in keeping the body in balance. It makes bile and proteins, stores nutrients and, importantly, clears drugs and toxins from the circulation¹. Because of this detoxifying role, it is highly vulnerable to drug-induced liver injury (DILI), which is now a leading cause of acute liver failure and a common reason for limiting or stopping useful medicines.^{2,3}

Methotrexate (MTX) is widely used as an anticancer and immunosuppressive drug, especially in malignancies and autoimmune diseases. However, its benefits are often constrained by liver toxicity (4). Excess reactive oxygen species, mitochondrial dysfunction, lipid peroxidation and the release of pro-inflammatory cytokines all contribute to MTX-induced liver injury⁴. Prolonged or high-dose exposure can lead to fibrosis, typical histopathological changes and a rise in serum liver enzymes, together with disturbed redox balance and increased tumour necrosis

factor- α .⁵ Conventional measures such as dose reduction, temporary withdrawal and folate supplementation provide only partial protection.⁶

Natural products with antioxidant and anti-inflammatory properties are therefore being explored as adjunctive hepatoprotective agents. Saffron (*Crocus sativus* L.) contains crocin, crocetin, safranal and picrocrocin, which show strong antioxidant, anti-inflammatory and anti-apoptotic effects in experimental models⁷. Silymarin, a flavonolignan complex from milk thistle (*Silybum marianum*), promotes liver regeneration, scavenges free radicals, limits lipid peroxidation and stabilises hepatocyte membranes, and is already used clinically for various liver disorders.⁸

Despite this, saffron and silymarin have mainly been studied separately. To our knowledge, there has been no direct comparison of these agents, alone and in combination, against MTX-induced acute hepatotoxicity

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using biochemical, histopathological and immunohistochemical endpoints. This study therefore aimed to evaluate and compare the hepatoprotective effects of saffron and silymarin, individually and together, in a rat model of MTX-induced liver injury, with particular focus on modulation of interleukin-6 (IL-6) and transforming growth factor- β (TGF- β) signalling pathways.

METHODS AND MATERIALS

Preparation of the Ethanol Extract of Saffron:

Dry stigmas of saffron (Iranian, from Erbil local market) were finely powdered (20 g) and macerated in 200 mL 95% ethanol (1:10 w/v) at 25 °C for 48 h with orbital shaking. The extract was concentrated at 40 °C under decreased pressure utilizing a rotary evaporator. (650 rpm) and dried in an oven at 40 °C. The dried extract stored at -20 °C until use.⁹

Preparation of the Ethanol Extract of Silymarin Seeds:

Silymarin seeds purchased from local market in Erbil and (100 g) were ground and macerated in 1 Litter 95% ethanol (1:10 w/v) at 25 °C for 72 h under orbital shaking. The extract was then vacuum-filtered, concentrated at 40 °C under reduced pressure, dried at 40 °C using an oven dryer, and stored at -20 °C under nitrogen.

For GC-MS analysis of both saffron and silymarin separately, 100 mg of the dried out powder was sonicated in 10 mL of methanol for 60 minutes at 25 °C, subsequently centrifuged at 10,000 rpm for 15 minutes, and filtered using a 0.22 μ m syringe filter. The supernatant was concentrated at 50 °C and subsequently re-dissolved in methanol before to analysis.¹⁰

GC-MS analysis was conducted using a Shimadzu QP2010 Plus system outfitted with an HP-5MS column (30 m \times 0.25 mm \times 0.25 μ m). Helium served as the carrier gas at a flow rate of 1.0 mL/min. The temperature program commenced at 60 °C with a 2-minute hold and completed at 280 °C, increasing at a rate of 10 °C/min, followed by a final hold of 10 minutes. The temperatures of the injector and mass spectrometer were 250 °C and 280 °C, respectively. Electron impact ionization occurred at 70 eV. Compounds were identified by mass spectral comparisons with the NIST 17 and Wiley libraries, and quantification was performed using TIC peak area normalization.¹¹

Experimental Animals

Wistar adult male rats were from the Experimental Animal House at Hawler Medical University in the Kurdistan Region of Iraq. The Ethics Committee for Animal Experimentation at the College of Dentistry, Hawler Medical University with ethic approval (Ref. No. HMUD/2425183).

Animals were housed in a temperature-controlled environment (25 \pm 2 °C; 50-70% relative humidity) with a 12-hour light/dark cycle, maintained under sanitary conditions. Conventional pellet feed and tap water were provided and libitum. Initial body weights (150-200 g)

were taken after one week's procured acclimatization, and weights were then determined every three days.

Rats were randomly allocated to the following five experimental groups (n = 6):

Group 1 (Control): Standard diet for 14 days and normal saline intraperitoneal (I.P) on day 7

Group 2 (MTX): Intraperitoneal (IP) administration of methotrexate (20 mg/kg) on day 7.¹²

Group 3 (Saffron + MTX): oral saffron extract (100 mg/kg/day) administered for 14 days, alongside intraperitoneal methotrexate (20 mg/kg) on day 7.¹³

Group 4 (Silymarin + MTX): 14-day oral silymarin extract (100 mg/kg/day), with IP MTX (20 mg/kg) on day 7.¹⁴

Group 5 (Saffron + Silymarin + MTX): oral saffron (100 mg/kg/day) and silymarin (100 mg/kg/day) for 14 days, alongside methotrexate (20 mg/kg)IP on day7.

Blood Collection

On day 15 (24 hours after the preceding oral administration), rats were anesthetized with diethyl ether. Approximately 5 mL of blood samples were collected from the retro-orbital plexus into serum separation tubes and allowed to coagulate for 20 minutes at ambient temperature. Samples were centrifuged at 3,000 rpm for 15 minutes, and serum was collected for biochemical (ALT, AST, ALP, LDH) measured using commercial kits (HITACHI, Germany) and a COBAS 6000 analyzer (HITACHI, Germany) serological tests : C-reactive protein (CRP) was assessed with COBAS 6000 (module 501, Roche Diagnostics, Switzerland)while serum IL-6 and TGF- β levels were determined with ELISA kits (ELK Biotechnology, USA) following manufacture's guide. Malondialdehyde (MDA) and glutathione peroxidase-1 (GPX-1) were determined with ELISA kits from the same company.

Tissue Collection

On day 16, rats were anesthetized using ketamine (80 mg/kg) and xylazine (20 mg/kg) via intraperitoneal injection. Livers were excised and preserved in 10% neutral-buffered formalin for histopathological analysis and immunohistochemical assessment of TGF- β and IL-6.

Histopathological assessment of liver damage

Liver sections fixed in 10% formalin (72 h) were Processed, embedded in paraffin wax, sectioned to 4-5 μ m, and stained with hematoxylin-eosin (H&E). Histopathologic alterations were assessed semi-quantitatively for inflammation, hydropic degeneration, necrosis, congestion, hemorrhage, and sinusoidal dilatation in five randomly chosen high-power fields per.

The score criteria were from 0 (normal) to 3 (severe), as listed in Table 1. To avoid observer bias, evaluation was performed by a blinded histopathologist.¹⁵

Table 1. Microscopic scoring criteria that included in this study

Criteria	0	1	2	3
Inflammatory cellular infiltration	Absent 0 (cells/field)	Weak 1 to 10 (cells/field)	Moderate 11 to 30 (cells/field)	Severe More 30 (cells/field)
Hydroptic degeneration	Absent 0% (cells/field)	Few 1-10% (cells/field)	Spread 11-25% (cells/field)	Intensive 25-50% (cells/field)
Necrosis	Absent 0% (cells/field)	Few 1-10% (cells/field)	Spread 11-25% (cells/field)	Intensive 25-50% (cells/field)
Congestion and hemorrhage	Absent 0 (blood vessels and bleeding/field)	Weak 1 to 2 (blood vessels and bleeding/field)	Moderate 2 to 5 (blood vessels and bleeding/field)	Severe More 5 (blood vessels and bleeding/field)
Sinusoidal dilatation	Absent 0% (field)	Few 1-10% (field)	Spread 11-25% (field)	Intensive 25-50% (field)

Immunohistochemistry for IL-6 and TGF- β genes

Paraffin sections (4 μ m) were de-paraffinized, rehydrated, and incubated with 3% H₂O₂ in methanol (10 min, RT) to inactivate endogenous peroxidase activity. Following PBS washing, slices were blocked with 0.5% normal goat serum for 30 minutes at room temperature and subsequently incubated overnight at 4 °C. using rabbit polyclonal anti-IL-6 (1:100; E-AB-51672, Elabscience, USA) or anti-TGF- β (1:100; E-AB-30977, Elabscience, USA). Bound antibodies were revealed using poly-HRP-conjugated goat anti-rabbit IgG (1:400; SE134, Wuhan Fine Biotech, China) and then visualized using DAB. Sections were counter-stained using hematoxylin.

Statistical Analysis

Data were analyzed on SPSS version 26.0 and GraphPad Prism v9.0. We present continuous variables as mean \pm SD, and ordinal scores as median (IQR). We examined parametric data utilizing one-way ANOVA succeeded by Tukey's HSD post hoc test, and non-parametric data employing the Kruskal Wallis test accompanied by Dunn's post hoc test. Statistical significance was established with

a p-value of less than 0.05 and EndNote version 21 used for citation of references.

RESULTS

GC-MS analysis of saffron and silymarin extracts

GC-MS of the ethanolic extracts showed that saffron and silymarin have different but complementary phytochemical profiles (Table 2). The saffron extract was rich in phenolic and terpenoid compounds, along with unsaturated fatty acids such as linoleic and oleic acids, which support antioxidant and anti-inflammatory effects. In contrast, the silymarin extract contained more esters and long-chain fatty acids, with a dominant lipid ester peak and detectable vitamin E, consistent with strong antioxidant and membrane-stabilising activity. These profiles provide a clear chemical basis for the hepatoprotective effects observed in the biochemical and histological results.

Table 2. Major bioactive compounds (\geq 2% relative peak area) identified in ethanolic extracts of saffron and silymarin by GC-MS

Plant extract (Source)	Class of Bioactive Compounds	Bioactive Compound	% Area
Saffron (<i>Crocus sativus</i>)	Phenolic compounds	4-Hydroxy-2-nitro-m-anisaldehyde	29.67
Saffron	Phenolic compounds	4-Hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-dienecarbaldehyde	2.10
Saffron	Fatty acids	n-Hexadecanoic acid	3.56
Saffron	Fatty acids	9,12-Octadecadienoic acid (Z,Z)-	8.51
Saffron	Fatty acids	9-Octadecenoic acid, (E)-	6.14
Saffron	Terpenoids	Neophytadiene	11.72
Saffron	Terpenoids	Tricyclo[6.3.0.0(1,5)]undecan-10-one, 4-hydroxy-5,9-dimethyl-	8.25
Saffron	Esters and ethers	Tetrapentacontane, 1,54-dibromo-	2.56
Silymarin (<i>Silybum marianum</i>)	Fatty acids	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	4.67
Silymarin	Fatty acids	9-Octadecenoic acid, (E)-	2.28
Silymarin	Fatty acids	Eicosanoic acid	6.14
Silymarin	Fatty acids	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	2.05
Silymarin	Fatty acids	9,12-Octadecadienoic acid (Z,Z)-, TMS derivative	2.04
Silymarin	Esters and ethers	i-Propyl 14-methylpentadecanoate	48.11
Silymarin	Alcohol and diols	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	13.55
Silymarin	Alcohol and diols	Vitamin E	2.03

Effect of Saffron and Silymarin extract on liver enzymes:

As shown in table (3) in comparison to controls, methotrexate (G2) significantly decreased serum albumin and total protein and increased all hepatic enzymes (p < 0.05). While silymarin (SLB) alone (G4) normalized ALT and ALP but was unable to significantly lower LDH, Saffron (CS) alone (G3) returned ALT, ALP, and LDH to control-equivalent values. Although the combined regimen

(G5) produced intermediate ALT and ALP values, it was just as successful in lowering LDH as Saffron alone.

Table 3. Liver Function Biomarkers and Serum Protein Analysis

Groups	AS T (U/L)	AL T (U/L)	AL P (U/L)	LD H (U/L)	S. Albu min (g/dL)	Total Serum Protein (g/dL)
Control (G1)	67.33 ± 35.20 ^a	23.67 ± 4.03 ^a	164.67 ± 35.59 ^a	493.17 ± 365.93 ^a	3.93 ± 0.03 ^a	7.6 ± 0.5 ^a
G2 (MXT)	115.72 ± 43.28 ^b	44.38 ± 23.51 ^b	204.37 ± 68.71 ^b	730.17 ± 392.24 ^b	1.57 ± 0.26 ^b	2.9 ± 0.3 ^b
G3 (saffron +MXT)	80.65 ± 16.46 ^a	22.17 ± 4.46 ^a	147.32 ± 44.20 ^a	304.67 ± 136.08 ^c	3.43 ± 0.23 ^{ab}	6.8 ± 0.4 ^{ab}
G4 (Silymarin+MXT)	82.52 ± 26.88 ^a	22.48 ± 3.14 ^a	143.70 ± 28.49 ^a	466.33 ± 219.88 ^{ab}	3.00 ± 0.33 ^{ab}	6.53 ± 0.03 ^{ab}
G5 (saffron + Silymarin +MXT)	88.92 ± 9.35 ^{ab}	27.28 ± 12.62 ^a	154.57 ± 70.27 ^a	304.67 ± 136.08 ^c	3.20 ± 0.33 ^{ab}	6.64 ± 0.12 ^{ab}

Data were presented as Means ± SD, Different superscripts (a, b) indicate significant differences (p < 0.05; one-way ANOVA followed by Tukey's test)

Serum inflammatory and oxidative stress markers in the different experimental groups

Serum inflammatory and oxidative stress markers are summarized in Table 4, Methotrexate administration (G2) caused a marked inflammatory response, as evidenced by a significant rise in CRP (13.67 ± 5.39^b mg/L) and IL-6 (64.0 ± 12.0^b pg/mL) compared with the control group (0.06 ± 0.00^a mg/L and 24.0 ± 2.5^a pg/mL, respectively). TGF-β and MDA levels were also significantly elevated in the MTX group (54.0 ± 10.0^b and 90.0 ± 12.0^b pg/mL) relative to controls (12.0 ± 1.5^a and 52.0 ± 5.0^a pg/mL), while GPX1 activity was significantly reduced (50.0 ± 6.0^b vs. 75.0 ± 7.0^a pg/mL).

Pretreatment with saffron (G3), silymarin (G4), or their combination (G5) markedly attenuated these MTX-induced alterations. In all treated groups, CRP and IL-6 values returned to levels comparable to the control (p > 0.05 vs. G1), and MDA concentrations were significantly

lower than in the MTX group and close to baseline. TGF-β levels in G3–G5 were significantly reduced compared with G2 but remained slightly higher than control, reflecting partial normalization. GPX1 activity was restored in all treated groups, with values not significantly different from the control and clearly higher than in the MTX group.

Table 4. Serum inflammatory and oxidative stress markers in the different experimental groups

Groups	CRP (mg/L)	IL-6 (pg/mL)	TGF-β (pg/mL)	MDA (pg/mL)	GPX1 (pg/mL)
Control (G1)	0.06 ± 0.00 ^a	24.0 ± 2.5 ^a	12.0 ± 1.5 ^a	52.0 ± 5.0 ^a	75.0 ± 7.0 ^a
G2 (MXT)	13.67 ± 5.39 ^b	64.0 ± 12.0 ^b	54.0 ± 10.0 ^b	90.0 ± 12.0 ^b	50.0 ± 6.0 ^b
G3 (CS)	2.08 ± 0.85 ^a	22.0 ± 4.5 ^a	38.0 ± 12.0 ^{ab}	50.0 ± 10.0 ^a	80.0 ± 10.0 ^a
G4 (SLB)	2.01 ± 0.63 ^a	25.0 ± 5.0 ^a	36.0 ± 13.0 ^{ab}	60.0 ± 11.0 ^a	77.0 ± 12.0 ^a
G5 (CS + SLB)	2.35 ± 1.52 ^a	28.0 ± 4.0 ^a	38.5 ± 17.0 ^{ab}	55.0 ± 10.0 ^a	80.0 ± 13.0 ^a

Values are expressed as mean ± SD (n = 6). Different superscript letters within the same column indicate statistically significant differences between groups (p < 0.05)

Effects of MTX, CS and SLB on liver histopathology

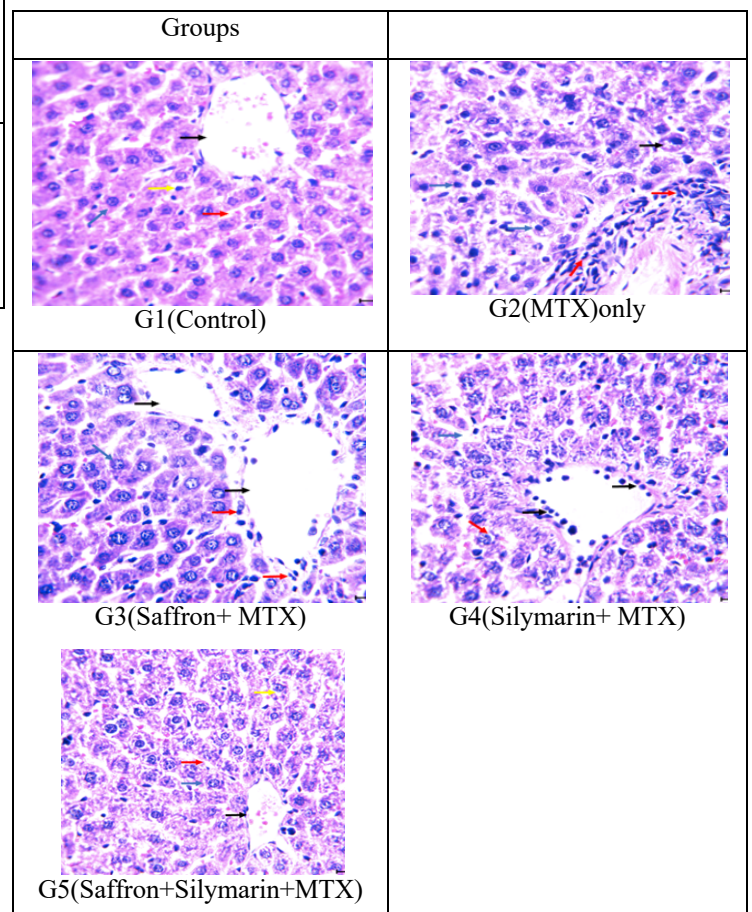


Figure 1. Histopathological appearance of rat liver sections stained with Hematoxylin and Eosin (H&E).

- G1(control): Histological section of liver of the normal rats showing normal appearance of portal vein (black arrow), surrounded by normal hepatocytes (blue arrow) and Kupffer cells (yellow arrows).
- G2 (Methotrexate group) showing abnormal histological appearance of liver tissues, showing congestion of the portal vein (black arrow), hydropic degeneration in many of the hepatocytes which appear as vacuolization and cell swelling (blue arrows), There is infiltration of inflammatory cells inside and surrounding the portal area (yellow arrows).
- G3(CS+MXT) Liver section of rats received saffron (100mg /kg) pre and post treatment by (20mg/kg) MXT (showing normal portal vein without congestion (black arrows), normal hepatocytes (blue arrow). There is few infiltrations of inflammatory cells (red arrows).
- G4(SLB+MXT): Histopathology of liver section received Silymarin (100mg/kg) pre and post treatment by (20mg/kg) of MXT Showing infiltrated of mononuclear cells at the portal area (black arrows) and dilated hepatic sinusoids with few bleeding (blue arrows), hydropic degeneration in some of the hepatocytes (red arrows).
- G5 (CS+SLB+ MXT) Histopathology of liver section of rats receiving combined treatment of saffron (100mg/kg) and Silymarin (100mg/kg) pre and post treatment of (20mg/kg) of MTX(E)) showing normal appearance of portal vein (black arrow), surrounded by normal hepatocytes (blue arrow) and normal Kupffer cells (yellow arrow). The spaces between cells are normal hepatic sinusoids (red arrows). H&E. (400x)

Figure (1) show that the microscopic examination of liver sections from the control group demonstrated a normal structure. however in the MTX group, showing disrupted hepatic architecture with marked inflammatory cell infiltration while in MTX + CS group (G3): showing partial improvement with reduced inflammatory infiltration, restoration of sinusoidal structure, and moderate hepatocellular regeneration. in group 4(MTX + SLB)group: demonstrating considerable histological recovery with clearer sinusoidal spaces, mild cellular infiltration, and preserved hepatocyte morphology. also in group 5(MTX + CS + SLB)group: revealing near-normal hepatic tissue with intact central vein, well-organized hepatic cords, minimal inflammatory infiltration, and regular sinusoidal spaces, indicating synergistic hepatoprotective effect. In general, significant improvement was evident in the Pre & Post treatment with CS and Silymarin and both.

Table 5. Semi-quantitative histopathological scoring of hepatic lesions in different experimental groups

Histopathologic Parameter	G1	G2	G3	G4	G5	p-value
Inflammatory cellular	0,0, 0	2,3, 3	1,0, 1	2,1, 1	0,0, 0	0.014* *

infiltration						
Hydropic degeneration	0,0, 0	3,2, 3	0,1, 0	2,1, 1	1,0, 0	0.024* *
Necrosis	0,0, 0	1,3, 3	0,0, 0	1,1, 0	0,0, 0	0.019* *
Congestion and hemorrhage	0,0, 0	2,1, 3	0,0, 1	2,1, 2	1,0, 0	0.035* *
Sinusoidal dilatation	0,0, 0	0,1, 3	1,1, 0	1,1, 2	1,1, 0	0.19* *

P value>0.05 = non-significant *

P value<0.05 = significant**

In Table (5) it shows the findings of the histopathology tests. The scores for each of the three liver sections from each animal (n = 3) are displayed as independent numbers. A score of 0 means there is none, a score of 1 means there is mild, a score of 2 means there is moderate, and a score of 3 means there is severe.

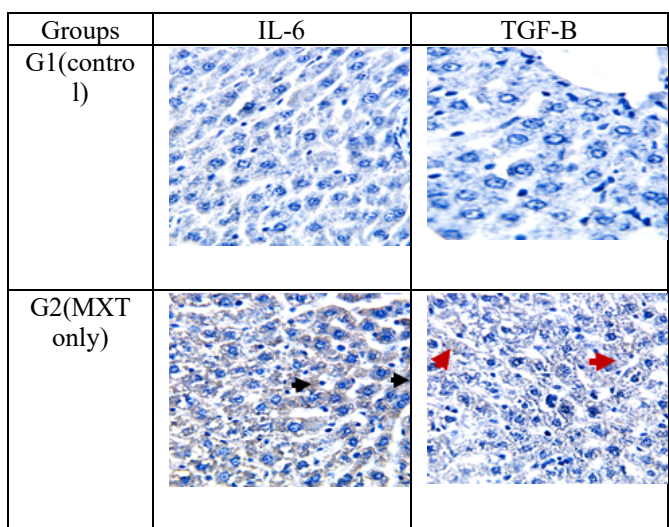
The liver structure in Group 1, which was the control group, was normal, with scores of 0 for all metrics.

Group 2 experienced a lot of damage, such as inflammatory cell infiltration (scores 2, 3, 3), hydropic degeneration (3, 2, 3), necrosis (1, 3, 3), and congestion/hemorrhage (2, 1, 3).

In Group 3 the number of damages decreased a lot. Most of the scores were 0 or 1, which suggests that there were only minimal or no lesions.

Group 4 provided some protection, with moderate infiltration (2, 1, 1), hydropic alterations (2, 1, 1), necrosis (1, 1, 0), and congestion/hemorrhage (2, 1, 2). Compared to the control group, Group 5 didn't have any notable alterations.

Immuno-histochemical study of liver tissue:



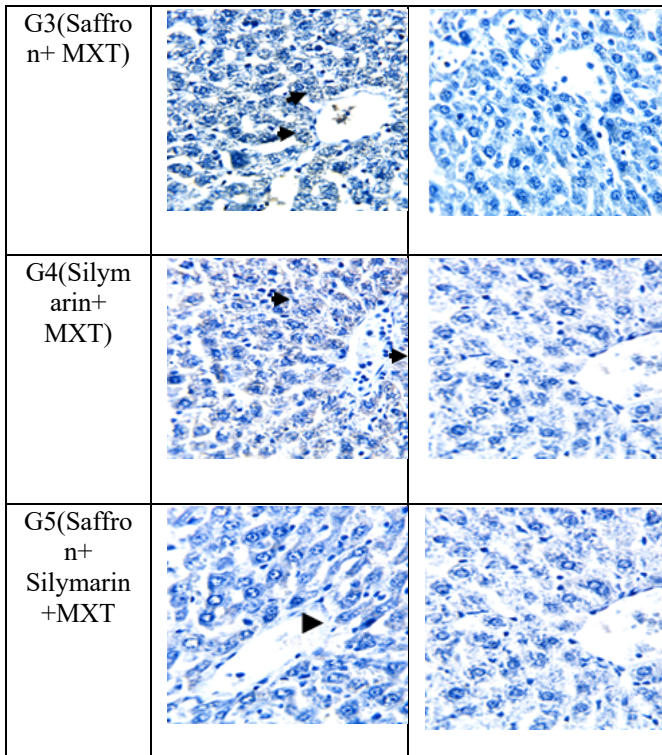


Figure 2: Effect of saffron and Silymarin on the expression of IL-6 and TGF- β gene in Methotrexate induced acute liver damage in rats.

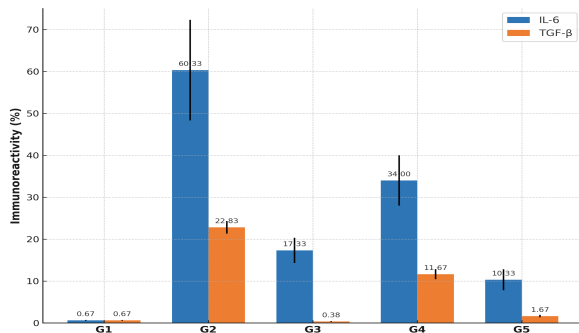


Figure 3: Quantification of the percentage of IL-6 and TGF- β immunostaining in the positive stain hepatic cells in different groups by using ImageJ software.

As shown in Figure 2 and Figure 3 Immunohistochemical analysis revealed no IL-6 and TGF- β staining in control livers (G1). In G2 Methotrexate (20 mg/kg single dose IP) evoked moderate cytoplasmic IL-6 immunoreactivity (mean score 2.0 ± 0.2) and low TGF- β expression (1.2 ± 0.1). Saffron (100 mg/kg) pre- and post-treatment in (G3) markedly reduced IL-6 to low levels (0.8 ± 0.1) and abolished TGF- β (0.1 ± 0.1). Silymarin alone (100mg/kg) in (G4) produced intermediate suppression of IL-6 (1.4 ± 0.2) and very low TGF- β (0.4 ± 0.1). Co-administration of saffron plus silymarin (G5) virtually eliminated both cytokines (IL-6 0.3 ± 0.1 ; TGF- β 0.0 ± 0.0), confirming synergistic anti-inflammatory and anti-fibrotic activity.

DISCUSSION

GC-MS showed that saffron and silymarin extracts have different but complementary phytochemicals, which likely explain their strong liver-protective actions.

Saffron contained many diverse compounds, including phenolics and unsaturated fatty acids such as linoleic and oleic acids, which support antioxidant, anti-inflammatory and membrane-stabilising effects^{16,17} Silymarin had fewer but more lipid-rich components, dominated by long-chain esters and fatty acids that also enhance antioxidant defence and protect hepatocyte membranes.^{18,19}

Methotrexate (20 mg/kg, IP) caused clear liver injury: liver enzymes and MDA increased, IL-6 and TGF- β were elevated, while albumin, total protein and GPX-1 fell. Histology showed distorted architecture, congestion and inflammatory infiltration, and immunohistochemistry revealed strong IL-6 and TGF- β staining, confirming active inflammation and early fibrogenesis.^{20,21}

Methotrexate harms the liver by blocking folate-dependent DNA synthesis and triggering excess reactive oxygen species. This causes mitochondrial dysfunction, oxidative DNA damage and hepatocyte death. At the same time, IL-6, TNF- α , NF- κ B and TGF- β drive inflammation and early fibrosis, while loss of antioxidant enzymes such as GPX-1 and SOD leaves the liver less able to defend itself, in line with previous reports.^{7,22}

Methotrexate, utilized for years as an immunosuppressant for rheumatoid arthritis and malignancies, may cause liver damage by increasing transaminase levels or causing moderate-to-severe fibrosis; however, it does not lead to liver failure, cirrhosis, or mortality when compared to other anticancer drugs.²³ However,²⁴ Demonstrated that intraperitoneal dose of methotrexate at 20 mg/kg markedly elevated serum ALT, AST, ALP, TBL, and lactate dehydrogenase (LDH) levels in Wistar rats relative to the control group. Methotrexate markedly elevated oxidative stress by increasing liver MDA levels in comparison to control rats.²⁵

Numerous studies demonstrate that the injection of MTX elevates the generation of reactive oxygen species (ROS), alongside nitric oxide (NO) creation and lipoperoxidation in the liver.²⁶

Derived from the latest search results (2023-2025), the hepatic levels of IL-6, TGF- β , MDA and GPX1 are now recognized as a compact biomarker panel that simultaneously captures inflammation, fibrogenesis, lipid peroxidation and antioxidant competence in liver injury models as well as IL-6 & TGF- β – Both cytokines are consistently elevated in toxin- or diet-induced hepatic damage and are down-regulated by antioxidant interventions such as selenomethionine (SeMet) and silybin, confirming their utility as sensitive inflammatory/fibrotic read-outs²⁷, MDA – A direct product of lipid peroxidation; its hepatic concentration rises in parallel with oxidative insult and falls when GPX1 activity is restored.²⁸

GPX-1; a key seleno-enzyme located predominantly in hepatocyte mitochondria and cytosol; its decrease signals compromised glutathione-dependent antioxidant defenses and is rapidly reversed by silymarin supplementation²⁷, thus, quantifying IL-6, TGF- β , MDA and GPX1 in liver tissue or serum provides a concise, mechanistically coherent snapshot of oxidative-inflammatory injury and the efficacy of hepatoprotective treatments.

Histological examination revealed restoration of normal hepatic architecture, with minimal necrosis and reduced inflammatory cell infiltration. IHC staining of IL-6 and TGF- β also showed marked downregulation, especially in the combination group. These outcomes support a synergistic hepatoprotective effect when saffron and silymarin are used together.

Pharmacodynamically, saffron's efficacy is largely attributed to its phenolic aldehydes and carotenoid derivatives such as crocin and crocetin, which inhibit lipid peroxidation, modulate pro-inflammatory signaling, and enhance mitochondrial integrity. Silymarin, by contrast, acts through its rich content of flavonolignans (e.g., silybin), which not only neutralize ROS but also stabilize hepatocyte membranes, stimulate ribosomal RNA synthesis, and inhibit the TGF- β /Smad pathway. Furthermore, silymarin enhances intracellular glutathione levels, thereby supporting phase II detoxification reactions in the Liver.²⁹

Conversely, MTX induces the depletion of beneficial antioxidants, such as glutathione (GSH), and inhibits free radical scavenging enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx).³⁰

Silymarin exhibits hepatoprotective effects through multiple pathways, primarily by modulating enzymatic and nonenzymatic biochemical indicators in the liver³¹ and upregulation of nuclear factor erythroid 2-related factor 2 (Nrf2) expression³². The anti-inflammatory activities of silymarin have been demonstrated in many models of liver injury. In a rat model of alcoholic fatty liver, silymarin functioned by downregulating the expression of nuclear factor kappa B (NF- κ B), interleukin-6 (IL-6), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-13 (MMP-13), transforming growth factor beta-1 (TGF- β 1), tumor-suppressor Krueppel-like factor, collagen α 1, and platelet-derived growth factor (PDGF) signaling in hepatotoxic damage animal models.³³

Saffron and silymarin counteract MTX-induced liver injury through complementary mechanisms. Saffron suppresses the expression of pro-inflammatory genes and decreases free radicals³⁴, while silymarin fortifies membrane stability, enhances antioxidant defenses, and inhibits cytokine-mediated fibrotic responses.³⁵

CONCLUSION

GC-MS and other findings show that saffron and silymarin protect the liver from methotrexate by lowering IL-6, reducing inflammation, and restoring GPX1. Their

different but complementary phytochemicals work together, and the combination gives slightly better protection, supporting their use as a natural add-on therapy against drug-induced liver damage.

Declaration section

Conflict of Interest

The authors declare the absence of any conflict of interest.

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Ethical Approval

Approval obtained from basic sciences department, College of Dentistry, Hawler Medical University Approval No: HMUD/2425183 and it is part of my PH.D thesis

Limitations and future directions

This study was limited by its single-dose MTX model and short observation period. Chronic models could better assess long-term fibrotic outcomes. Future research should explore chronic dosing regimens, herb-drug interactions, and clinical translation in MTX-treated patient populations.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SMZA performed animal experiments, carried out biochemical and histological analyses, and drafted the manuscript. HTN conceived and designed the study, supervised the experiments and contributed to data interpretation and manuscript revision. Both authors read and approved the final manuscript.

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