

Evaluation of the Effects of TAK-242 and GIT-27 on Methotrexate-Induced Liver Injury

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Abstract

Background: Methotrexate (MTX)-induced liver injury is a common problem that is described as increased level of hepatic biomarkers that is seen in 14%–25% of patients with inflammatory bowel disease and 49% of patients with rheumatoid arthritis or as idiosyncratic induced liver injury that is seen in 1% of patients with inflammatory bowel disease, or as fibrosis and cirrhosis in 17% of rheumatoid arthritis patients and 25% of psoriatic patients. This profile may rarely progress to acute liver failure. **Aim:** The aim is to study the effect of TAK-242 and GIT-27 on MTX-induced liver injury. **Materials and Methods:** Thirty-five Albino-Wistar rats were divided into five groups: the first group was maintained on distilled water, the second group was administered intraperitoneal (I.P.) dimethyl sulfoxide followed by oral MTX, the third group was administered oral MTX, the fourth group was administered I.P. TAK-242 followed by oral MTX, and the fifth group was administered I.P. GIT-27 followed by oral MTX. **Results:** The significant increase in markers of hepatic function, inflammatory and oxidative stress markers, as well as severe liver histopathologic change “steatosis” induced by methotrexate were alleviated in the animals pretreated with the drugs TAK-242 and GIT-27. With significant improvement in serum level of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, interleukin-6, tumor necrosis factor- α , malondialdehyde and reduced glutathione; beside an improved histopathologic profile of moderate steatosis. **Conclusion:** This study suggests that both TAK-242 and GIT-27 protect against liver injury induced by MTX depending on their antagonism of the inflammatory Toll-like receptors 4 and 2/6.

Keywords: Drug-induced liver injury, GIT-27, Methotrexate, TAK-242, Toll-like receptors

INTRODUCTION

Drug-induced liver injury (DILI) points to any liver injury caused by xenobiotics or chemicals including drugs or medicinal herbs, whether introduced in therapeutic doses or in overdose.^[1,2] DILI is the most common reason of drug withdrawal after preclinical or clinical studies for example (bromofenac and troglitazone), denied approval for example (ximelagatran), and cessation of development for example (fialuridine), also DILI is the most frequent reason of admission to hospital, liver transplantation, acute liver failure, and acute hepatitis.^[3,4] Methotrexate (MTX) is used to treat various cancers and neoplasms,^[5] rheumatoid arthritis-naïve patients, eczema, psoriasis,^[6,7] inflammatory bowel disease, ulcerative colitis, and steroid-dependent Crohn’s disease.^[8-10] Off-label MTX is indicated in acute graft versus host disease after allogeneic hematopoietic stem cell transplantation.^[11] In combination, it is used in tubal ectopic pregnancy with

mifepristone,^[12,13] narrowband ultraviolet phototherapy, and adalimumab or infliximab.^[7,14] MTX-induced liver injury present as hepatic fatty infiltration, fibrosis, and steatohepatitis. MTX increase cellular sensitization to free radicals (FR) leading to stimulation of immune system starting with hepatic satellite cells (HSCs) which result in leukocyte accumulation, neutrophils secretion of pro-inflammatory enzymes and cytokines like nuclear factor- κ B (NF- κ B) and tumor necrosis factor- α (TNF- α). This in turn cause more production of FR which leads to sinusoidal congestion, dilation, hepatic fatty vacuolation focal necrosis and portal inflammation which is the typical pattern of drug induced steatohepatitis produced by FR.^[15-17] This participation of immune system that results

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in the production of pro-inflammatory CKs is the link between MTX-induced toxicity and Toll-like receptor (TLR) pathways, which are the common participant receptors of the immune system, the activation of which is required for CK production.

TAK-242 is also known as resatrovid. A cyclohexene derivative with chemical structure of Ethyl-(6R)-6-[N-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate^[18] is a selective inhibitor of TLR4 signal transduction pathway that interferes with Intracellular TLR/interleukin-receptor domain (TIR IC) adaptor molecules' interaction, thus preventing monocyte and macrophage (MQ) pro-inflammatory CK and nitrous oxide (NO) production both *in vitro* and *in vivo*.^[19,20] It was designed as a novel antiseptic agent,^[18] with anti-inflammatory action that protects against hypertension-related cardiac changes, cardiac apoptosis, and microinfarction after coronary microembolization^[19,21] and also protects nerves against central nervous system ischemia/reperfusion (I/R) and traumatic injury.^[22] Furthermore, it ameliorates the low-grade inflammatory process accompanied by insulin resistance in diabetes.^[18,21,22] GIT-27 is a small isoxazoline compound (4,5-dihydro-3-phenyl-5-isoxazole acetic acid), also known as VGX-1027, which possesses very interesting immunomodulatory effect throughout antagonizing the action of ligand-stimulated TLR4 and TLR2/6, with preferable low toxicity and high efficacy.^[23-25] This drug has been developed for treating miscellaneous inflammatory disorders such as type 1 diabetes mellitus and decreasing diabetic neuropathy and pancreatic insulinitis,^[25-27] colitis, inflammatory bowel disease, pleurisy, modulation of systemic lupus erythematosus even at genetic level, and rheumatoid arthritis.^[28,29]

Aim of the study

This study was performed to investigate whether treating the animals with TAK-242 and GIT-27 could reverse liver injuries induced by MTX or the tested drugs have a valuable hepatoprotective potential, especially considering that both drugs are anti-inflammatory and immunomodulating agents.

MATERIALS AND METHODS

Experimental design

Thirty-five male Albino-Wistar rats (4–6 months) (125–225 g) obtained from Kut technical Institute, University of Wasit, were maintained under nonspecific pathogen-free conditions under a constant temperature $24^{\circ}\text{C} \pm 3^{\circ}\text{C}$ with 12:12 h light–dark cycle in wire-meshed cages (seven rats in each cage) with *ad libitum* access to water and regular rat diet. Animal handling and housing were preceded in accordance with the International Guidelines for the care and use of laboratory animals of the National Research Council.^[23,30,31] The animals were divided randomly into five groups as follows: control group: rats were kept on distilled water (D/W) throughout the treatment; vehicle pretreated group: rats were administered intraperitoneal (I.P.) dimethyl sulfoxide (DMSO) diluted with D/W 1:12.5 with a final concentration of 8% (the same concentration was used to

dissolve both the drugs TAK-242 + GIT-27 according to their protocols and rats' weight) for 7 days^[32,33] followed by 7 days of oral MTX 0.2 mg/kg;^[30] MTX group: rats left untreated for 7 days followed by 7 days of oral MTX 0.2 mg/kg (dependent on the adult dose for rheumatoid arthritis stated by the previous literature) that is diluted with D/W at a final concentration of 0.333 mg/ml and administered via rat oral gavage according to rat weight to stimulate DILI;^[30] TAK-242 pretreated group: animals were administered I.P. TAK-242 5 mg/kg for 7 days,^[32] which was dissolved with DMSO D/W at a final concentration of 17 mg/ml (DMSO solubility of ≥ 360 mg/ml according to the manufacturer) 1 h before its administration^[33] followed by 7 days of oral MTX 0.2 mg/kg;^[30] and GIT-27 pretreated group: rats were administered 4 I.P. challenge doses of GIT-27 25 mg/kg at 168, 120, 72, and 24 h^[23,29] before starting treatment with oral MTX 0.2 mg/kg for 7 days.^[30] GIT-27 has been dissolved in DMSO D/W at a final concentration of 7 mg/ml (DMSO solubility ≥ 65 mg/ml according to the manufacturer) 1 h before its administration.^[33] After 24 h of the end of treatment, the rats were anesthetized with intramuscular (I.M.) ketamine 91 mg/kg–xylazine 9 mg/kg.^[34,35] Heart blood was obtained using direct needle puncture after sacrifice.^[36] Blood samples were allowed to be settled in 10 ml sterile labeled gel tubes and then centrifuged at 4000 rpm for 10 min at 25°C . The collected serum was stored in 2 ml Eppendorf tubes at -20°C until used for further analysis.^[23,30]

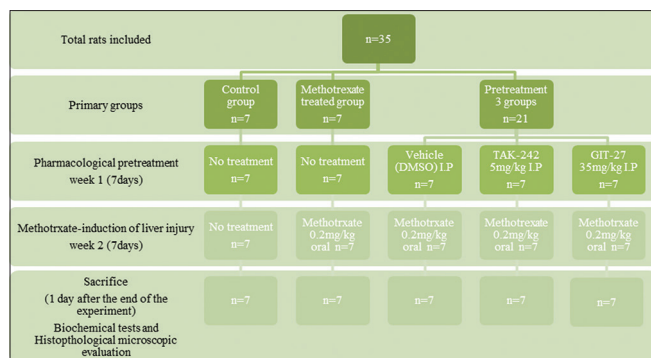
Chemicals and drugs

DMSO was purchased as 99.5% solution (Central Drug House [P] Ltd., New Delhi, India) and MTX was purchased from a local pharmacy as 50 mg/5 ml injectable solution (KOÇAK pharma, İstanbul, Turkey). TAK-242 was purchased as white crystalline powder (MedChemExpress, New Jersey, USA) and GIT-27 was supplied as off-white crystal (MedChemExpress, New Jersey, USA). Ketamine was purchased as 10% injectable solution (Alfasan woerden, JA Woerden, Holland) and xylazine as 20% injectable solution (Kepro, ZA Deventer, Holland).

Chemical analysis

Estimation of serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALPL) (hepatocellular markers), Interleukin-6 (IL-6), TNF- α (inflammatory markers), and level of lipid peroxide (LPO) was done via sandwich-enzyme-linked immunosorbent assay (ELISA) kits. Hepatobiliary bilirubin (Bb) and malondialdehyde (MDA) were measured by competitive ELISA kits, while total serum protein (TSP) and reduced glutathione (GSH) were measured via assay kits and their content was estimated depending on Equations 1 and 2.^[30,33,37] All kits were purchased from Elabscience, Georgia, USA, and were performed according to the manufacturers' procedure.

$$\text{TSP } (\mu\text{g/mL}) = \frac{\text{OD Sample} - \text{OD blank}}{\text{OD Standard} - \text{OD blank}} \times \text{Concentration of standard (563 } \mu\text{g/mL)} \times \text{Dilution factor of sample before tested (50)} \quad \text{— Eq-1}$$

**Figure 1:** Consort flow diagram of the study

$$\text{GSH content (mg GSH/L)} = \frac{\text{OD Sample} - \text{OD blank}}{\text{OD Standard} - \text{OD blank}} \times \text{Concentration of standard (20} \times 10^{-6} \text{ mmol/L)} \times \text{GSH molecular weight (307)} \times \text{Dilution factor (10)} - \text{Eq-2}$$

where the term OD refers to the optical densities measured by the spectrophotometer of the sample, the blank, and the standards for both Equations 1 and 2.

Tissue sample collection and histopathological study

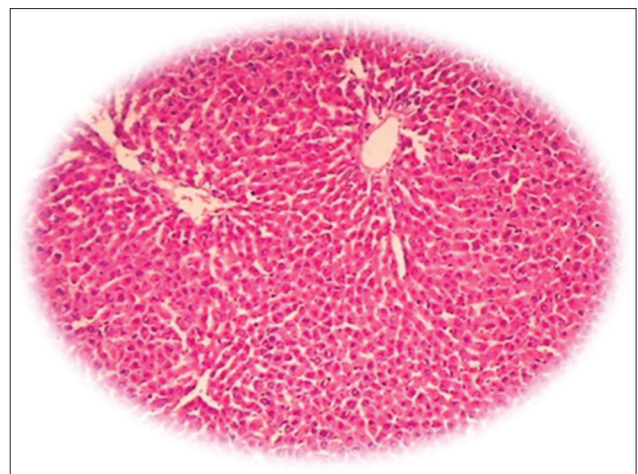
A cut was done to the rat's abdomen using a sharp scissor, and the liver was dissected out immediately. Liver tissue samples were fixed in containers with 30 ml of 10% formalin and then stored until they were processed.^[32] Liver sectioning and embedding was done according to the traditional processing procedure (paraffin-embedded method) described by Bancroft and Stevens to prepare liver tissue for microscope evaluation, then the tissue was stained with hematoxylin and eosin (H and E)^[37,38] Liver structure evaluation after MTX-induced injury was done utilizing the histological scoring system for nonalcoholic fatty liver disease (NAFLD) (NAS score) which comprehend three main changes in the liver: steatosis (S), lobular inflammation (L), and ballooning of hepatocytes (B). Total NAS score represents the sum of scores for steatosis, lobular inflammation, and ballooning (S + L + B) and ranges from 0 to 8.^[39]

Statistical analysis

Statistical analysis was done using International Business Machines Corp. [IBM] SPSS v20 package for windows 8, New York, USA. The resulted data were presented as mean ($\bar{x}$) $\pm$ standard deviation (S.D).^[25,32] Statistical differences among groups of data were determined using ANOVA test followed by least significant difference test. Pearson's correlation was measured to estimate the correlation among the measured markers.^[32,37,40] $P < 0.05$ was considered statistically significant.^[19,23]

RESULTS

This study involved 35 male Albino-Wistar rats; there was no loss in sample because of death or any other causes as shown in Figure 1.

**Figure 2:** Liver section of normal control rats (no abnormality) showing normal lobular rearrangement (H and E, $\times 100$)**Table 1: Changes in serum level of biochemical markers between rats treated with MTX and control group for 14 days, (n=7 each group)**

Parameters	Groups ($\pm$ S.D)		P*
	Control	MTX	
ALT (ng/ml)	41.55 $\pm$ 15.49	56.31 $\pm$ 10.87	0.022*
AST (ng/ml)	25.94 $\pm$ 3.05	32.50 $\pm$ 3.46	0.000*
ALPL (ng/ml)	13.22 $\pm$ 3.80	19.61 $\pm$ 4.49	0.009*
TSP (ng/ml)	2419.39 $\pm$ 340.80	2009.44 $\pm$ 313.96	0.030*
Bb (mcg/ml)	1.23 $\pm$ 0.18	1.80 $\pm$ 0.85	0.047*
IL-6 (pg/ml)	50.21 $\pm$ 13.04	76.60 $\pm$ 14.92	0.006*
TNF- α (pg/ml)	137.77 $\pm$ 38.39	196.18 $\pm$ 65.56	0.012*
LPO (ng/ml)	181.24 $\pm$ 9.04	199.76 $\pm$ 7.89	0.002*
MDA (ng/ml)	50.59 $\pm$ 6.28	65.35 $\pm$ 16.24	0.035*
GSH (mg/ml)	0.25 $\pm$ 0.05	0.19 $\pm$ 0.07	0.039*

* $P < 0.05$ considered significant. ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALPL (alkaline phosphatase), TSP (total serum protein), Bb (bilirubin), IL-6 (interleukin-6), TNF- α (tumor necrosis factor- α), LPO (level of lipid peroxide), MDA (malondialdehyde), GSH (reduced-glutathione)

Methotrexate effect on markers of hepatic function and inflammatory and oxidative stress

In comparison with the control group, treating rats with 0.2 mg/kg MTX only for 1 week^[30] resulted in a significant increase in the serum level of hepatocellular and hepatobiliary markers. ALT, AST, ALPL, and Bb were increased significantly ($P < 0.05$), while TSP was decreased significantly ($P < 0.05$). Furthermore, there was a significant increase in serum level of IL-6 and TNF- α , as well as LPO and MDA ($P < 0.05$), while GSH was decreased significantly [$P < 0.05$, Table 1].

Correlation coefficient among study markers

The measured inflammatory and oxidative stress markers were analyzed for association with hepatic function tests to clarify treatment effect throughout alteration in their level, which was found to be significant. It seems that decrement of serum GSH

Table 2: Serum liver enzymes changes among rats treated with MTX, TAK-242 and GIT-27 (pre-treatment groups) for 14 days, (n=7 each group)

Parameters	Groups ($\pm$ S.D)		P*	Groups ($\pm$ S.D)		P*
	MTX	TAK-242+MTX		MTX	GIT-27+MTX	
ALT (ng/ml)	56.31 $\pm$ 10.87	37.50 $\pm$ 10.11	0.005	56.31 $\pm$ 10.87	44.19 $\pm$ 6.29	0.057
AST (ng/ml)	32.50 $\pm$ 3.46	28.94 $\pm$ 3.17	0.042*	32.50 $\pm$ 3.46	28.98 $\pm$ 2.76	0.044*
ALPL (ng/ml)	19.61 $\pm$ 4.49	13.82 $\pm$ 2.79	0.016*	19.61 $\pm$ 4.49	14.60 $\pm$ 3.56	0.035*
TSP (ng/ml)	2009.44 $\pm$ 313.96	2439.57 $\pm$ 294.28	0.024*	2009.44 $\pm$ 313.96	2357.62 $\pm$ 414.52	0.063
Bb (mcg/ml)	1.80 $\pm$ 0.85	1.19 $\pm$ 0.64	0.035*	1.80 $\pm$ 0.85	1.23 $\pm$ 0.11	0.046*
IL-6 (pg/ml)	76.60 $\pm$ 14.92	55.95 $\pm$ 22.19	0.029*	76.60 $\pm$ 14.92	54.53 $\pm$ 18.96	0.020*
TNF- α (pg/ml)	196.18 $\pm$ 65.56	150.39 $\pm$ 23.76	0.045*	196.18 $\pm$ 65.56	149.12 $\pm$ 34.52	0.040*
LPO (ng/ml)	199.76 $\pm$ 7.89	189.76 $\pm$ 7.31	0.075	199.76 $\pm$ 7.89	188.89 $\pm$ 14.14	0.054
MDA (ng/ml)	65.35 $\pm$ 16.24	43.68 $\pm$ 14.81	0.003*	65.35 $\pm$ 16.24	45.51 $\pm$ 9.59	0.006*
GSH (mg/ml)	0.19 $\pm$ 0.07	0.26 $\pm$ 0.02	0.008*	0.19 $\pm$ 0.07	0.25 $\pm$ 0.03	0.031*

*P<0.05 considered significant. ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALPL (alkaline phosphatase), TSP (total serum protein), Bb (bilirubin), IL-6 (interleukin-6), TNF- α (tumor necrosis factor- α), LPO (level of lipid peroxide), MDA (malondialdehyde), GSH (reduced-glutathione)

Table 3: The assessment of liver injury according to NAFLD histopathological grading scores among the treatment groups MTX, TAK-242 and GIT-27 (pre-treatment groups) for 14 days, (n=7)

NAFLD Activity Score (NAS) Components					
Score	Components	Groups			
		Control	MTX	TAK-242	GIT-27
Steatosis	Score	0	3	2	2
	Extent	<5%	>66%	33-66%	33-66%
Lobular inflammation	Score	0	2	0	1
	Extent	None	2-4 foci /200x	No inflammation	<2 foci /200x
Ballooning degeneration	Score	0	2	1	1
	Extent	None	Many	Few cells	Few cells
Total scores		0	8	3	4

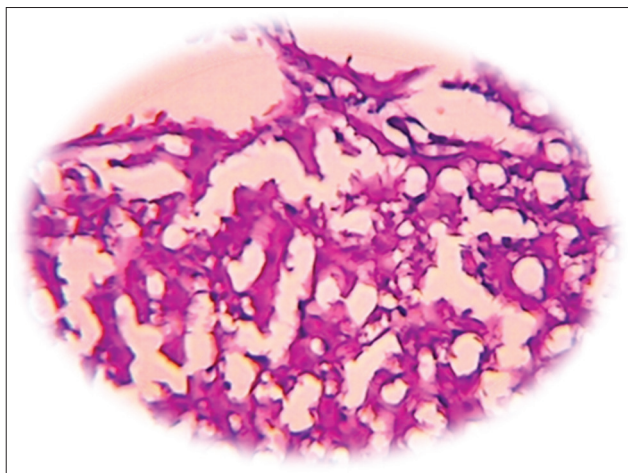


Figure 3: Liver section of methotrexate-treated rat (moderate-to-severe steatosis) showing hepatocyte degeneration and microvesicular and macrovesicular fat vacuoles connecting and opening onto each other forming fatty cystic chains (H and E, $\times$ 100)

was associated with a statistically significant increase in serum ALT and ALPL. GSH level showed moderate negative correlation with both ALT ($R = -0.421$) and ALPL ($R = -0.356$), while

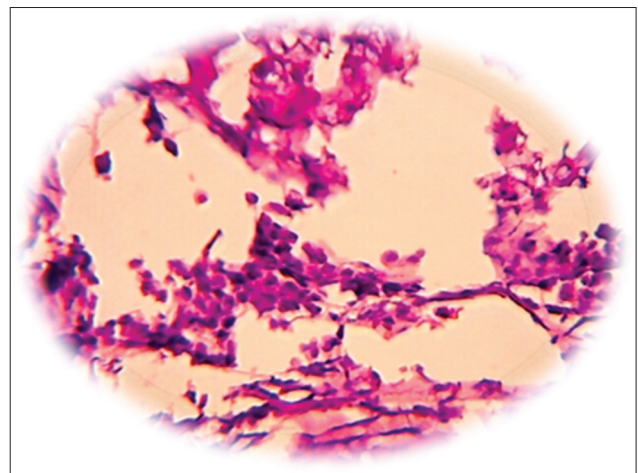


Figure 4: Liver section of methotrexate-treated rat (moderate-to-severe steatosis) showing hepatocyte fatty degeneration with moderate inflammatory cell infiltration (H and E, $\times$ 100)

increment in both serum LPO and MDA was associated with a statistically significant increase in serum ALPL; both showed a moderate positive correlation with a value of ($R = 0.384$) between LPO and ALPL and ($R = 0.381$) between MDA and ALPL.

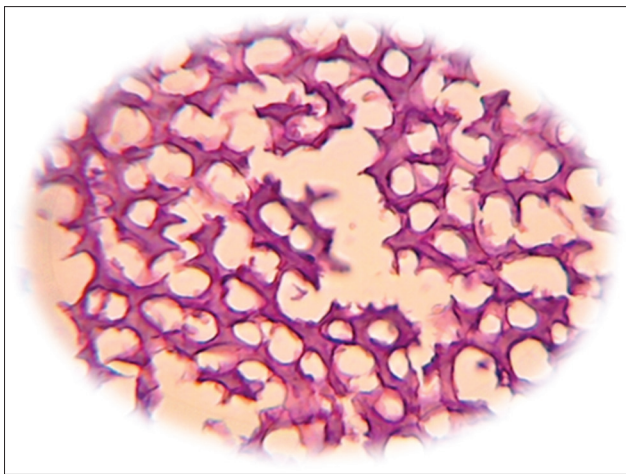


Figure 5: Liver section of TAK-242 pretreated rats (moderate) showing hepatocyte fatty degeneration. No inflammatory cells shown (H and E, ×100)

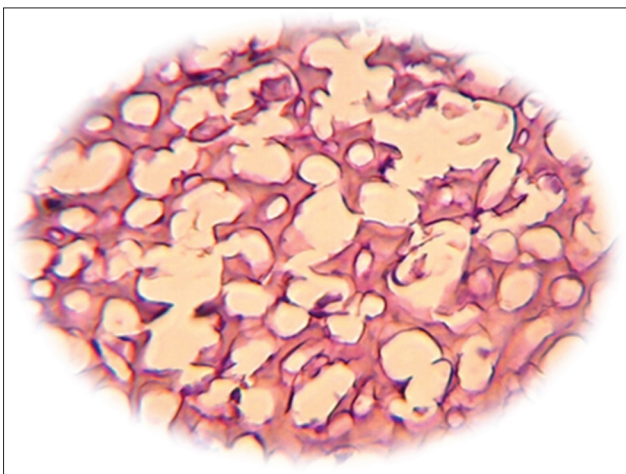


Figure 6: Liver section of GIT-27 pretreated rats (moderate) showing hepatocyte degeneration and microvesicular and macrovesicular fatty cysts. No inflammatory cells shown (H and E, ×100)

Effect of dimethyl sulfoxide on chemical parameters

DMSO pretreated rats showed nonsignificant changes ($P > 0.05$) in serum level of hepatic function markers and serum level of inflammatory and oxidative stress markers similar to those treated with MTX (data not shown).

TAK-242 and GIT-27 pretreatment effect on markers of hepatic function and inflammatory and oxidative stress

This study displays that the two pretreated rats' groups with TAK-242 and GIT-27 in comparison with hepatotoxic MTX-treated group resulted in the following changes in markers of hepatic function [Table 2].

Pretreatment with TAK-242 caused a significant decrease ($P < 0.05$) in serum level of ALT (by a mean decrease of 18.80 ng/ml), AST (by a mean decrease of 3.55 ng/ml), ALPL (by a mean decrease of 5.79 ng/ml), and Bb (by a mean decrease of 0.60 mcg/ml) and a significant increase ($P < 0.05$)

in TSP (by a mean increase of 430.13 mcg/ml). It also causes a significant decrease ($P < 0.05$) in serum level of the inflammatory markers IL-6 (by a mean decrease of 20.65 pg/ml) and TNF- α (by a mean decrease of 45.79 pg/ml), decrease in the oxidative stress markers MDA (by a mean decrease of 21.66 ng/ml), and significant increase ($P < 0.05$) in GSH (by a mean increase of 0.07 mg/ml), while decrement in LPO level is still nonsignificant ($P > 0.05$, by a mean decrease of 10.00 ng/ml).

Pre-treatment with GIT-27 causes significant ($P < 0.05$) decrease in serum level of AST (by mean decrease of 3.51 ng/ml), ALPL (by mean decrease of 5.01 ng/ml), and Bb (by mean decrease of 0.56 mcg/ml). Also significant decrease ($P < 0.05$) in serum level of inflammatory markers IL-6 (by mean decrease of 27.06 ng/ml) and TNF- α (by mean decrease of 47.06 pg/ml) and significant decrease in the oxidative stress markers MDA (by mean decrease of 19.84 ng/ml) and significant increase ($P < 0.05$) in GSH (by mean increase of 0.05 mg/ml) whilst it causes nonsignificant decrement in serum level of ALT (by mean decrease of 12.11 ng/ml), and LPO (by mean decrease of 10.87 ng/ml), and nonsignificant ($P > 0.05$) increase in TSP (by mean increase of 348.17 mcg/ml).

Treatment effects on liver histopathological findings

The histopathological findings from DILI were graded as mild, moderate, and severe [Table 3]. They were examined in five treatment groups, each containing seven rats. According to NAFLD component scoring system, [Figure 2] shows no liver abnormality in hepatic architecture and normal lobular rearrangement in control group animals, while the highest grading score (severe) was found in MTX-treated animals as shown in [Figures 3 and 4], respectively, and groups pretreated with the drugs TAK-242 and GIT-27 [Figures 5 and 6] show lower grade scores with moderate hepatic changes. The lowest grading scores were seen in TAK-242 pretreated animals. Note that pretreatment with vehicle (DMSO) shows the same changes as seen in MTX-treated animals (data not shown).

DISCUSSION

MTX-induced liver injury is proposed to be resultant from its effect on *de novo* synthesis of folate, hepatic drug metabolism and accumulation, oxidative stress, inflammation, and apoptosis.^[41,42] Treatment of the animals with 0.2 mg/kg oral MTX for 7 days^[30] resulted in significant increase in serum levels of ALT, AST, ALPL, and Bb with significant decrease in TSP, in accordance with previous studies.^[17,30,42-44] Serum transaminase elevation is assumed to be a mark of hepatocytes damage since they are considered intracellularly concentrated enzymes.^[41] This is proposed to be a result of MTX metabolism which proceeds primarily in the liver with concurrent reactive oxygen species (ROS) production and its intracellular retention as polyglutamate (MTX-PG), which causes rapid folate depletion due to the proliferative nature of hepatocytes.^[30,45,46] The scenarios associated with ROS

production would change hepatocellular biological membranes, affecting their permeability and structural proteins causes leak and thus high level of hepatic enzymes.^[44,47] While ALPL and Bb serum elevation would reflect MTX hepatobiliary injury since they are concentrated in both liver spleen,^[30] reduction of TSP is proposed to be resultant of MTX possible renal tubular injury that leads to loss of proteins as well as formation of protein adducts with the FRs.^[40,41]

Oxidative stress is proposed to be a major contributor of MTX-induced liver injury.^[30,47] FRs are generated first during MTX hepatic metabolism to 7-hydroxy metabolite, second from MTX and MTX-PG metabolite-mediated consumption of cellular antioxidants leading to mitochondrial dysfunction, third by MTX increasing plasma cysteine level producing superoxide and reactive nitrogen species.^[15,16] In accordance with previous studies, MTX-significant elevation of pleiotropic inflammatory CKs IL-6 and TNF- α ^[42,48,49] would suggest an oxidative stress-induced inflammation throughout activation of neutrophil, monocyte, and leucocyte accumulation in the hepatic tissue resulted from MTX-induced liver injury.^[46,50] The increment in the serum level of TNF- α would result from MTX-induced imbalance in TNF- α /NF- κ B and hepatocyte inflammation, while IL-6 high serum level is proposed to be induced by MTX-stimulated IL-1 β secretion.^[42,49]

In accordance with the previous studies, MTX-induced oxidative stress was reflected by a significant increase in the level of two sensitive products of lipid peroxidation (MDA and LPO) besides a significant decrease in the level of the antioxidant GSH.^[40,45,47] This was assumed to result from and yet stimulated by MTX-induced FR which attacks cellular lipid bilayer membrane, increasing the content of unsaturated fatty acid; disturbing surface negative charges, membrane permeability, and fluidity; as well as sensitizing the membranous proteins to oxidative damage; also a high MDA would alkylate mitochondrial enzymes rendering them inactive.^[45,50,51] The significant decrease in serum GSH level would be attributed to MTX-induced oxidant/antioxidant imbalance due to increasing rate of FR-macromolecular structure adduct formation, MTX-PG formation, and cellular accumulation, thus directly interacting and increasing the consumption of NADPH which maintains the reduced state of GSH.^[37,44,51] MTX-induced steatosis and inflammation is observed in histological section of treated animals possibly attributed to MTX increase in both oxidative and nitrosative stress which stimulates inflammatory response by activating Kupffer cells and MQ.^[17,31,46]

In agreement with previous studies, pretreatment with TAK-242 first significantly decreases serum level of ALT and AST, TNF- α , IL-6 and MDA and also significantly improved serum level of GSH.^[38,52-56] This is further confirmed by the histopathological assessment of this study which reveal decreasing in the severity of drug-induced steatohepatitis (DISH) from severe to moderate.^[57] TAK-242 blockade of TLR4 is reported to decrease the serum level of hepatic transaminase

and hepatocyte damage after acetaminophen-induced liver injury and bile duct ligation in animal models with significant modulation in liver histopathologic alteration seen after I/R injury in Rats' model.^[38,52,53] TAK-242 inhibited both TLR4-myeloid differential 88 (MyD88)-dependent TIR domain containing adaptor protein (TIRAP/Mal) and MyD88 independent TNF receptor-associated factor 6 (TRAF6) signaling pathways. This result in the induction of NF- κ B, expression of co-receptor proteins lymphocyte antigen 96 (MD2), cluster of differentiation 14 (CD14) as well as activator protein-1 (AP-1) which stimulate inflammatory immune response and release of CK as TNF- α and IL-6^[55,58,59] as well as stimulate mitogen activated protein kinase (MAPK) and c-Jun phosphorylation which are involved in ROS generation.^[59,60] While TAK-242 effect on oxidative stress is also proposed to be due to the downregulation of TLR4 itself since it is expected to be upregulated by oxidative stress through release of intracellular and extracellular damage-associated molecular patterns (DAMPs) that function as "alarmins" after been recognized by TLRs.^[53,61] This is further confirmed by total relief of inflammation assessed by histological examination of the pretreated animals after exposure to methotrexate. This action of TAK-242 was described in both murine and cellular-human models irrespective of the kind of stimuli.^[20,62]

In agreement with previous studies, GIT-27 caused a significant decrease in serum TNF- α and IL-6^[23,25,63,64] and also a significant decrease in serum AST, ALPL and Bb as well as almost borderline significant decrement in ALT. This could be attributed to its anti-inflammatory action, so its nonselective inhibition of TLR would decrease inflammation as well as the resultant ROS generation which leads to hepatocyte injury and death.^[25,65] This effect on ROS is also achieved in this study by GIT-27 significant decrease in serum level of MDA 1st and improvement of GSH level 2nd. This is consistent with another study which revealed that GIT-27 treatment in murine diabetic model improved the associated hepatic steatosis and induced restoration of smaller size fat vacuoles. This hepatic alteration was almost exactly achieved in this study confirmed by GIT-27 reduction of hepatic histopathological changes to moderate grade after exposure to methotrexate; though not to the mild grade, this could be due to the short course of pretreatment of the study in comparison with the same study.^[63] Furthermore, nonsignificant improvement of GIT-27 in serum level of LPO could be attributed to this moderation of but not the total amelioration of DISH mentioned before. Accordingly, the significant decrement of pro-inflammatory CK in GIT-27 pretreated rats is attributed to its inhibition of TLR4-, TLR6-, and rather TLR2-activation by the DAMPs resulted from damaged cells that would increase in NF- κ B and p38 MAPK especially by TLR4, but unlike TAK-242, it has no effect on AP-1 or c-Jun singling cascade.^[66,67] This inhibition would decrease MQ activation, migration to the site of inflammation, as well as secretion of pro-inflammatory CK such as TNF- α and IL-6; this is further confirmed by the decrease in inflammatory

cell infiltration to a few cell grading seen in histopathologic evaluation.^[25,29,63]

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Nil.

Conflicts of interest

There are no conflicts of interest.

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