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# Hepato-protective effects of Silymarin against methotrexate-induced hepatotoxicity in rat model

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## ABSTRACT

**Aim:** Assessment of the possible Hepatoprotective effects of Silymarin against hepatotoxicity induced by MTX in rats.

**Materials and methods**: An 24 Albino Wister rats were included in this study, they were randomly divided into 4 groups each of 6. Group 1: control group (administered 0.9% N/S 1ml/kg/d ip) for 5 days. Group 2: MTX group (received MTX in a dose of 20mg/kg/day ip) as a single dose at day 1 and observed till day 5 of the experiment. Group 3: Silymarin group (received MTX in a dose of 20mg/kg/d ip as a single dose then Silymarin 300 mg /kg/ d ip for 5days). Group 4: vehicle group received 1ml/kg ip for each rat (10%DMSO + 40% PEG300 + 5% Tween400 + 45% Normalsalin) vehicle solution for 5 days. At day 5 all animals were sacrificed, serum was aspirated for the assessment of ALT, AST, ALP, TSB levels by colorimetric assay. Liver was removed and divided into 2 parts for histopathological examination and for assessment of tissue TNF-alpha, caspase-3, MDA, total antioxidant capacity, and TLR4 levels by ELISA method.

**Results:** Mean serum levels of ALT, AST, ALP and TSB as well as tissue MDA, TNFα, TlLR4, Caspse3 were significantly increased in MTX group compared with the control and vehicle groups together with significant reduction in total antioxidant capacity and obvious histological damage, whereas meanwhile treatment of rats with Silymarin together with MTX resulted in significant reduction in mean serum levels of ALT, AST, ALP and TSB as well as tissue MDA, TNFα,TlLR4,Caspse3 together with significant increment in total antioxidant capacity and near normal restoration of hepatic tissue architecture.

**Conclusions:** Silymarin has Hepatoprotective effect against MTX induced hepatotoxicity by its antioxidant, anti-inflammatory, and anti- apoptotic properties

**Keywords:** *Methotrexate, Silymarin (silly), Hepatotoxicity, rats*

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## INTRODUCTION

Methotrexate is a folic acid antagonist a widely used medication as a cytotoxic and immune modulator, it is the first-line drug for the treatment of connective tissue disorders such as psoriasis, juvenile idiopathic arthritis, rheumatoid arthritis (RA), vacuities, multiple sclerosis, and systemic lupus erythematous also in virally induced arthritis [1]. MTX act as folate antimetabolite get into the cell by certain carrier molecules then transforms into methotrexate-polyglutamate molecule that inhibit the enzyme dihydrofolate reductase, which is responsible for the conversion of dihydrofolate into tetrahydrofolate (the active form of folic acid). The reduction in tetra-hydrofolate levels will prevent the production of DNA, RNA, and other nuclear proteins [2]. Extracellular MTX is eliminated by Glucarpidase that detoxifying it and preventing its accumulation. It is very effective for managing different tumors and autoimmune disorders. MTX use is frequently hindered by its hepatotoxicity which is a major and recognizable adverse reaction that complicate its clinical use one of the major chemotherapeutic drugs used to treat various tumours is methotrexate. Patients with psoriasis, systemic lupus erythematous, inflammatory bowel disease, vacuities, rheumatoid arthritis, juvenile idiopathic arthritis, psoriasis, and numerous other connective tissue illnesses can also benefit from [3]. It is contraindicated in pregnant women due to high teratogenicity index, it is also effective in organ transplantation because of its anti-inflammatory and immunomodulatory properties. Methotrexate can be combined with anti-TNF agents in managing patients with ulcerative colitis [4]. Methotrexate is used in off-lain cases of mycosis fungoid, dermatomyositis, pityriasis rubra pilaris, eczema, and sarcoidosis, MTX induce hepatotoxicity by multiple mechanisms such as inflammation, oxidative stress, as well as apoptosis Methotrexate Induced Hepatotoxicity by its effect at the cellular level. Methotrexate enters the cell linked to the folate transporter and is then carried out of the cell by the ATP-binding cassette (ABC) family of transporters as a polyglutamate that inhibits DHFR enzyme. Methotrexate reduces the formation of pyrimidine and purine with generation of methionine from homocysteine as an indirect effects. Oxidative stress, lipid peroxidation and reactive oxygen radicals all are implicated in hepatic toxicity together with homocysteine overload, hepatic tissue infiltration with fat, and release of proinflammatory cytokines with consequent liver fibrosis [5]. Inflammation is also implicated in the mechanism of Methotrexate induced hepatotoxicity, pro-inflammatory signaling pathways and cytokines produced by MTX-PG include tumor necrosis factor, nuclear factor kappa B, and IL-1 and IL-12 [6] as well as increase the level of IL6. Apoptosis also has been involved in the mechanism of Methotrexate induced hepatotoxicity, Caspase-3 is activated by both intrinsic and extrinsic pathways. Both paths cause the intracellular enzymes such as proteases and endonucleases, which are involved of cell disintegration, to become active. The extrinsic pathway necessitates activation of a death receptor, such as the Fas receptor, which causes the creation of a signaling complex that causes death with FADD and procaspase-8 or -10 [7]. Silymarin, a lipophilic extract from the plant Silybum marianum known as milk thistle is a natural substance, the plant with large brig and purple flowers in areas with enough sunlight exposure. Silymarin has the empirical formula C25H22O10 and is a complex combination of the flavonolignans isomers silibinin, isosilibinin, silidianin, and silichristin [8]. Silymarin was found to have Hepatoprotective effects by many clinical and experimental studies, it can increase the Glutathione levels, stabilize cell membranes, scavenging the free radicals, and enhancing the DNA polymerase to protect the liver against chemical injuries [9]. By preventing the production of free radicals and reactive oxygen and nitrogen species, as well as by supporting antioxidant enzymes like GSH, catalase, and SOD that are all involved in the neutralization of free radicals and by lowering the activation of the inflammatory mediator NFkB, Silymarin has powerful anti-oxidant properties [10]. According to research, Silymarin reduces the production of inflammatory cytokines such TNF-alpha, IL-2, IL-6, IL-8, and IL-12. The level of TLR4, a crucial mediator protein for the transmission of inflammatory signals, is ameliorated by Silymarin. It plays a critical role in immune response and inflammatory reactions by being able to recognize xenoantigens and acting through signaling pathways to create proinflammatory mediators [11].

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The advantages of SM are related to the beneficial effects of SM are associated with reduction in TLR4 activation and expression with improvement of Liver Function Tests parameters in experimental animals treated with Methotrexate [12]

## MATERIALS AND METHODS

### *Animals*

A total of 24 adult Wister Albino rats with age of 7-8 weeks, weighting 150-250 g were obtained from Animal house at the Kufa University College of Sciences. The study protocol was approved by the central bioethical Committee of Kufa University. Animals were settled in the animal house of university of Kufa in a temperature-controlled (22+-2 C) room, with alternating 12hr light: 12hr dark cycles. Animals had free access to water and chow diet until the start of the experiments.

### *Study design*

After two weeks of acclimatization, the rats were randomized into 3 groups (n=6) as following Control group 1: Rats received 0.9% sodium-chloride (1ml/kg/day, via IP route) for5days. MTX treated group 2: Rats received MTX (20mg/kg/day, via IP route) for5days Sily- treated group 3: Rats received MTX (20ml/kg/day, via IP route) + Silymarin (300mg/ daily dose of Silymarin sol (300 mg/kg/day, ip) for 5 days

Vehicle group 4: 1ml/kg from the stock suspension was administrated ip for each rat (10% DMSO + 40% PEG300 + 5% Tween400 + 45% Normalsalin) for 5 days. At day 5 following variables were assessed: Serum ALT, AST, ALP, TSB concentration, Tissue MDA level, Tissue TNFα level, Tissue Caspas3 level, Tissue TLR4 level, and tissue TAC level, and histopathological examination

### *Blood Sample preparation*

At day 5, Ketamine 100mg/kg and xylazine 10mg/kg were given IP according to the weight of rats [32-33].Following this, blood samples were drawn directly from the left ventricle of the heart via a cardiac puncture. Blood samples were then placed in tubes containing clot activator gel and allowed to coagulate at 37C before being centrifuged at 3000 rpm for 10 minutes to extract the serum [13]. The subsequent serum collected is used to determine the levels of total serum bilirubin (TSB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

### *Hepatic tissue preparation for ELISA measuring of TLR4, TNFα, MDA, Caspse3, and TAC activities*

Liver was rinsed with sodium chloride solution 0.9% to remove the clots and then placed in a deep freezer at -80 degrees Celsius. Following that, a liver segment was removed and homogenized using a high-intensity ultrasonic liquid processor in phosphate buffered saline that is 1:10 W/V and contains 1% Triton X-100 and 1% protease inhibitor cocktail [35] According to the manufacturer of the Elisa kits, the homogenates were centrifuged at 3000 rpm for 20 minutes at 4centigrade, and the supernatant was used to determine the levels of measuring of TLR4, Caspase3 , MDA,TAO and TNF- α (Bioassay Technology Laborator) [14].

### *Preparation of hepatic tissue samples for Histopathology*

Liver tissue were kept in 10% formalin and fixed. Then the samples were immersed in ethanol for two hours for each concentration (70, 80, 90, and 100%), the samples were then cleaned using xylene (an organic solvent) and prepared for examination. The samples were then cleaned using xylene (an organic solvent) which was used to remove the alcohol from the samples so that they could soak in paraffin wax (an embedding agent).Histological sections from all groups were analyzed, and the histopathology test was performed in X100 to semi-quantify the difference in liver damage [15]. Score of the percentage of tissue damage as follows:

Score 0: No damage, normal architecture
Score 1 (mild): Less than 25% damage
Score 2 (moderate): 25-50% damage
Score 3 (severe): 50-75% damage
Score 4 (highly severe): 75-100% damage.

### *Statistical Analysis*

Data were analyzed using SPSS programmer version20.Statistical analysis of the experimental results was conducted according to GraphPad prism where one way (ANOVA) were used to assess the significance of differences between groups .

The data were expressed as mean ± standard errors (SE) and P value < 0.05 was considered statistically significant.

## Results

***Effect of Methotrexate, and Silymarin on Toll like Receptor (TLR4) level***

The amount of TLR4 in hepatic tissue was significantly higher in the Methotrexate group p ≤ 0.01 than in the control and vehicle groups, Silymarin significantly reduced the level of TLR4, figure (1)

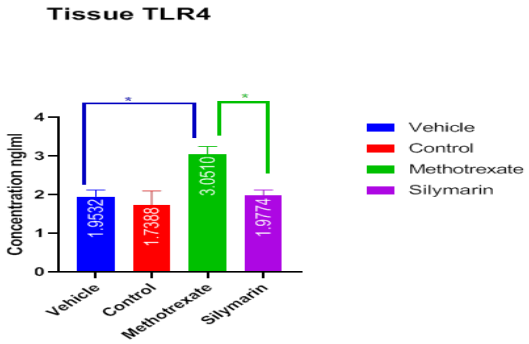


**FIGURE 1**: Mean tissue level of TLR4 (ng/l) in control and treatment groups

Values of ≤ 0.05 were considered statistically significant
*Significant ** Significant

***Effect of Methotrexate, and Silymarin on inflammatory parameter (TNF -α) level***

The amount of (TNF-α) in hepatic tissue was substantially higher in the Methotrexate group p ≤ 0.01 than in the control and vehicle groups, while in Silymarin group TNF level was significantly low, figure (2).

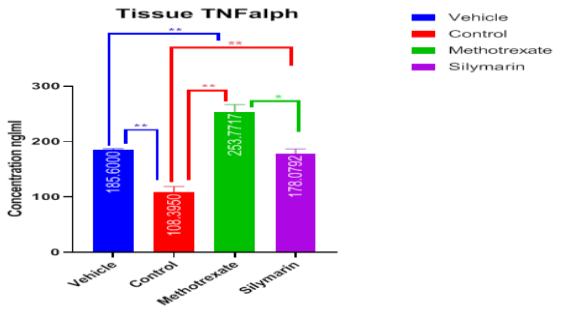


**FIGURE 2:** Mean tissue level of (TNF -α) (ng/l) of the four groups

Values of ≤ 0.05 were considered statistically significant
*Significant ** Significant

***The level of oxidative stress parameters in hepatic tissue (MDA and TAC)***

***Oxidative stress parameter MDA***

The amount of MDA in hepatic tissue was substantially higher in the Methotrexate group p ≤ 0.01 than in the control and vehicle groups while in Silymarin treated group the MDA level was significantly reduced, figures (3-4)

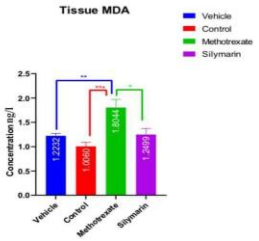


**FIGURE 3:** Mean tissue level of MDA (ng/l) of the four groups

Values of ≤ 0.05 were considered statistically significant
*Significant ** Significant

***Total antioxidant capacity level in control and treatment groups (TAC)***

The amount of TAC in hepatic tissue was substantially lower in the Methotrexate group p ≤ 0.01 than in the control and vehicle group while in Silymarin treated group TAC was significantly higher, figure (4)

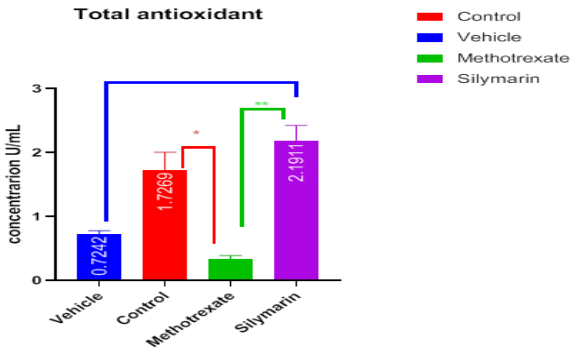


**FIGURE 4:** Mean tissue level of TAC (U/L) of the four groups

Values of ≤ 0.05 were considered statistically significant
*Significant ** Significant

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***Effect of Methotrexate, and Silymarin on the apoptotic parameter caspase-3***

The amount of casp-3 in hepatic tissue was substantially higher in the Methotrexate group $p \leq 0.01$ than in the control and vehicle groups while in Silymarin group caspase-3 level was significantly reduced, figure (5)

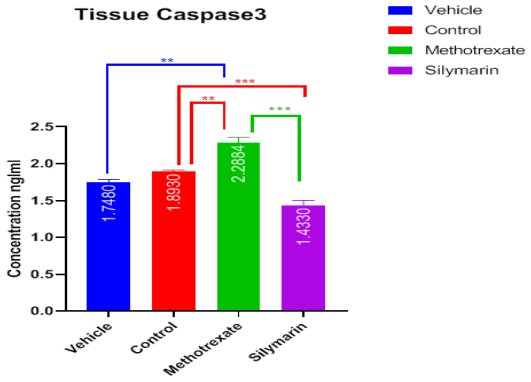


**FIGURE 5:** Mean tissue level of CASP-3 (ng/l) of the four groups

Values of $\leq 0.05$ were considered statistically significant
*Significant ** Significant

***Effect of methotrexate and Silymarin on liver biochemical markers (ALT, AST, ALP, TSB)***

The level of (ALT, AST, ALP, and TSB) in hepatic tissue was substantially higher in the Methotrexate group $p \leq 0.01$ than in the control and vehicle groups whereas in Silymarin group their level was significantly reduced, figures (6-9)

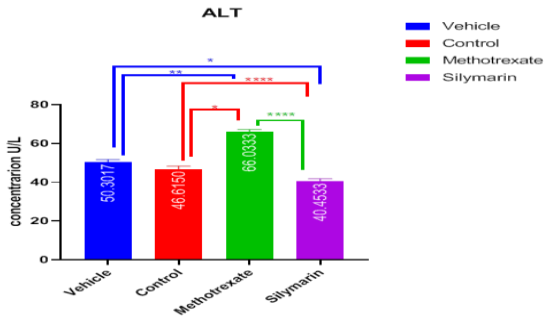


**FIGURE 6:** Mean serum level of ALT (U/l) of the four groups

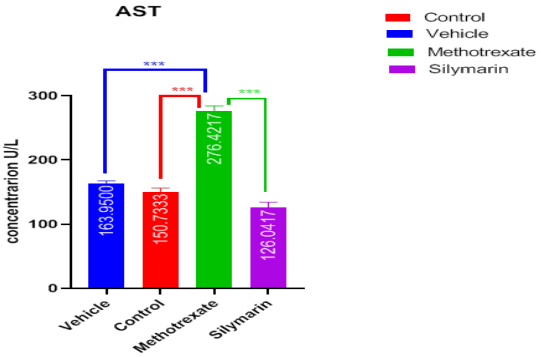


**FIGURE 7:** Mean serum level of AST (U/l) of the four group

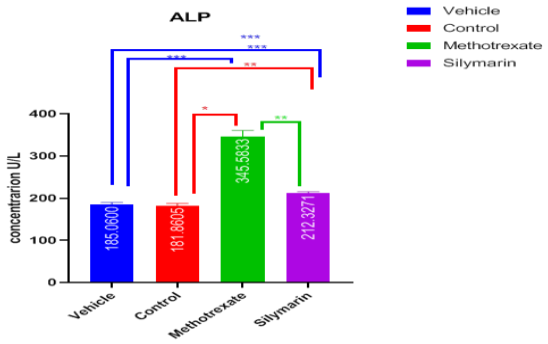


**FIGURE 8:** Mean serum level of ALP (U/l) of the four groups

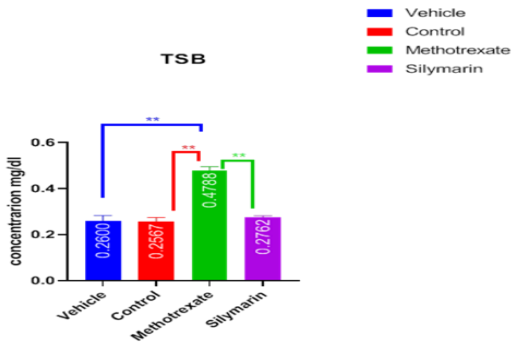


**FIGURE 9:** Mean serum level of TSB (U/l) of the four groups

Values of $\leq 0.05$ were considered statistically significant
*Significant ** Significant

***Effect of Methotrexate, and Silymarin on hepatic histology***

The histology of the hepatic tissue in control group showed normal morphological appearance with intact hepatic tissue architecture. Rat liver tissue in the Methotrexate treated group displayed significant histological abnormalities that included hepatic cellular necrosis, invasion of mononuclear cells, sinusoidal enlargement, and an increase in the number of kupffer cells. In the Silymarin-treated group there was restoration of nearly normal hepatic tissue architecture manifested by reduction in focal mononuclear cell infiltration, sinusoid enlargement with inflammatory cells infiltration together with an increase in hepatocyte mitotic activity were observed, figures (10-15).

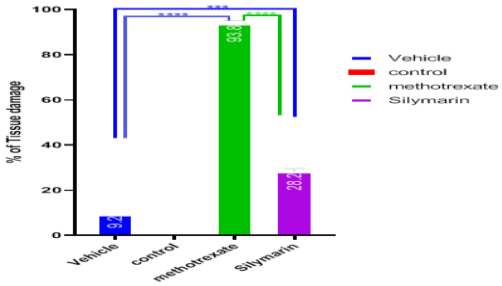


**FIGURE 10:** Percentage of tissue damage in each group

Values of ≤ 0.05 were considered statistically significant

*Significant ** Significant

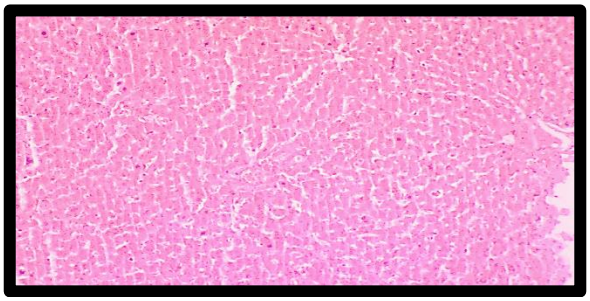

**FIGURE 11:** The histological section in the liver of rat in control group with normal histological texture

The tissue is stained by H&E stain and captured using light microscope at 10X magnifier scale

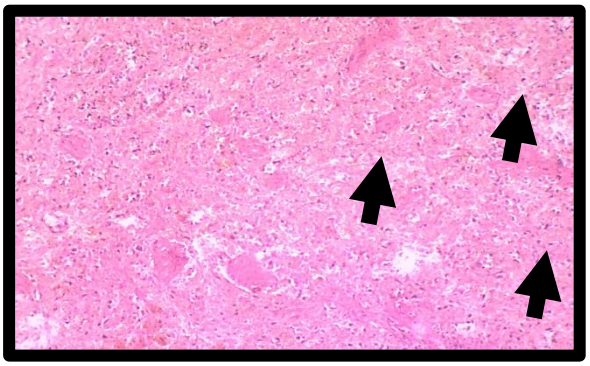

**FIGURE 12:** MTX group

The histopathological section in the liver shows clear damage in the hepatocytes (liquefactive necrosis, Black arrows). The tissue is stained by H&E stain and the section is captured using light microscope at 10X magnifier scale

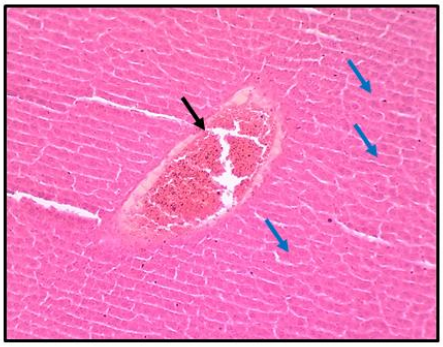

**FIGURE 13:** MTX group

The histopathological section in the liver shows clear blood vessels congestion (Black arrow) and hypertrophy of hepatocytes (Blue arrows)

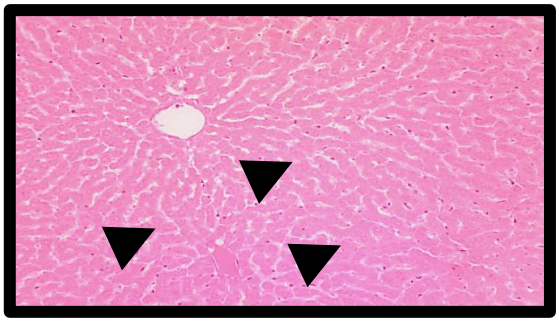

**FIGURE 14:** Silymarin group

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The histological section in the liver shows mild hydropic degeneration in the hepatocytes (Black arrows) with reduction in vascular congestion. The tissue is stained by H&E stain and the section is captured using light microscope at 10X magnifier scale.

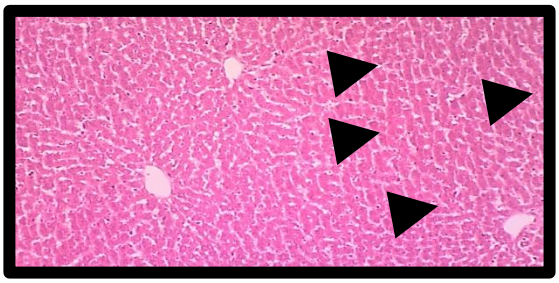

**FIGURE 15:** DMSO vehicle group: The histopathological section in the liver shows mild fatty changes (fat droplet infiltration, Black arrows) with inflammatory cell infiltration

## DISCUSSION

Methotrexate (MTX) is a common anti-metabolite medication widely applied in chemotherapy and autoimmune illnesses. Hepatotoxicity which ranges from mild hepatitis and cholestasis to abrupt liver failure and cirrhosis is one of the most dangerous side effects of methotrexate [16]. As a result, methotrexate's usage is hampered. In order that MTX to be administered safely, adjuvant substances having hepato-protective potential must be found. A substantial researches demonstrated that Silymarin has a powerful antinflammatory, anti-oxidant, and anti-apoptotic properties in vitro and in vivo studies making it a promising product that can minimizing and ameliorating the MTX hepatotoxicity.

### *Effect of MTX, Silymarin on TLR4 level in hepatic tissue*

In our study, TLR4 levels in liver tissue was significantly higher in the Methotrexate-treated group. MTX may cause inflammation by activating the TLR4 NF-B signaling system and increasing the production of inflammatory mediators in a variety of organs. Our findings are consistent with many studies [17-19] who reported that MTX induced an inflammatory response in cardiac and hepatic tissues via up-regulation of TLR4 and NF-Kappa B as well as additional activation of the Nucleotide-binding domain receptor family (inflammasome). Rats treated concurrently with Silymarin and MTX had significantly lower tissue TLR4 levels, which in turn resulted in a diminished inflammatory cascade. Silymarin is well known for its powerful pleotropic effects, which include reducing TLR4 and its downstream transcription factors to reduce inflammation which is attributed in part to its protective features this is in agreement with previous studies [20-22] who found that Silymarin can reduce the expression of TLR4 in hepatic, neuronal tissues and monocyte cell culture respectively.

### *Effect of Methotrexate, & Silymarin on inflammatory parameter (TNFα)*

As demonstrated by previous researchers [23-24] who all demonstrated that MTX-induced hepatotoxicity would increase in TNF-Alpha level and the downward inflammatory pathway, our results also showed that TNF-Alpha was significantly elevated in MTX-intoxicated rats. According to a previous researcher who claimed that Silymarin had reduced tumor necrosis factor (TNF)-alpha mRNA expression in the liver toxicity model, concurrent treatment of MTX-intoxicated rats with Silymarin resulted in a considerable drop in the TNF alpha level.In an animal model of insulin resistance[48], Silymarin was found to lower the amount of TNF alpha in hepatic cells which is in agreement with our findings [25] who indicated that Silymarin had lowered TNF alpha levels in acute hepatotoxicity brought on by triptolide toxicity in rat models.

### *Effect of Methotrexate, & Silymarin on oxidative stress MDA and total antioxidant capacity (TAC)*

While the TAC was significantly decreased in the MTX group which is consistent with pervious study [26] who found that treatment of rats with MTX resulted in a significant reduction in TAC. Hepatic tissue toxicity in the MTX intoxicated group showed increased MDA tissue level, which is attributed to increased oxidative stress and tissue injury carried on by free radicals, while the TAC was significantly higher in Silymarin group in comparison to the MTX group, this was consistent with studies [52,53,54,55] who all stated that significantly reduced lipid peroxidation with the suppression of excessive ROS production and preservation of the liver's antioxidant status can be produced by Silymarin.

Also our results are in agreement with [27] who showed that Silymarin have reduced the level of oxidative stress markers and increased TAC [28] stated that Silymarin is a potent antioxidant against carbon tetrachloride, alcohol, oxidative stress brought on by iron, acetaminophen, and bleomycin.

***Effect of Methotrexate, Silymarin &Montelukast on apoptotic (Caspase3)***

Similar findings were made by [29] who discovered that administering MTX to rats caused an increase in caspase3 level, hence accelerating the process of apoptosis. Treatment with MTX resulted in a significant rise in the incidence of the apoptotic marker caspases 3. Rats given Silymarin concurrently saw a marked decrease in caspases 3 levels, which is consistent with the findings of [64] who discovered that Silymarin lowered the production of pro-apoptotic genes to lessen the harmful effects of lead on hepatic tissue.

***Effect of MTX & Silymarin on liver function test***

***Effect of MTX on liver function test***

In the present study, MTX treatment for 5 day has been shown to induce liver dysfunction, which was distinguished by a significantly increased serum level of liver enzymes ALT, AST, ALP and TSB in contrast to the monitoring collective. Since these enzymes are found in the cytosol and released into the blood after liver damage, this spike is attributed to damaged liver cells. This outcomes was in agreement with the results reported by [30]. The elevation in Liver enzyme since they are located in the cytoplasm and released into the bloodstream following cellular damages signaling the development of hepatotoxicity, level has been linked to the damage to the structural integrity of the liver.

***How Silymarin works on liver function test (ALT, AST, ALP and TSB)***

It has been demonstrated in the current study that taking Silymarin for five days causes Hepatoprotective activity. Silymarin's antioxidant action on MTX-induced hepatic dysfunction results in a notable drop in AST, ALT, ALP, and TSB levels. This outcome was consistent with earlier research [31] which demonstrated that administering Silymarin to rats might lower the amount of these enzymes. The Hepatoprotective effect of Silymarin, which reacts to receptors on cell membranes to reduce the binding of toxins in these sites, may be the cause of the protective action of Silymarin as indicated by the reduction of these enzymes and return to nearly normal values. SLM also lowers the levels of ALT, AST, ALP, and TSB in hepatic diseases and reduces drug-induced hepatocellular damage. This outcomes also was reported by [32] who was demonstrated that, the membrane structure and integrity of the liver cells, were unharmed and uncongested in Silymarin group which would otherwise have been destroyed by MTX

***Effect of MTX, and Silymarin on liver histology***

Hepatic tissue in the control group had a normal morphological appearance according to histology. Significant histological abnormalities in the rats' livers of the MTX group included hepatocellular damage evidenced by hydropic degeneration, congestion of the central vein, dilatation of the sinusoid, inflammation, bile duct injury, and necrosis, which is consistent with [33]. In agreement with [34] who discovered the same histopathological alterations that are in line with the numerous earlier studies of MTX-induced hepatotoxicity in rat models, in which the Methotrexate group displayed significant histological abnormalities, including hepatic cellular necrosis and an invasion of mononuclear cells, along with sinusoidal enlargement, mononuclear cell infiltration, and an increase in the number of kupffer cells of kupffer cells. In the Silymarin-treated group, reduced focal mononuclear cell infiltration and sinusoid enlargement together with an increase in hepatocyte mitotic activity were all observed which are in agreement with [35].

## CONCLUSIONS

Silymarin has a Hepatoprotective effect against MTX induced hepatotoxicity mediated by its antioxidant, anti-inflammatory, and antiapoptotic properties together with ameliorative effect on TLR4 signaling pathway.

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