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Research Article

Antioxidant, anti-inflammatory and anti-apoptotic impact of Moringa leave extract supplementation in livers of methotrexate treated rat

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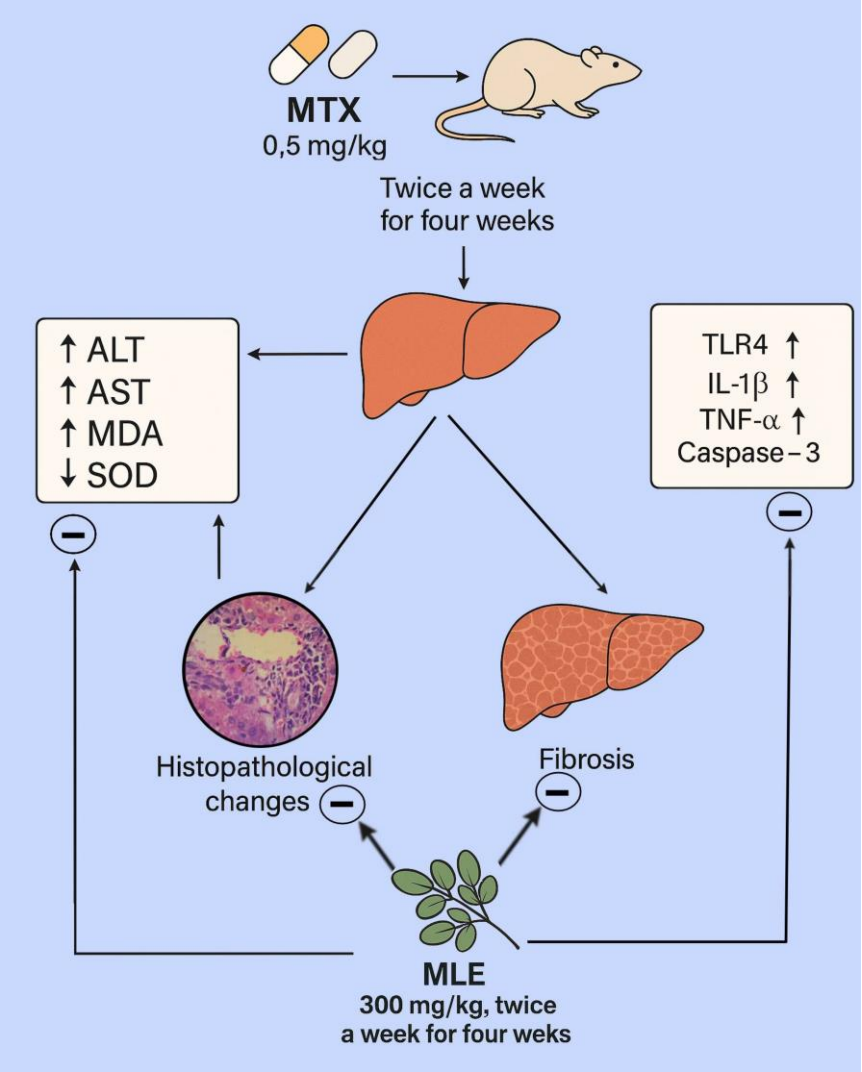
Abstract

Background: Methotrexate (MTX), a chemotherapeutic medication, is known to cause hepatic damage a potentially harmful adverse effect that limits its therapeutic applications. Moringa leaf extract (MLE) has antioxidant and anti-inflammatory properties. However, its role in MTX-induced liver damage has not been fully studied. **Objective:** The present investigation was designated to determine possible hepato-protective effects of MLE against MTX-induced hepatic damage in rat animals. **Materials and Methods:** Rats were given MLE orally twice a week for four weeks at a dose of 300 mg/kg of total rat body weight, with or without an intra-peritoneal injection of 0.5 mg/kg MTX administered twice a week for four weeks. Blood samples and liver tissues were collected for biochemical and histopathological analysis. **Results:** MTX impairs liver function, as shown by elevated serum levels of ALT and AST. In addition it caused oxidative stresses through raising MDA and reducing SOD. It also triggered an inflammatory response by increasing Toll-like receptor 4 (TLR4), interleukin 1β (IL-1β) and Tumor Necrosis Factor Alpha (TNF-α) levels, with subsequence increased Caspase 3 immuno- expressions in liver tissues. Interestingly, MLE combined with MTX significantly reduced all forms of oxidative stresses and inflammation-related signaling, resulting in improved liver function and structure. This was demonstrated by lowering liver oxidation and inflammation indicators and decreasing the Caspase3 expression. **Conclusion:** MLE has antioxidant, anti-inflammatory and anti-apoptotic properties that could possibly mitigate MTX-induced hepatotoxicity.

Keywords: Methotrexate, Moringa leave extract, TLR4, Caspase 3



Graphical Abstract

Introduction

Chemotherapy -induced hepatic damage is one of the most prevalent life-threatening adverse reactions. MTX a chemotherapeutic medication, used to treat cancer when given in large doses [1] In addition, MTX can also be used at a lower dose as a disease modifying agent to treat autoimmune illnesses. However, it continues to cause liver damage as an adverse effect, implying that MTX-induced hepatotoxicity is not dose-dependent [2]

Because of its structural resemblance to folic acid, MTX competes with the dihydrofolate reductase enzyme [3], making it an effective anticancer/ disease modifying agent. Folic acid and its derivatives are thought to reduce the therapeutic efficacy of MTX [4]

MTX induce toxicity in various organs. Therefore, strategies to reduce MTX-induced toxicity while retaining MTX efficacy are important [5]. Supplementing with natural phytobioactive compounds that combat oxidation and inflammation has recently been proven to minimize MTX-induced multiple organs damage including liver [6].

The liver is the organ most susceptible to medication-induced toxicity since it is the major location of medications metabolism. Oxidative stress, xenobiotic toxicity, and drug toxicity all have a substantial effect on liver cells. Liver cell toxicity causes abrupt liver failure, and hepatocellular death with subsequent systemic inflammation [7]. Drug-induced

liver damage is the most major consequence of pharmaceutical post-approval investigations [8] .

The exact mechanism MTX-induced hepatotoxicity is still unknown. The deposition of MTX polyglutamate metabolites within hepatic cells may be the cause. As it leads to oxidative stress, that is evident by a decrease in hepatic antioxidant capacity and an increase in liver lipid peroxidation products. Additionally, inflammation of liver cells which is followed by hepatocellular death, fibrosis, and liver steatosis [9]

Many drugs have a great tendency to harm liver mitochondria. The failure of liver mitochondria which are ATP-producing organelles impairs energy metabolism. This consequence generates intracellular oxidation stress due to an overabundance of reactive oxygen compounds and peroxynitrite. Hepatotoxicity continues to be a main reason of drug withdrawal from the pharmaceutical industry and clinical applications [10]

Moringa oleifera is an edible tree that is widely cultivated in both tropical and subtropical regions of Asia as well as Africa. It belongs to the Moringaceae family, which contains only one genus. Moringa products have diverse applications in agriculture, industry, and medicine. Moringa leaves have rather high crude protein content, ranging from 25% to 32%.[11]

As the plant mitigates oxidation and inflammatory processes, it is widely used in traditional medicine. The Moringa oleifera plant's leaves are high in vitamins, and extracts from Moringa pterygosperma root have antimicrobial properties. Moringa oleifera leaves and seeds contain a significant amount of antioxidant compounds, vitamins such as A, D, E, C, and γ-carotene [12]

Moringa leaf extract is used topically to treat dermatitis caused by insect bites, fungal even infections caused by bacteria [13]
When MLE is consumed in a larger quantity, it revealed harmless impacts. Therefore, it is safe, with no to minimal toxicity rate [11]. .
MTX-induced hepatocytes toxicity mechanisms is not fully investigated; nonetheless, oxidative, inflammatory, and apoptotic impacts may be mediate toxicity signaling pathways [14]
In addition, MTX may deplete numerous protective antioxidant mediators and impede the actions of certain free radical scavengers [15,16]
The current work was designated to investigate the efficacy of MLE on liver cells damage provoked by MTX-administration in rat animals, and elucidate its role in modulating oxidation, inflammation, fibrosis and apoptosis processes.

Materials and methods

Animals

Thirty-two male albino rat animals were provided from The National Research Center present in Cairo. Rats were housed and acclimatized for 7 days prior to the start of the experiment at Minia-University's Histology & Cell Biology Department. Experiment received authorization from the animal care ethical committee (Faculty of Medicine, Minia University, Egypt, Approval number: 1051// 2024) following to the international criteria (Act -1986) addressing animal welfare requirements.

Drugs

MTX was purchased from Minapharm Pharmaceuticals (Cairo, Egypt). It was dissolved in a freshly prepared normal saline solution. The estimated amount was 0.5 mg/kg two times per week with duration between the two doses of three days for four weeks long, delivered by (I.P) injection[17]
MLE from El-Shekh Zoed Station, Desert Research Centre, North Sinai, Egypt in the form of dried ethanol extraction of fresh Moringa oleifera leaves. It was delivered by mouth two times per week with duration between the two doses of three days for four weeks long, at a dosage of 300 mg/kg rat body weight [18]

Experimental design:

Group 1 (Control) (n=8 rats) were provided ordinary laboratory food and drink.
Group 2 (MLE positive control, n=8) received the estimated MLE amount orally.
Group 3 (MTX therapy) (n=8) received the estimated MTX dose via the I.P injection.
Group 4 (MTX+MLE) (n=8) received MTX and MLE simultaneously for four weeks at the same time, using the same dose and technique of administration as previously mentioned in other groups.
Rats' body weight was determined at the start

and after end of the experiment duration.

After four weeks, all animals of each group were weighed then sacrificed by anesthesia. Blood samples were obtained from retro orbital veins; sera were stored for biochemical investigations. Livers were carefully extracted.

Sampling

Livers were cut into slices, preserved for 24 hours duration in a 10% formaldehyde solution, dried using a series of increasingly alcohols concentration, rinsed with xylene, and subsequently placed within paraffin-based blocks for histological and immunohisto-chemical analysis. The other liver specimens have been subjected homogenization in 20% w/v cold potassium phosphate buffer solution (0.01 M, pH 7.4) and spun at 5,000 rpm for ten minutes at 4°C then subjected to centrifugation to obtain centrifuge that utilized to measure SOD and MDA liver content.

To perform the biochemical analysis, blood sera were subjected to centrifugation process at 4000 g for ten minutes at room temperature then preserved at -80°C. Serum concentrations of ALT, AST, and LDH were estimated using commercial colorimetric kits.

To determine oxidative stress variables, the activity of SOD was estimated at 420 nm using colorimetric methods, the MDA quantity was determined using a thiobarbituric acid reactive material in the chemical composition of 1, 1, 3, 3-tetra methox.

To determine inflammatory mediators, TNF-α level (Elabscience Biotechnology Inc., USA, E-EL-R0019 E), IL-1β level (E-EL-R0012 96T) and TLR4 level (E-EL-R0990 E) in liver tissues were estimated using ELISA kits in accordance to instructions of their manufacturer.

For histological examination, Liver tissues were divided, quickly maintained in 10% buffered formalin, and then prepared to form paraffin made blocks. Five µm thick sections were cut and mounted. Hematoxylin and Eosin stain (H&E) was employed to stain some sections. For other sections Masson's Trichrome has been employed to demonstrate collagen fibers [19].

For immunohistochemical examination, Anti-cleaved caspase-3 immunohistochemistry was used (Catalog No. PA1-29157, Thermo Fisher Scientific Biotechnology, dilution 1:100). Sections were cleaned of paraffin and rehyd-rated, and in order to recover antigen, they had been immersed in absolute methanol with 0.3% H_2O_2 and treated with 0.1% trypsin and Tris buffer [20] .These sections were incubated with goat sera at the room temperature, further, incubation for thirty minutes at the room temperature with the (1: 100) for cleaved caspase-3, and then rinsed three times for 30 minutes with the phosphate-buffered saline. These rinsed sections were subjected to Vector's avidin/biotin peroxidase complex in Burlingame treatment, California, USA. The chromogenic 3,30-diaminobenzidine tetra hydrochloride substrate was used to identify the sites of peroxidase binding. Hematoxylin counterstain was applied to tissue slices.

Capturing photographs

In addition to examining stained sections, digital photos were taken using an Olympus microscope (Olympus, Japan) with color digital camera with a high-resolution. The images were then connected to a computer and reviewed using Adobe Photoshop (2021 pour Windows).

Histopathological evaluations and morphometric study

For each group, H&E sections of six rats were examined histopathologically in three fields with avoiding of overlapping at a magnification of X400 utilizing a light microscope. The Ishak modified histological activity index was applied for estimating the final numerical score. The following criteria were used to determine the score: periportal or periseptal interface hepatitis (0–4); confluent necrosis (0–6); focal lytic necrosis, apoptosis, and focal inflamm-ation (0–4); portal inflammation (0–4) [21,22] .

Masson's Trichrome-stained sections were subjected to quantitative analysis: under light microscope magnification X100, light micro-scopic taken micrographs were examined in three fields with avoiding of overlapping per rat for six rats in each experiment group [23,24] .

Caspases-3-immunolabeled cells in ten adjacent none overlapping areas of each rat's tissue slices underwent quantitative analysis by counting the overall number of hepatocytes using hepatocyte's nucleus. In each experimental group, the proportion of hepatocytes with caspase-3-immunolabeled cells to all hepatocytes was computed. For every group, the percentage range was computed [25,26]

Data management and statistics:
Graph Pad Prism (version 5.01 for Windows, Graphpad Software, San Diego, California, USA, www.graphpad.com) has been utilized for data analysis. The mean and standard deviation were utilized for analyzing quantitative data. Statistical differences between different studied groups were evaluated through one-way ANOVA, followed by Tukey-Kramer post hoc analysis to allow comparisons. P value less than 0.05 were regarded as significant difference.

Result

The impact of MLE on the MTX-induced reduction in rat body weight:
There were no significant differences in rat body weights across all groups at the start of the trial. However, at the end of the study, the MTX-exposed group had considerably lower rat body weights than the control and MLE groups. MTX+ MLE showed significant rise compared to MTX-exposed group (Figure 1).

The impact of MLE on MTX-induced hepatic function impairment:
MTX treatment caused significant ($P < 0.05$) elevations in serum liver enzymes AST, ALT, and LDH compared to the control and MLE groups. In contrast, Treatment with 300 mg/kg MLE concomitant with MTX for 4 weeks resulted in a significant reduction in AST, ALT, and LDH levels when compared to MTX individually (Figure 2).

The impact of MLE on MTX-induced oxidative stresses:
MTX treatment caused a significant ($P < 0.05$) increase in MDA, a lipid peroxidation product, and a significant decrease ($P < 0.05$) in liver cells antioxidant SOD activity compared to the control and MLE groups. Concomitant treatment with MLE significantly decreased liver cells contents of MDA and increased activity of liver SOD ($P < 0.05$) compared to the MTX group (Fig. 3).

The impact of MLE on MTX-induced liver cells inflammation mediators; TLR4, TNF-α and IL-1β:
To investigate the inflammatory mediators in MTX-induced liver cells toxicity, their liver contents were measured using an ELISA kit. Treatment with MTX significantly leads to increase in hepatic TLR4, TNF-α, and IL-1β levels ($P < 0.05$). On the other hand, concomitant treatment with MLE significantly abolished the MTX-induced elevation in these inflammation mediator cytokines (Fig. 4).

The impact of MLE on MTX-induced liver histopathological alterations:
Sections of the liver from the MLE and control groups displayed normal hepatic architecture. Blood sinusoids were well recognized between cords of hepatocytes.
The MTX group displayed atypical morpholo; hepatocytes displayed a number of degenerativ e characteristics, such as vacuolated or apoptot ic hepatocytes, vascular congestion hemosiderin loaded Kupffer cells, inflamematory cells in sinusoids, and inflammatory cell infiltrations surrounding the portal tract.
The architecture was restored to normal in the MTX and MLE groups (Fig. 5A, B, C and D). Histopathological scoring confirmed these results (Table1).

Table I: Histopathological scoring of rat livers in the studied groups (n=6) according to Modified Ishak histology activity index.

Ishak modified histological activity index	Control	MLE group	MTX group	MTX and MLEgroup
Periportal or periseptal interface heptatitis (piecemeal necrosis)	0	0.5	3.2	1.5
Confluent necrosis	0	0	4.8	1.1
Focal (spotty) lytic necrosis, apoptosis and focal inflammation	0.5	0.6	3.2	2.4
Portal inflammation	1	1.1	3.4	1.8
	1.5	2.2	14.6	6.8

The impact of MLE on MTX-induced hepatic fibrosis (Fig.6):

Masson trichrome-stained slices from the various groups revealed that the control and MLE groups had no to very faint perivascular collagen staining. MTX caused moderate to high collagen deposition in the periportal and perivascular areas. In the MTX and MLE groups, fibrosis scores reduced significantly indicating low fibrosis (Fig. 6E).

The impact of MLE on MTX-induced increased hepatic expression of activated Caspase 3 (Fig.7):

Hepatic activated caspase-3 immunoreactivity in the MLE and control groups revealed either minimal or no immunopositive caspase 3 expression. On the other hand, Hepatic activated caspase-3 immunoreactivity in the MTX treated group revealed significantly greater immunopositive cytoplasmic staining and invasive inflammatory cells. Hepatocytes in the MTX and MLE group displayed significantly less immunopositivity staining (Fig.7E).

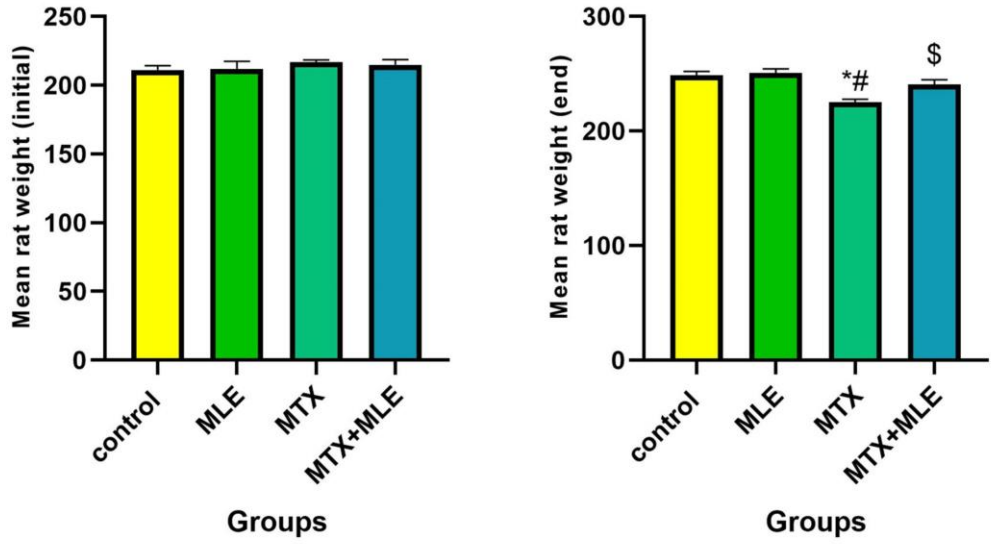


Fig. 1: quantitative examination of mean rat weight at start and end of study. Data are given as mean ± SD. *, #, and $ indicate significant changes from control, MLE, and MTX groups at P<0.05 (n=8).

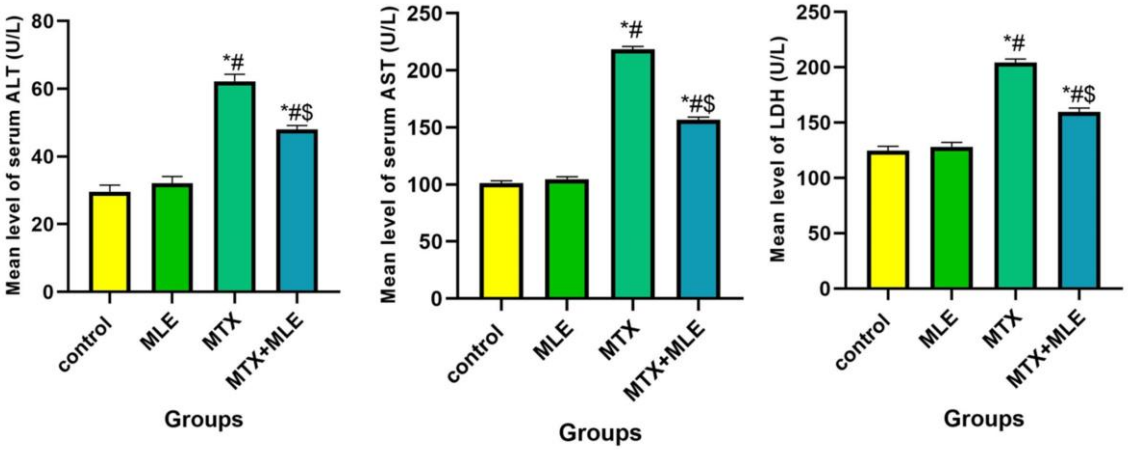


Fig. 2: Quantitative examination of ALT, AST, and LDH mean levels (mean ± SD). *, #, and $ indicate significant differences from control, MLE, and MTX groups at P < 0.05 (n=8).

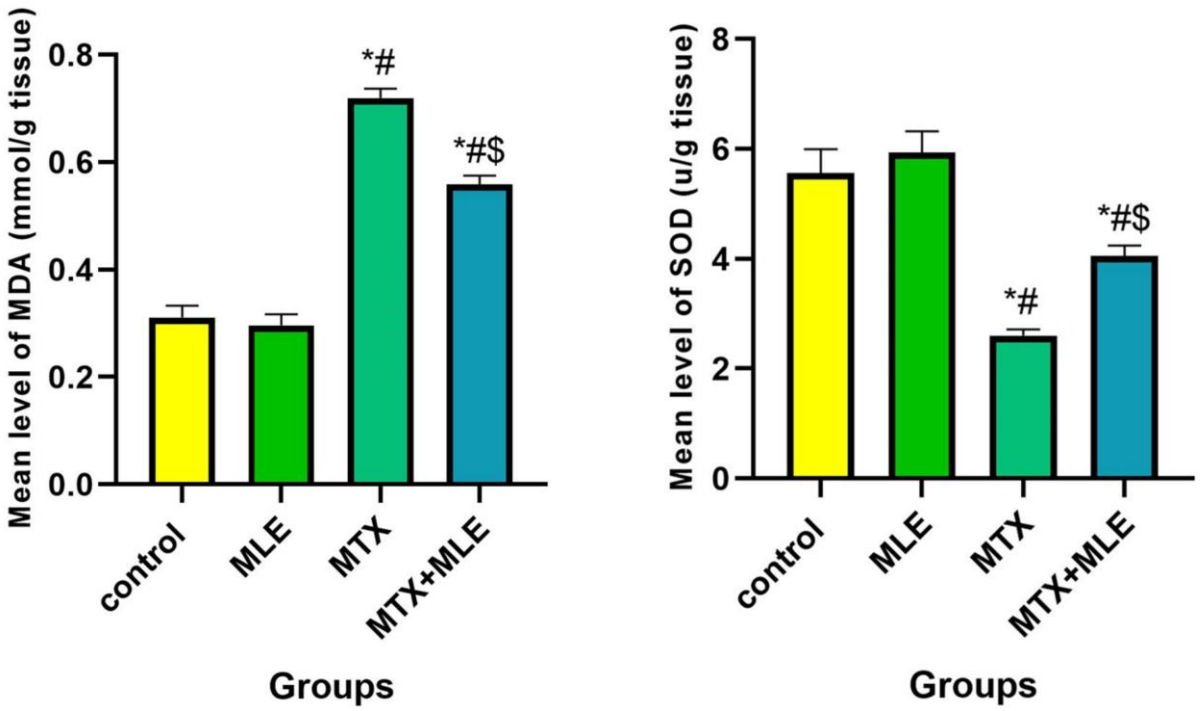


Fig. 3: Quantitative examination of MDA and SOD mean levels. *, #, and $ indicate significant differences from control, MLE, and MTX groups respectively at P < 0.05 (n=8).

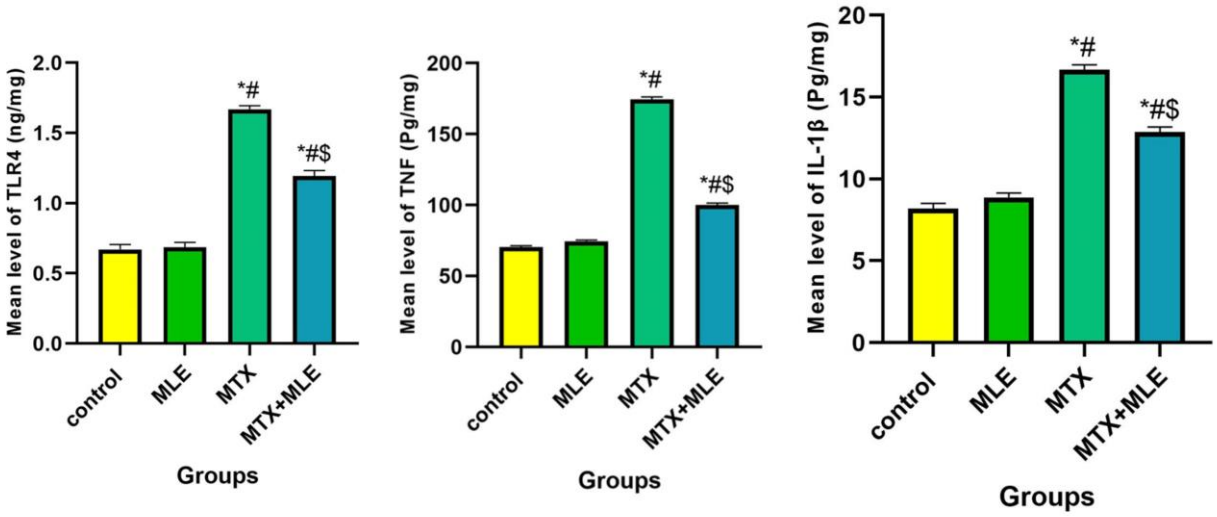


Fig. 4: Quantitative examination of TLR4, TNF-α and IL-1β mean levels, *, #, and $ Significance differences from control, MLE, and MTX groups respectively at P<0.05 (n=8).

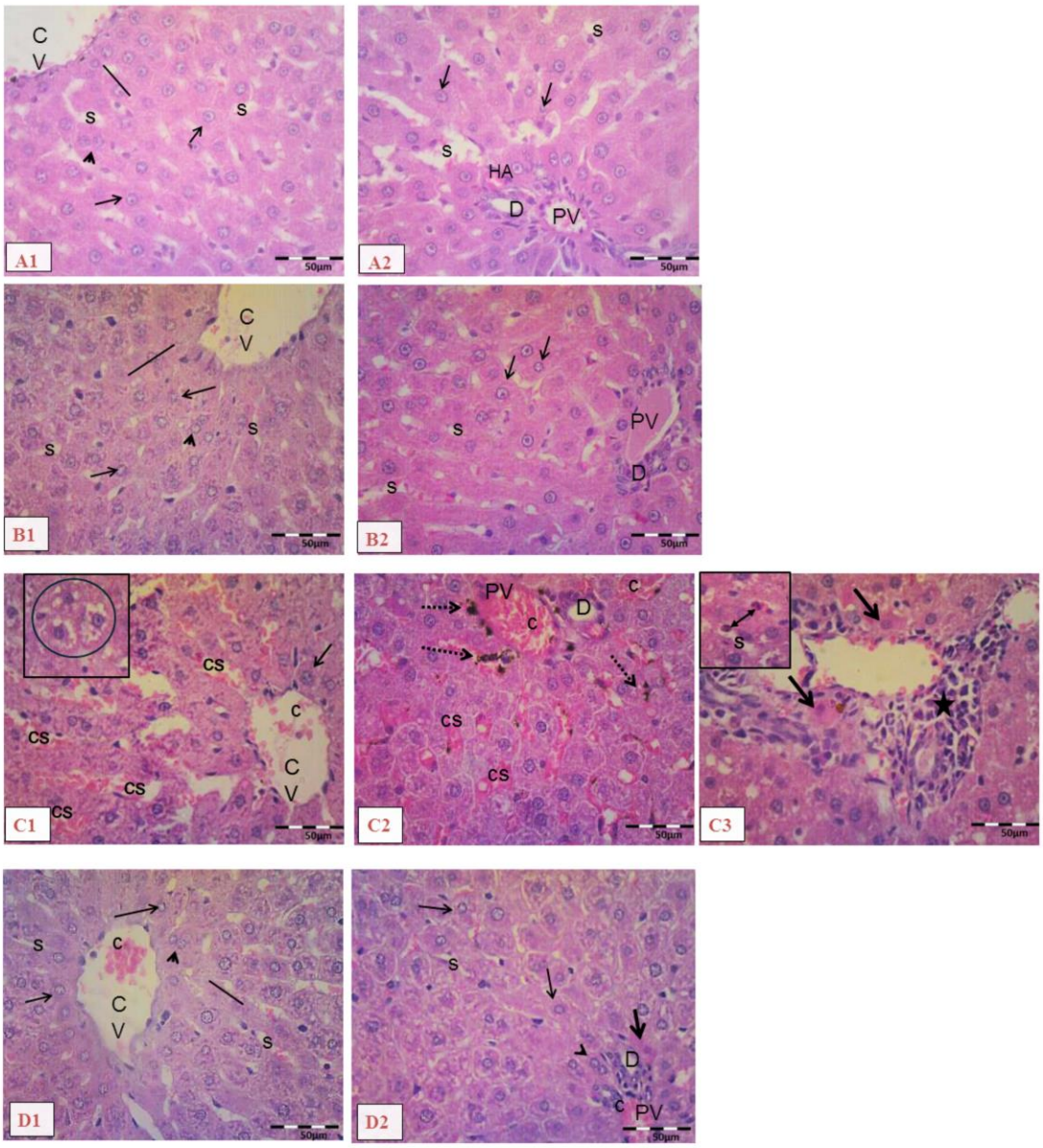


Fig. 5: Representative H&E photomicrographs of rat animal hepatic tissues from the Control (A) and MLE (B) groups, demonstrating typical lobular configuration encircling the major veins (1) and portal areas (2). C) A disrupted normal architecture is displayed by the MTX group. D) The restored normal architecture is displayed by MTX and MLE. CV; central vein, lines; plates of hepatocytes, arrows; vesicular nuclei, arrowheads; binucleated hepatocytes, S; blood sinusoids, PV; portal vein, HA; hepatic artery, D; bile duct; cS; dilated congested sinusoids, circles in inset; vacuolated hepatocytes, thick arrows; apoptotic hepatocytes, c; vascular congestion, dashed arrow; hemosiderin loaded Kupffer cells, double head arrow in inset, inflammatory cells in sinusoids and star; inflammatory cell infiltrations around portal tract. H&E × 400

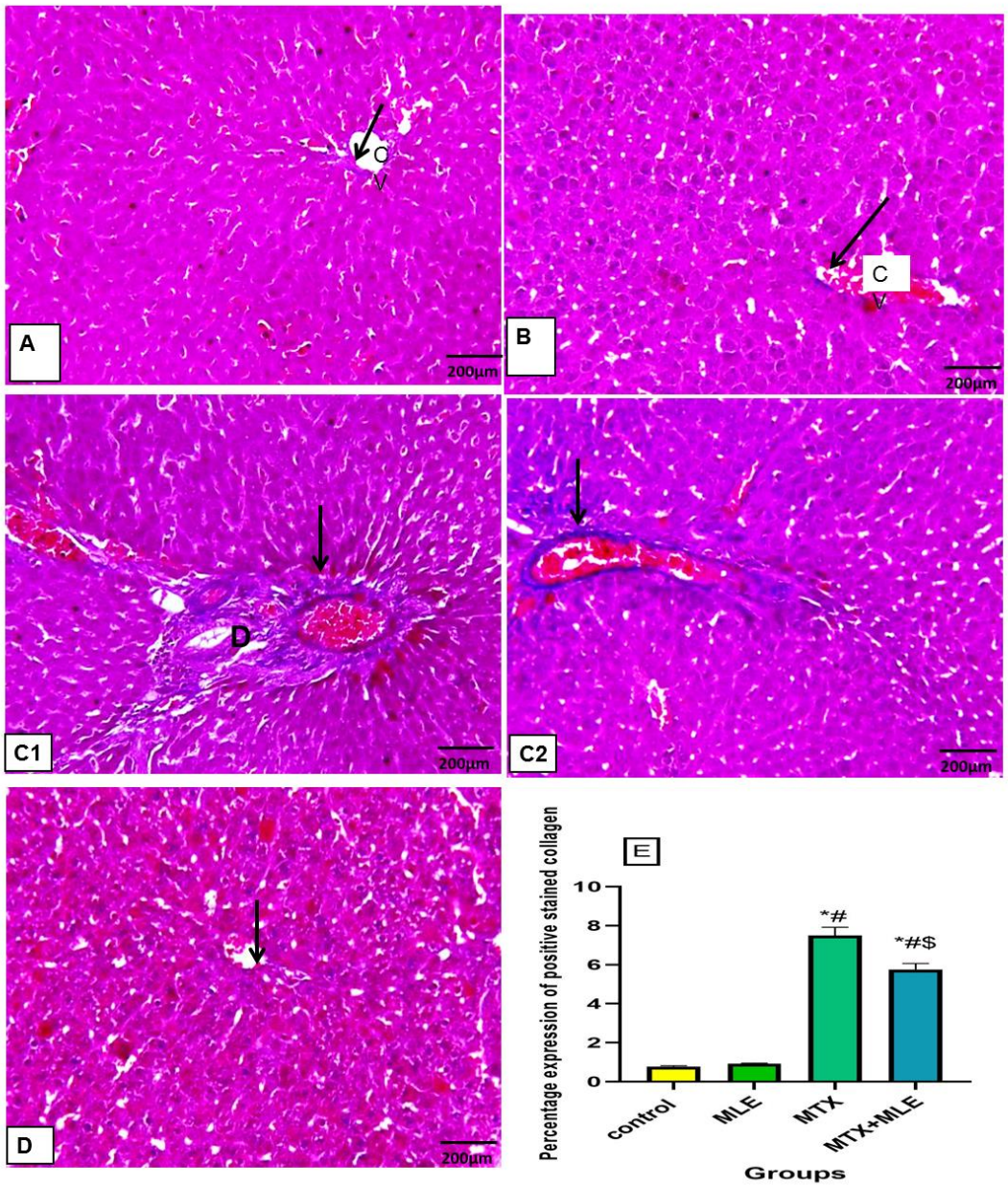


Fig. 6: Representative Masson trichrome staining micrograph of hepatic tissues sections from different groups. A, B) Control and MLE group showing none to few faint perivascular collagen staining. C1,2) MTX group showing periportal and perivascular moderate to high collagen deposition. D) MTX and MLE showing few, faint thinner collagen threads. Thin arrow= positive collagen deposition, CV; central vein, D; bile duct. Image magnification= 100x. E) *,#, and $ Significance differences from control, MLE, and MTX groups respectively at P<0.05.

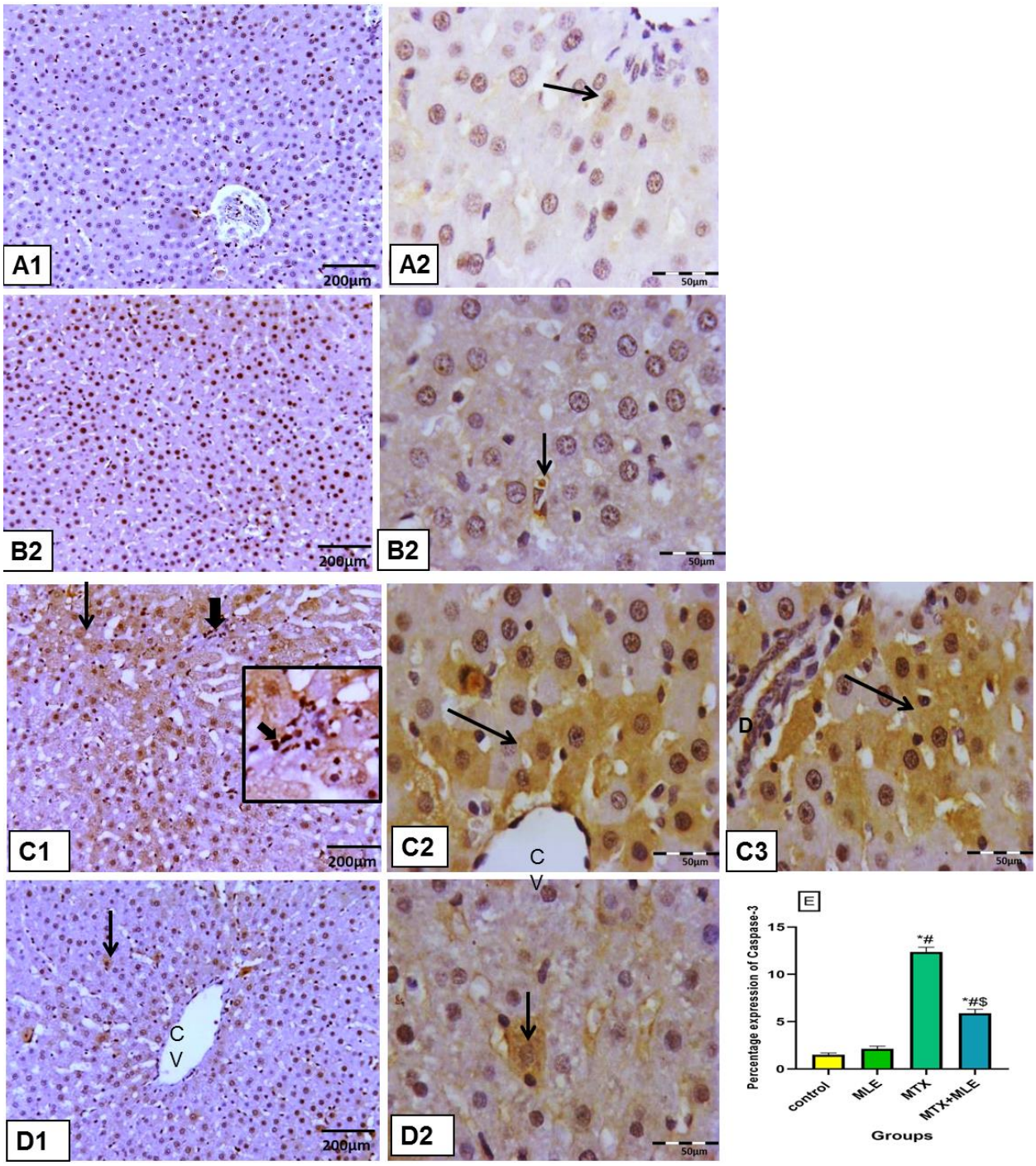


Fig. 7: Representative IHC of activated Caspase expression in hepatic section of different treatment groups. A, B) Control and MLE group are showing few to mild faint (thin arrow) immunopositive expression of caspase. C1, 2, 3). MTX group showing moderate to severe immnopositive cytoplasmic and nuclear staining in hepatocytes (thin arrow) with invading inflammatory cells (thick arrow,inset). D) MTX and MLE showing few, minimal cytoplasmic immunopositivity staining in hepatocytes (thin arrow). CV; central vein, D; bile duct. Image magnification 1 X100; inset, 2&3X400. E) *,#, and $ Significance differences from control, MLE, and MTX groups respectively at P<0.05.

Discussion

Methotrexate, previously known as amethopterin, is a successful treatment for a variety of inflammatory and immunological illnesses, as well as specific types of cancer [27] However, its liver-related morbidity limited its application. Additionally, MTX dramatically increased serum cytoplasmic enzymes; AST, ALT, and LDH concentrations. When liver cells subjected to damage, these cytoplasmic enzymes are produced in excess quantities and enter the bloodstream, reflecting a decline in liver cells proper function. Furthermore when, hepatocellular function is compromised, bile acid deposition starts resulting in further stress and cytotoxicity [28] .

However, the toxic impacts of MTX on the liver cells function were considerably mitigated by concomitant administering MLE for four weeks. Similarly, other previous studies reported hepatic cells protective impact of MLE [29]

MTX administration decreased hepatic activity of SOD while increasing hepatic lipid peroxidation content marker; MDA, which is used as an indicator of increased ROS formation, According to the present work, MTX-induced liver cells oxidative stress environment as it disrupts the physiological balance between the free radicals production process and the antioxidants activities. On the other hand, MLE corrected the oxidative equilibrium state that was disrupted by MTX.

When MLE and MTX were administered concurently, the hepatic contents, MDA, and the hepatic activity of SOD returned to nearly normal values. MLE has been demonstrated to exhibit antioxidant activity in the hepatocytes and other organs, including the digestive tract [30].

The decreased intracellular antioxidant enzyme activity in the present study is either due to excess free radicals release or by the inhibitory impact of MTX on the antioxidant response element (ARE) - gene expression [31]

In addition, the formation of MTX-polyglutamate (MTX-PG) inside body cells, which is a MTX metabolite that causes oxidation of hepatocytes, possibly explains the mechanism of MTX-induced hepatic injury. MTX-PG disturb oxidative equilibrium state in the liver cells by promoting peroxidation of lipid, with subsequent free radicals release and direct inhibitory impact on cellular antioxidant components [9]

Studies indicate that MTX-induced hepatotoxicity is primarily caused by the generation of inflammatory mediators as TLR4, TNF-α, and IL-1β. However, the particular inflammatory signaling route that causes MTX hepatotoxicity is yet unknown [32]

The present investigation revealed that toll-like receptors 4 (TLR4) may play a role in MTX hepatotoxicity cascades.

TLR4 is one of the groups of pattern recognition receptors (PRRs) that trigger inflammatory reactions. PRRs can detect both exogenous pathogen-associated molecular patterns (PAMPs) present in gram-negative bacteria and endogenous damage-associated molecular pattern receptors (DAMP) present in apoptotic or injured cells. Nevertheless, it has been discovered that ROS are produced by NADPH oxidase when TLR4 is activated, suggesting an unexpected connection between ROS and TLR4 receptors. In addition, TLR4 triggers generation of tumor necrosis factor-α and other inflammatory molecules [33].

A study by Narendra and others [10] indicates that medicines connected to direct hepatic injury alter cytokine levels, by creating an imbalance state between both pro-inflammatory and anti-inflammatory molecules as a result of IL-1β up-regulation [34]. In addition MTX-PG enhances the signaling pathways of pro-inflammatory molecules and cytokines, including TNF-α, NF-kappa B, IL-6, IL-1, and IL-12[9]

The release of inflammation mediator molecules during hepatotoxicity is related to release of IL-1 β and TNF-α [10]
In the current study, 300 mg/kg MLE given twice a week for four weeks resulted in decrease the generation of MDA and inflammatory mediators as TLR4, TNF-α, and IL-1β. This antioxidant and anti-inflammatory impact may be due to MLE contents of phenolic compounds as they have multifactorial actions against oxidation and inflammation [30].

In the current study, 0.5 mg/kg MTX given twice a week for four weeks using the (I.P) injection caused damage to hepatic histological architecture, which involves vacuolation and/ or apoptosis of hepatocytes, congested dilated blood sinusoids, vascular congestion, hemosiderin-loaded Kupffer cells, inflammatory cells that reside in sinusoids, and inflammation-related cell infiltrations around the portal tract. These findings are consistent with those published by [35], who noticed that nodular regeneration, sinusoidal obstruction, and acute fatty liver are additional pathologies for direct methotrexate induced hepatotoxicity.

Likewise, Laskin and Laskin[36] discovered that certain medications with induced hepatotoxicity are associated with both general and local inflammatory process characterized by mobilization of both neutrophils and macrophages in liver vascular system. As Liver damage causes Kupffer cells activation and neutrophils are drawn into the liver. In certain cases, these inflammatory cells cause more liver damage even though they are in charge of clearing away cell debris and are a component of the host defense system.

Furthermore, liver nonparenchymal cells such as Kupffer cells, sinusoidal endothelial cells, and stellate (fat-storing or Ito) cells, as well as monocytes and neutrophils, play a role in the pathophysiology of liver cells damage. Kupffer cells and neutrophils generate proinflammatory cytokines and chemokine, as well as reactive oxygen and nitrogen species, which contribute to oxidative stress process induced by toxins and ischemia/reperfusion procedure [37]

In the current study, MTX group had moderate to high periportal and perivascular collagen deposition. This may be explained by the accumulation of MTX metabolites, polyglutamates (MTX-PGs), with the reduction of folic acid level in hepatocytes, these factors further trigger oxidative stress, fibrotic changes, apoptosis, and inflammation of liver cells. Furthermore, sinusoidal endothelial cells are unusually fenestrated and preferentially sensitive to cancer chemotherapeutic drugs, resulting in veno-occlusive illness. Overproduction of collagen by activated stellate cells leads further fibrosis of liver cells [37]. In addition MTX-PG inhibits 5-aminoimidazole-4-carboxamide ribonucleotide transformylase enzyme, causing accumulation of intracellular adenosine, activation of hepatic stellate cells, extracellular matrix formation, and hepatic fibrosis[9]. Furthermore, excessive collagen expression induces apoptosis [37].

On the other hand, MLE administration resulted in significant reduction in Masson trichome in the form of few, faint thinner collagen threads. This suggests that ML has an anti-fibrosis impact, which may be linked to reduced collagen expression [37].

In the current work, MTX group showed significantly increased in hepatic activated caspase-3 cytoplasmic and nuclear immnopositive staining with invading inflammatory cells. MTX induces hepatocytes apoptosis is either by activating caspase 3 intrinsic pathways or may be due to MTX-PG that inhibits RNA and DNA formation process through its folic acid lowering mechanism [9]

The current work demonstrated significant improvements in light microscopic examination of liver tissue performed on animal samples treated with MLE. Various researches confirmed the administration of MLE extract resulted in significant decreases in fibrosis, hepatic necrosis, lipid accumulation, infiltration of inflammatory cells, hepatocellular degeneration, and sinusoidal distortion. Similar researches found that MLE dosages more than 200 mg/kg body weight had a more significant impact on liver histopathological features and liver biomarkers than smaller levels [38].

Conclusion

The normalization of the evaluated chemical variables suggests that MLE scavenges the harmful metabolite, protecting the tissues. This is due to the fact that MLE reduces oxidative stress, inflammatory, fibrotic, and apoptotic impacts brought on by MTX. Thus, the role of MLE in protecting tissue from MTX-induced liver damage must be taken into account.

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