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Hepatoprotective Effects of Montelukast Against Methotrexate Toxicity in Rat– An Experimental Research

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Abstract

Methotrexate, a widely utilized antineoplastic and immunosuppressive agent, is associated with significant adverse effects including hepatotoxicity and myelosuppression across various malignancies and autoimmune conditions. This experimental investigation evaluated the potential hepatoprotective properties of montelukast in mitigating methotrexate-induced hepatic injury. Thirty male Wistar rats were randomly allocated into five groups (n=6 per group). Group 1 served as the negative control, receiving equivalent volumes of normal saline. Group 2 (montelukast control) received montelukast 10 mg/kg body weight intraperitoneally from day 4 to day 10. Groups 3, 4, and 5 received a single intraperitoneal dose of methotrexate 20 mg/kg body weight on day 0 to induce hepatotoxicity. Groups 4 and 5 additionally received montelukast 5 mg/kg and 10 mg/kg body weight respectively, administered intraperitoneally from day 4 for seven consecutive days following methotrexate treatment. Biochemical analysis revealed that montelukast co-administration resulted in dose-dependent improvements in hepatic function parameters. Serum levels of total cholesterol, high-density lipoprotein cholesterol, total bilirubin, alanine aminotransferase, aspartate aminotransferase, total proteins, albumin, and globulin demonstrated significant amelioration correlating with increasing montelukast dosage compared to methotrexate monotherapy. These findings suggest that montelukast exhibits hepatoprotective activity against methotrexate-induced liver injury, potentially through modulation of hepatic enzyme levels and preservation of hepatocellular function. Montelukast may represent a promising adjuvant therapy to mitigate hepatotoxicity associated with methotrexate treatment. Further clinical investigation is warranted to validate these preclinical observations in human subjects.

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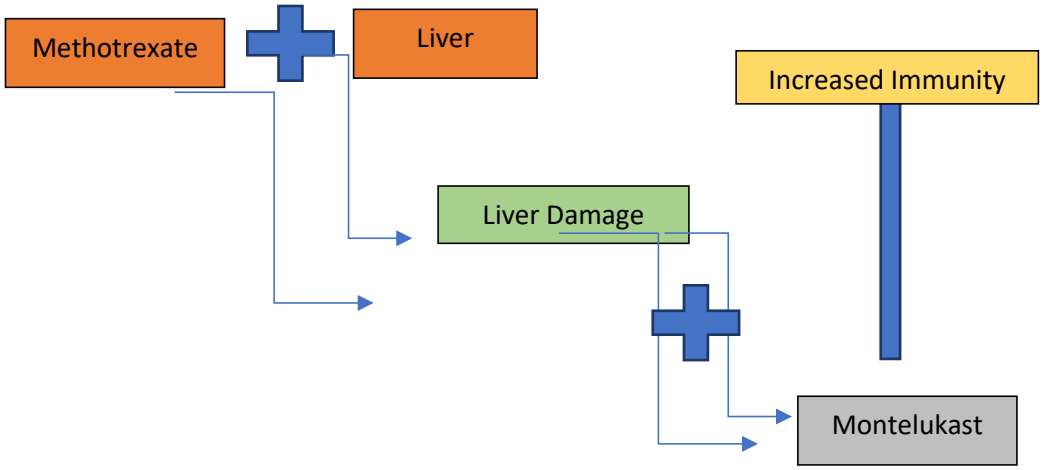
GRAPHICAL ABSTRACT



HIGHLIGHTS

- Montelukast decrease serum total cholesterol, HDL-Cholesterol, total bilirubin, alanine aminotransferase, aspartate aminotransferase, total proteins, albumin, and globulin levels.
- After Methotrexate drug treatment, montelukast decreases liver enzymes.
- Montelukast can be used against anti-cancerous drug methotrexate.

1. INTRODUCTION

Methotrexate is a widely used chemotherapeutic and immunosuppressive agent, initially developed for the treatment of malignancies such as leukemia and lymphoma, and later applied in solid tumors, psoriasis, and rheumatoid arthritis. As a structural analogue of folic acid, it interferes with purine and pyrimidine synthesis, a mechanism that underlies both its effectiveness in cancer therapy and its associated toxicities [1]. At therapeutic concentrations below cytotoxic levels, methotrexate exerts anti-inflammatory properties through suppression of T-lymphocyte activation and clonal expansion, while

concurrently reducing the expression of cellular activation markers and adhesion moleculess [2].

The hepatic parenchyma serves as the primary site for drug metabolism and detoxification, rendering it particularly vulnerable to chemotherapy-induced injury. Prolonged methotrexate exposure has been documented to cause a continuum of liver pathology, encompassing initial biochemical abnormalities through advanced structural changes including hepatic fibrosis, cirrhosis, and associated portal hypertension. Elevated serum transaminase concentrations represent early markers of methotrexate-induced hepatotoxicity, often preceding clinically significant liver dysfunction [3]. The literature on methotrexate is extensive, but the effects on liver tests and biopsies can vary significantly depending on factors such as dosage, treatment regimen, and duration of therapy. Methotrexate works by inducing apoptosis in cancerous cells and inhibiting cell proliferation. However, its use can lead to elevated levels of liver enzymes. Prolonged treatment with high doses of methotrexate may result in fatty liver. In cases of high-dose methotrexate intake, potential consequences include cirrhosis, fibrosis, liver damage, and hepatic steatosis. [4].

Montelukast is used in the management of bronchial asthma as an anti-inflammatory drug [5]. Montelukast is a selective antagonist that acts on the cysteinyl leukotriene (CysTL) receptor, thereby blocking its activity and reducing the levels of inflammatory agents in the airways [6]. Leukotrienes (LTC4, LTD4, LTE4), are mainly secreted by eosinophils, macrophages, monocytes, and mast cells. They give different actions that highlight its pathogenicity in the inflammatory conditions [7]. Montelukast is a selective antagonist of the cysteinyl leukotriene (CysTL) receptor, specifically targeting the CysTL1 subtype. It inhibits the function of leukotrienes, such as LTE4 and

LTC4, which are derived from arachidonic acid and play a crucial role as mediators of asthma. By blocking the activity of these molecules on the lungs and bronchial tubules, Montelukast helps reduce inflammation and improve respiratory symptoms[8].

Methotrexate-induced hepatotoxicity involves oxidative stress mechanisms that promote lipid peroxidation cascades, resulting in hepatocellular inflammation and depletion of endogenous antioxidant enzyme systems. Experimental studies in rabbit models demonstrate significant elevations in hepatic biomarkers, including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, indicative of compromised hepatocellular membrane integrity and progressive hepatocyte necrosis. [9].

Montelukast, through antagonism of the cysteinyl leukotriene receptor 1 (CysLT1), modulates oxidative processes in ischemic cells, influencing the generation of reactive oxygen species. Therapeutically, montelukast administration has been associated with reductions in serum liver enzymes such as ALT and AST, reflecting decreased oxidative stress and mitigation of ischemic liver injury. By preserving hepatic structure and function, and through its antioxidant and anti-inflammatory properties, montelukast contributes to protecting the liver from oxidative damage. Its high affinity for CysLT1, along with its regulation of pro-inflammatory mediators and interferon activity, further supports its role in dampening inflammatory responses [10]. Methotrexate, a chemotherapeutic agent, can have toxic effects on multiple target organs in the body, including the bone marrow, gastrointestinal tract, mucosal membranes, and hair follicles. These side effects can lead to various complications and require careful monitoring during treatment. [11].

Leukotriene antagonists and inhibitors are a strong anti-inflammatory effect in rat's models, such as in gastric mucosal damage induced by ethanol [12], in renal sepsis reperfusion injury, burn, and sepsis in different types of body organs [13]. The treatment of asthma with selective reversible CysLT 1 antagonist, montelukast decreases the eosinophilic level of airways inflammation [14]. Ethanol-induced GIT mucosal damage and colitis is treated with biosynthesis inhibiting of CysTL 1 by the neutrophil dependent mechanism. In previous studies the antagonist receptors, montelukast reduced the multiple organ damage, burn, and sepsis [14].

Previous research has demonstrated that anti-asthma medications, such as montelukast, possess anti-inflammatory and anti-oxidant properties. Recent studies have focused on developing strategies to mitigate the harmful effects of methotrexate on the liver and improve overall health and adherence to this potent medication. Montelukast's anti-oxidant and anti-inflammatory characteristics have been shown to reduce ischemic-reperfusion oxidative damage in the kidneys and bladder. In this study, we aimed to investigate the protective effects of montelukast against oxidative stress-induced liver tissue damage. Montelukast suppresses the release of inflammatory and oxidative stress markers by increasing the activity of enzymatic anti-oxidant systems and blocking lipopolysaccharide-induced markers of liver injury.

2. MATERIAL AND METHODOLOGY

2.1. EXPERIMENTAL ANIMALS

Thirty albino rats of comparable age were obtained from the animal facility of the University of Health Sciences, Lahore. The animals were housed in stainless-steel cages within the Experimental Animal Shed of the Department of Physiology, University of Veterinary and Animal Sciences, Lahore. Environmental conditions were maintained at

22 ± 2 °C with a 12-hour light/dark cycle. Prior to experimentation, both cages and housing areas were thoroughly cleaned, and animals were allowed a one-week acclimatization period. During this time, rats had unrestricted access to standard rat chow and water. Following acclimatization, the animals were randomly assigned into five groups, each consisting of six rats, and the experimental protocol was carried out over a 10-day period.

The rats were equally distributed in five groups; each group contained six rats. Rats of G1 (negative control) were injected with a volume of normal saline as a negative control. Montelukast (10mg/kg BW) was administered I/P to the G2 (MK positive control) from day 4 today ten. 20 mg/kg BW, methotrexate I/P at day zero, a single dose was injected to induce its toxicity in rats of G3 (Methotrexate) positive control. G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10). Montelukast will be administered I/P to the G4 and G5 on the fourth day for seven consecutive days after the administration of Montelukast. Rats were healthy and physically very active when housed in cages. Feed and water intake were also measured daily.The body weights of rats were recorded; the complete grouping and treatment plan is shown below (Table 1).

Table 1. Treatment Plan of the Study

Treatment Days	Groups				
	G1 Negative Control	G2 MK Positive Control	G3 METHOTREXATE Positive Control	G4 5-METHOTREXATE+MK*	G5 10-METHOTREXATE+MK
Day Zero	Normal Saline 1ml/kg BW (I/P)*	_	Methotrexate 20mg/kg BW (I/P) single dose/10days	Methotrexate 20mg/kg BW (I/P) single dose	Methotrexate 20mg/kg BW (I/P) single dose
Day four	2% ethanol 1ml/kg BW (I/P)	Montelukast 10mg/kg BW (I/P) for consecutive 7 days		Montelukast 5mg/kg BW (I/P) for consecutive 7 days	Montelukast 10mg/kg BW (I/P) for consecutive 7 days

I/P*=Intraperitoneal Methotrexate + MK* =Methotrexate+ Montelukast

2.2. INDUCTION OF METHOTREXATE TOXICITY

A toxic dose of 20 mg/kg body weight of methotrexate was administered to rats in group 3 as a positive control and group 4 at day zero to induce toxicity. Methotrexate (50 mg) under the brand name Unitrexate was purchased from Fazal Din & Sons (PVT) LTD, Chemist & Druggists, and The Mall Road, Lahore. The dose of methotrexate was administered individually based on the body weight of the rats at day zero. Methotrexate was administered intraperitoneally using insulin syringes. Rats had free access to food and water. After methotrexate treatment, rats exhibited diarrhea-like

symptoms, leading to dehydration, lethargy, and a reduction in feed and water intake. Toxicity was confirmed by collecting blood samples from group 3 rats on day four and performing a liver function test, which revealed abnormal serum levels of liver enzymes.

2.3. MONTELUKAST SOLUTION PREPARATION AND TREATMENT

Montelukast was purchased under the brand name Myteka sachet in the strength of 4 mg from Green plus Pharmacies, Outfall Road, Lahore. Ethanol was used as a solvent to homogenize the drug for administration to rats. Each dose was dissolved in 1 ml of 2% ethanol. Based on the body weight and the number of rats to which montelukast (MK) was to be administered, the required amount of MK was dissolved in the appropriate volume of 2% ethanol and stirred until a clear solution was obtained. The body weight of each rat was recorded on day four to assess the variation in body weight after confirming the toxicity of methotrexate. This allowed for the accurate calculation of doses: 10 mg/kg body weight (BW) for individual rats in group 2 (MK positive control) and group 5, and 5 mg/kg BW for individual rats in group 4. Subsequently, a specific dose of MK was administered to the rats in groups 2, 4, and 5 for consecutive 7 days.

2.4. BLOOD SAMPLING

At the conclusion of the 10-day experimental protocol, all animals were euthanized using diethyl ether anesthesia. Cardiac blood samples were obtained via direct ventricular puncture and collected in sterile borosilicate glass tubes. Following collection, samples underwent spontaneous coagulation at ambient temperature for 30 minutes. Serum separation was achieved through centrifugation at 3000 rpm for 15 minutes at room temperature. The resulting non-hemolyzed serum supernatant was carefully aspirated and stored at -20°C pending biochemical analysis [15].

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2.5. BIOCHEMICAL ANALYSIS

Serum samples were used to measure the liver function tests, including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin, globulin, albumin, and total proteins. Additionally, a lipid profile was determined using the enzymatic colorimetric kit method provided by the Randox Company. The summary of the biochemical analysis steps is as follows (Fig.1):

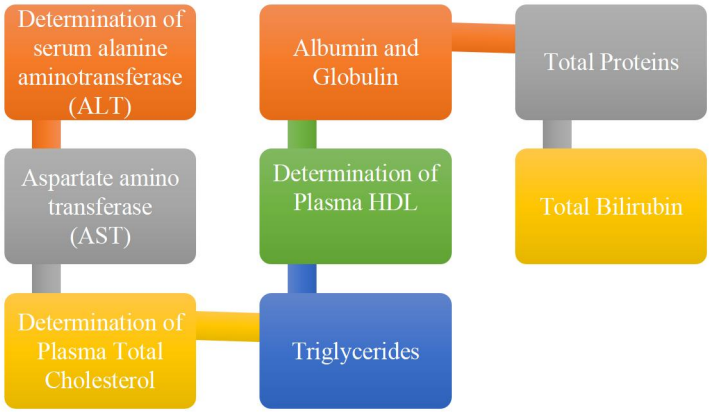

FIG.1. SUMMARY OF BIOCHEMICAL ANALYSIS STEPS

2.5.1. DETERMINATION OF SERUM ALANINE AMINOTRANSFERASE (ALT)

Alanine aminotransferase (ALT) is predominantly localized in the liver, with smaller amounts present in the kidneys, heart, skeletal muscles, and pancreas. Measurement of serum ALT serves as a sensitive marker of hepatocellular injury. Under normal physiological conditions, only low levels of ALT are detectable in the circulation; however, liver damage leads to the release of this enzyme into the bloodstream, resulting in elevated serum concentrations that strongly indicate hepatic dysfunction. Serum ALT activity was assessed using a commercially available diagnostic kit (RANDOX). Two sterilized test tubes were prepared and labeled as “blank” and “sample.” For the sample tube, 100 µL of serum was added, followed by 500 µL of buffer solution in both

tubes. The tubes were mixed thoroughly and incubated at 37 °C for 30 minutes. Subsequently, 500 µL of 2, 4-dinitrophenylhydrazine (2, 4-DNP) was added to each tube, and 100 µL of serum was then added to the blank tube. After thorough mixing, both tubes were incubated for 20 minutes at 25 °C. Finally, 5 mL of sodium hydroxide was added to each tube, and the absorbance of the sample was recorded against the blank at 546 nm.

2.5.2. ASPARTATE AMINOTRANSFERASE (AST)

Two sterile test tubes were prepared and designated as 'test' and 'blank' respectively. The assay procedure involved adding 100 µL of serum sample to the test tube, followed by 500 µL of phosphate buffer solution. After thorough vortex mixing, both tubes were incubated at 37°C for 30 minutes. Subsequently, 500 µL of 2, 4-dinitrophenylhydrazine reagent was introduced to each tube. The blank received 100 µL of sample at this stage to account for background interference. Following gentle agitation, the reaction mixture underwent incubation at 25°C for 20 minutes. The reaction was terminated by adding 5 mL of 1M sodium hydroxide solution to each tube with immediate mixing. Spectrophotometric analysis was performed at 546 nm wavelength using the blank as reference, and optical density values were recorded.

2.5.3. SERUM TOTAL CHOLESTEROL ANALYSIS

Total cholesterol concentration was determined using an enzymatic colorimetric method. The assay principle relies on the sequential enzymatic conversion of cholesteryl esters to free cholesterol via cholesterol esterase, followed by oxidation to cholest-4-en-3-one and hydrogen peroxide through cholesterol oxidase activity. The generated hydrogen peroxide subsequently undergoes a Trinder reaction with phenol and 4-aminoantipyrine in the presence of peroxidase, yielding a quinoneimine chromophore with absorbance

directly proportional to total cholesterol concentration. The analytical procedure involved preparation of duplicate tubes designated as reagent control and test sample. Each tube received 10 µL of distilled water (control) or serum sample, followed by addition of 1000 µL of cholesterol reagent (R1). After gentle mixing, tubes were pre-incubated at 37°C for 5 minutes to ensure thermal equilibration. Spectrophotometric measurement was performed using 1-cm path length cuvettes at 546 nm wavelength, with the reagent control serving as reference. Absorbance readings were obtained within 60 minutes of assay initiation to ensure measurement stability

2.5.4. TRIGLYCERIDES

The enzymatic hydrolysis of triglycerides by lipoprotein lipase (LPL) in the presence of water produces the chromogenic compound 4-(p-benzoquinone-monoamine)-phenazone. For the assay, three test tubes were prepared. In the sample tube, 10 µL of serum was added to 1000 µL of reagent, followed by thorough mixing and incubation at 25 °C for 30 minutes. The absorbance of the reaction mixture was then measured against a blank within 60 minutes at a wavelength of 546 nm.

2.5.5. DETERMINATION OF PLASMA HDL

The supernatant containing HDL (high-density lipoproteins) fraction is obtained by centrifugation and later assayed for HDL-cholesterol by using a cholesterol kit. 200 µL sample and 500 µL diluted HDL reagent were poured into a centrifuge tube. Following initial preparation, samples underwent room temperature incubation for 10 minutes with gentle agitation. Selective precipitation was achieved through centrifugation at 4000 rpm for 10 minutes, after which the clear supernatant containing HDL particles was carefully aspirated for cholesterol quantification using a commercial enzymatic assay kit. The HDL-cholesterol determination employed a standard three-tube analytical setup

comprising reagent control, calibration standard, and test specimen. Each tube received 100 µL of the respective solution: distilled water (control), cholesterol standard solution, or HDL-enriched supernatant (test sample). Subsequently, 1000 µL of cholesterol reagent was dispensed into each vessel. After thorough mixing, the reaction mixture was allowed to develop at ambient temperature for 20 minutes. Spectrophotometric quantification was performed at 500 nm wavelength using the reagent control as baseline reference, with HDL-cholesterol concentrations calculated from the standard calibration curve

2.5.6. ALBUMIN AND GLOBULIN

Serum albumin concentration was determined using the bromocresol green (BCG) method, in which albumin binds with the BCG indicator in an acidic medium to form a green-colored complex. The intensity of this color increases proportionally with albumin concentration. For the assay, three test tubes were designated as blank, albumin standard, and sample. Ten µL of albumin standard (4.5 g/dL) or serum sample was added to the respective tubes, followed by 3 mL of working reagent in each. After thorough mixing, the tubes were incubated at 25 °C for 5 minutes. Absorbance was recorded at 630 nm against the reagent blank within 30 minutes. Serum globulin concentrations were subsequently calculated by subtracting the measured albumin values from the total protein concentrations.

2.5.7. TOTAL PROTEINS

Serum protein concentration was determined using the biuret colorimetric method, which relies on the formation of a copper-peptide complex under alkaline conditions. This assay exploits the ability of peptide bonds to coordinate with cupric ions, yielding a violet-colored chromophore with absorbance intensity directly correlating with protein

content. The analytical procedure utilized a three-tube configuration: test specimen, protein calibrator (4.5 g/dL), and reagent control. Each tube received 1 mL of biuret reagent, followed by the addition of 25 µL of the appropriate solution—either protein standard or serum sample. After gentle agitation, tubes were incubated at ambient temperature (20-25°C) for 10 minutes to ensure complete color development. Spectrophotometric measurements were obtained at 540 nm wavelength using the reagent control as reference, with readings completed within the 30-minute stability window. Protein concentrations were calculated using standard calibration methodology based on the linear relationship between absorbance and protein content.

Total proteins were calculated using the following formula (1):

$$\text{Total proteins Conc.} = \frac{Abs\ Sample}{Abs\ Standadrd} x\ Standard\ conc. \quad (1)$$

2.5.8. TOTAL BILIRUBIN

Total bilirubin concentration was determined using the Jendrassik and Grof method. In this assay, bilirubin reacts with diazotized sulphanilic acid to form an azo dye. In the presence of the accelerator caffeine, albumin-bound bilirubin is released, producing a blue-colored complex in an alkaline medium. Direct (conjugated) bilirubin reacts directly with diazotized sulphanilic acid without the addition of caffeine, whereas indirect (unconjugated) bilirubin is calculated by subtracting the direct fraction from the total bilirubin. All reagents (hydrochloric acid, sodium nitrite, sodium benzoate, and serum) were equilibrated to room temperature before analysis. Test tubes were labeled as "sample blank" and "test." To each tube, 200 µL of sulphanilic acid with hydrochloric acid was added. For the test tube, 5 µL of sodium nitrite was also introduced, followed by 1.0 mL of caffeine-sodium benzoate solution and 200 µL of serum in both tubes. The mixtures were thoroughly mixed and allowed to stand for 5–30 minutes at room

temperature. Absorbance of the test solution was measured against the sample blank at 578 nm (range 560–600 nm).

2.5.9. STATISTICAL DESIGN

Results are expressed as mean ± standard error of the mean (SEM). Statistical evaluation was performed using one-way analysis of variance (ANOVA) with SPSS software version 20.0 (IBM Corporation, Armonk, NY, USA). Post-hoc comparisons between experimental groups were conducted using Duncan's multiple range tests to identify specific inter-group differences. Statistical significance was established at $p < 0.05$ for all analyses

3. RESULTS

The results of the effects of Methotrexate and Montelukast treatment on serum Biochemistry are summarized in Table 2.

TABLE 2. EFFECTS OF METHOTREXATE AND MONTELUKAST TREATMENT ON SERUM BIOCHEMISTRY

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Total cholesterol mg/dl	43.70 ±1.70^{a}	34.16± 2.60^{ab}	45±17.8^{a}	26.83±2.32^{b}	25.20±2.08^{b}	0.005
Triglycerides mg/dl	94.66±26.85	86±12.97	97.25±11.96	61.50±4.17	66±24	0.436
HDL mg/dl	15.16±1.50	8.18±1.01	7.86±1.48	9.05±6.050	12.46±2.61	0.138
Total Bilirubin mg/dl	0.25±0.01^{c}	0.80±0.08^{a}	0.58±0.07^{b}	0.36±0.03^{c}	0.38±0.26^{c}	0.001

ALT u/l	47± 4.41[a]	28.33± 7.53[abc]	22.33± 1.45[bc]	42±11.84[ab]	19.75±4.40[c]	0.031
AST u/l	118.75± 4.90[a]	112.66± 1.45[ab]	98.33± 9.90[ab]	94±1.73[b]	66.50±8.58[c]	0.001
Total proteins g/dl	4.71± 1.11	5.31± 0.83	6.24± 0.79	5.27± 0.29	5.41± 0.88	0.777
Albumin g/dl	1.17± 0.17	0.87± 0.21	1.15± 0.15	0.72± 0.11	0.89± 0.10	0.235
Globulin g/dl	3.53± 1.24	4.43± 0.89	5.08± 0.86	4.54± 0.23	4.52± 0.96	0.818

3.1. EFFECTS OF METHOTREXATE AND MK TREATMENT ON SERUM TOTAL CHOLESTEROL

Analysis of serum cholesterol concentrations revealed significant temporal variations across all experimental groups relative to controls at baseline, day 4, and day 10 timepoints. Montelukast (MK) administration (groups G4 and G5) demonstrated marked cholesterol-lowering effects by day 4, with both treatment cohorts exhibiting significantly reduced serum cholesterol levels compared to untreated controls (G1) and methotrexate monotherapy (G3).. While the results of G4 and G5 at day ten showed improvements in serum total cholesterol levels as compared to G2. Montelukast administration after methotrexate treatment resulted in a significant decrease in serum total cholesterol levels, with an increase in the dose of MTK leading to greater reductions..

TABLE 3. TOTAL CHOLESTEROL IN SERUM

Group	Serum total Cholesterol mg/dl
Group1	43.70 ±1.70[a]
Group2	34.16±2.60[ab]

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Group3	45±17.8[a]
Goup4	26.83±2.32[b]
Group5	25.20±2.08[b]
P-value	0.005

The total cholesterol levels are shown in Figure 2. In the figure, G1 represents the negative control, G2 represents the MK positive control, G3 represents the methotrexate positive control, G4 represents the methotrexate-MK 5 group, and G5 represents the methotrexate-MK 10 group

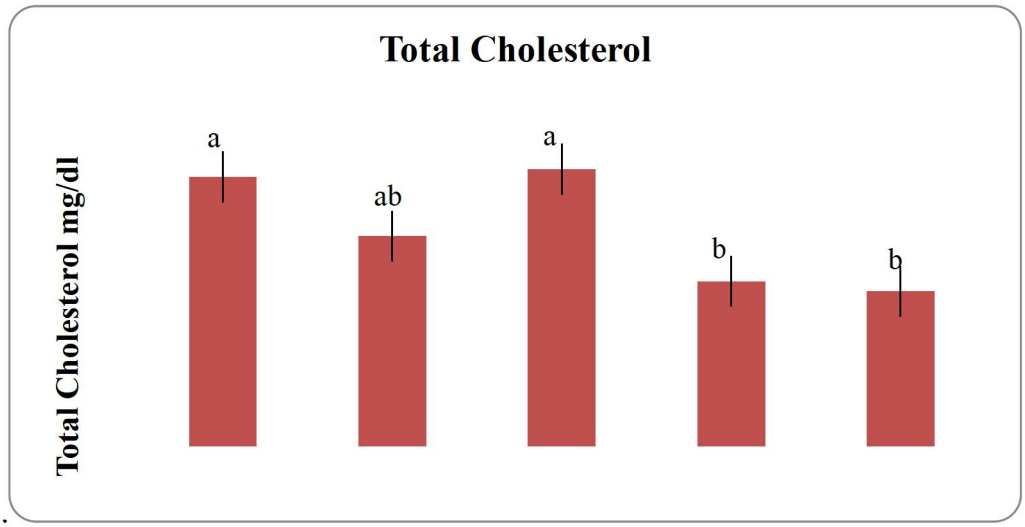

FIG.2. EFFECTS OF METHOTREXATE AND MK ON SERUM TOTAL CHOLESTEROL;

3.2. EFFECTS OF METHOTREXATE AND MK ON SERUM TRIGLYCERIDES

ANOVA shows non-significant changes in the groups; however, post hoc test, Duncan's test revealed that there was a reduction in serum triglycerides in G4 and G5 as compared to G1. G2 showed a reduction in Triglycerides as compared to G3 (Table 4).

TABLE 4. TRIGLYCERIDE CONCENTRATION IN SERUM

Group	Serum Triglycerides mg/dl
Group1	94.66±26.85
Group2	86±12.97
Group3	97.25±11.96
Goup4	61.50±4.17
Group5	66±24
P-value	0.436

Values are expressed as mean ± standard error of the mean. Statistical significance between treatment groups is denoted by different lowercase superscript letters (a-b), with groups sharing common superscripts showing no significant difference ($p \geqslant 0.05$). Experimental groups comprised: G1, untreated control; G2, montelukast control (10 mg/kg); G3, methotrexate control (20 mg/kg); G4, methotrexate plus montelukast (5 mg/kg); G5, methotrexate plus montelukast (10 mg/kg).

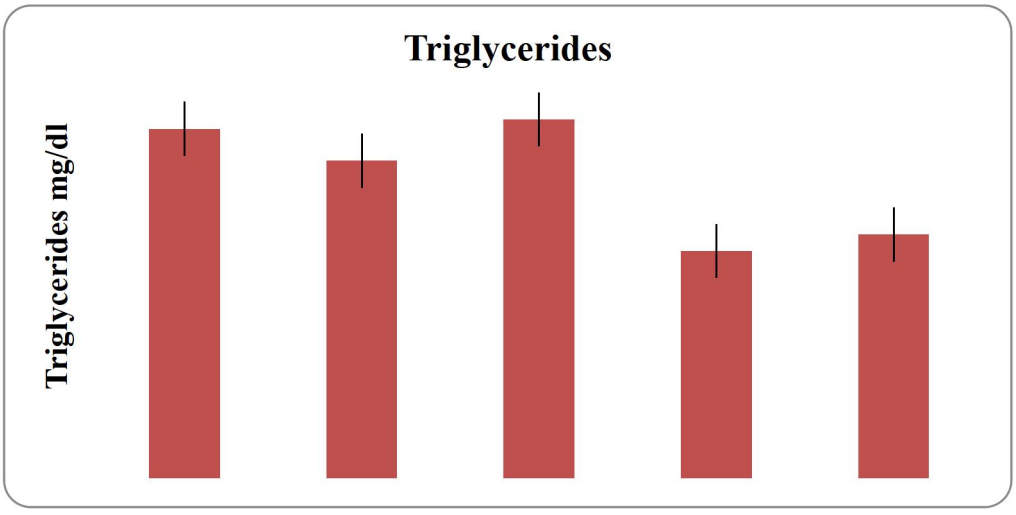

FIG. 3. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM TRIGLYCERIDES:

The figure shows G1 (Negative control), G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

3.3. EFFECTS OF METHOTREXATE AND MK ON SERUM HDL-CHOLESTEROL

While overall ANOVA yielded non-significant between-group differences, subsequent Duncan's post-hoc analysis identified specific inter-group variations. Notably, both the methotrexate control group (G3) and montelukast control group (G2) demonstrated significant reductions in serum HDL-cholesterol concentrations compared to baseline values. Conversely, the remaining experimental groups showed no statistically significant alterations in HDL-cholesterol levels relative to controls.(Table 6).

TABLE 5. HDL-CHOLESTEROL CONCENTRATION IN SERUM

Group	Serum HDL-Cholesterol mg/dl
Group1	15.16±1.50
Group2	8.18±1.01
Group3	7.86±1.48
Goup4	9.05±6.050
Group5	12.46±2.61
P-value	0.138

Data are expressed as Mean ± S.E.M. Groups within the same row that do not share a common superscript letter (a–b) differ significantly at $P < 0.05$. Experimental groups were defined as follows: G1, negative control; G2, montelukast-positive control; G3, methotrexate-positive control; G4, methotrexate + montelukast (5 mg/kg); and G5, methotrexate + montelukast (10 mg/kg).

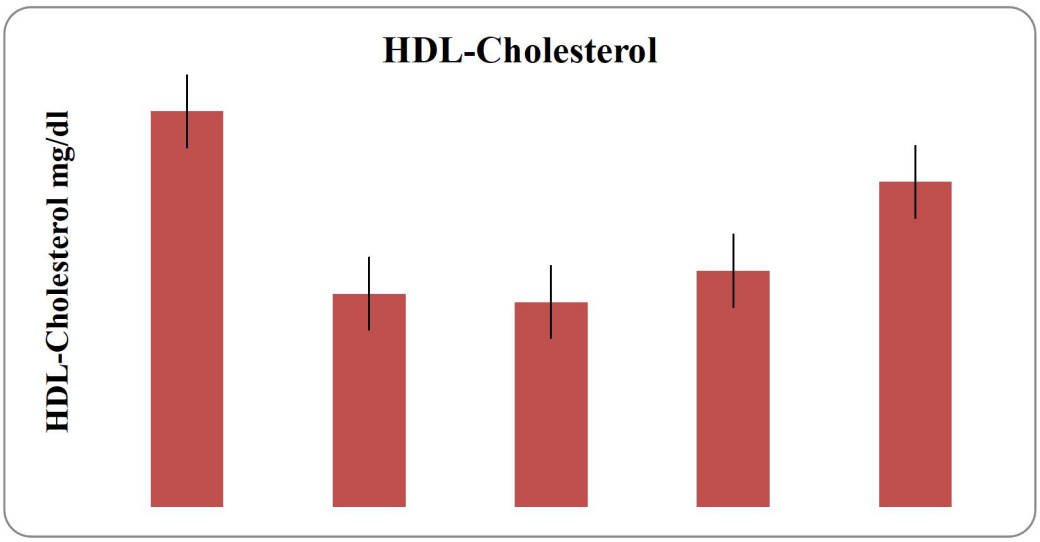

FIG. 4.EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM HDL:THE FIGURE SHOWS, G1(Negative control),G2 (MK +VE Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

3.4. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM TOTAL BILIRUBIN

Significant differences were observed in serum total bilirubin levels when compared to the negative control (G1). Similar variations were found in all groups. G1, G4, and G5 showed results that were comparable to those of G2 and G3 (Table 7).

TABLE 6. TOTAL BILIRUBIN CONCENTRATION IN SERUM

Group	Serum Total Bilirubin mg/dl
Group1	0.25 ± 0.01^{c}
Group2	0.80 ± 0.08^{a}
Group3	0.58 ± 0.07^{b}
Goup4	0.36 ± 0.03^{c}
Group5	0.38 ± 0.26^{c}
P-value	0.001

Data are reported as mean ± standard error of the mean. Within each parameter, significant inter-group differences ($p < 0.05$) are indicated by distinct lowercase superscript letters (a-b); groups bearing identical superscripts demonstrate no statistical difference. Treatment allocations: G1, vehicle control; G2, montelukast monotherapy; G3, methotrexate monotherapy; G4, combination therapy (methotrexate + montelukast 5 mg/kg); G5, combination therapy (methotrexate + montelukast 10 mg/kg)

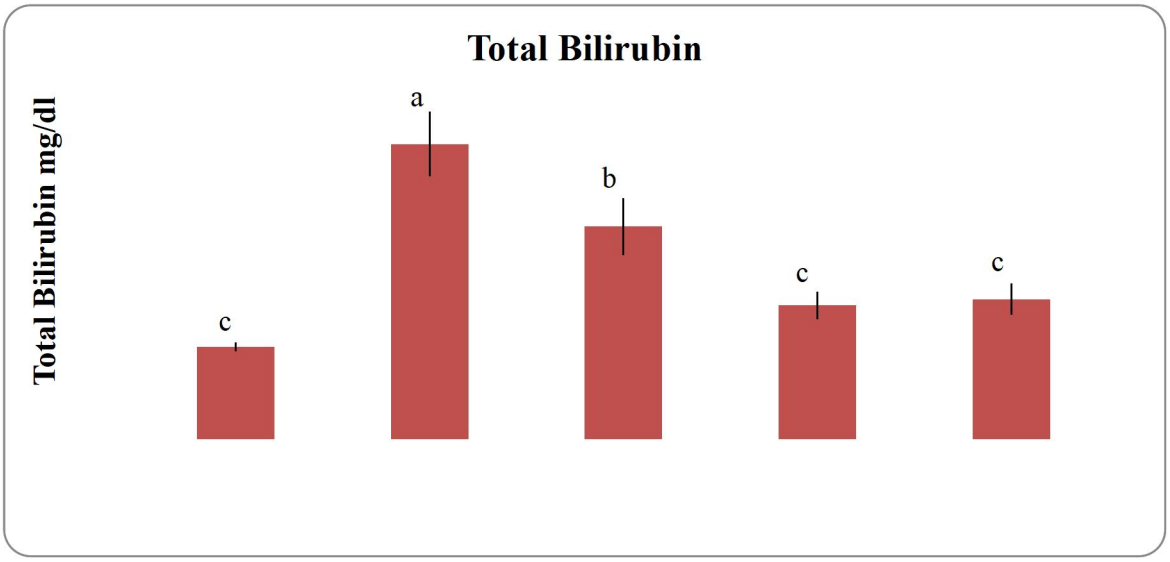

FIG. 5. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM TOTAL BILIRUBIN: G1(NEGATIVE control),G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

3.5. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ALANINE AMINOTRANSFERASE

Significant differences were observed in alanine aminotransferase (ALT) levels when compared to the negative control (G1). However, G3, G2, and G4 did not show any significant differences The high-dose montelukast combination group (G5)

demonstrated significantly decreased alanine aminotransferase levels relative to all other experimental cohorts.(Table 8).

TABLE 7. ALANINE AMINOTRANSFERASE CONCENTRATION IN SERUM

Group	Serum Alanine Aminotransferase u/l
Group1	47± 4.41[a]
Group2	28.33±7.53[abc]
Group3	22.33±1.45[bc]
Goup4	42±11.84[ab]
Group5	19.75±4.40[c]
P-value	0.031

Values are presented as mean ± S.E.M. Groups within a row that do not share the same superscript letter (a–b) differ significantly at $P < 0.05$. The experimental groups were defined as follows: G1, negative control; G2, montelukast control; G3, methotrexate control; G4, methotrexate + montelukast (5 mg/kg); and G5, methotrexate + montelukast (10 mg/kg).

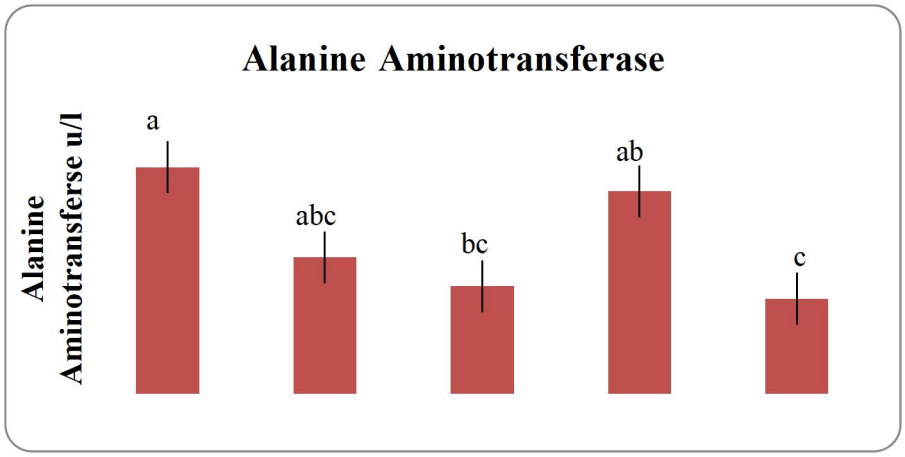

FIG. 6. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ALANINE AMINOTRANSFERASE

Figure show, G1(Negative control),G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

3.6. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ASPARTATE AMINOTRANSFERASE

There was a significant result found for aspartate aminotransferase level when compared with G1. No statistically significant difference in alanine aminotransferase levels was observed between the montelukast control (G2) and methotrexate control (G3) groups. For aspartate aminotransferase, the high-dose combination therapy group (G5) exhibited significantly reduced serum concentrations compared to all other treatment cohorts. The low-dose combination group (G4), montelukast control (G2), and methotrexate control (G3) demonstrated statistically equivalent aspartate aminotransferase levels.(Table 9).

TABLE.8 ASPARTATE AMINOTRANSFERASE CONCENTRATION IN SERUM

Group	Serum Aspartate aminotransferase u/l
Group1	118.75 ± 4.90^{a}
Group2	112.66 ± 1.45^{ab}
Group3	98.33 ± 9.90^{ab}
Goup4	94 ± 1.73^{b}
Group5	66.50 ± 8.58^{c}
P-value	0.001

All data are expressed as mean ± S.E.M. Groups within a row that do not share the same superscript letter (a–b) were considered significantly different at $P < 0.05$ G1 (Negative Control), G2 (MK+ve Control), G3 (Methotrexate +ve Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

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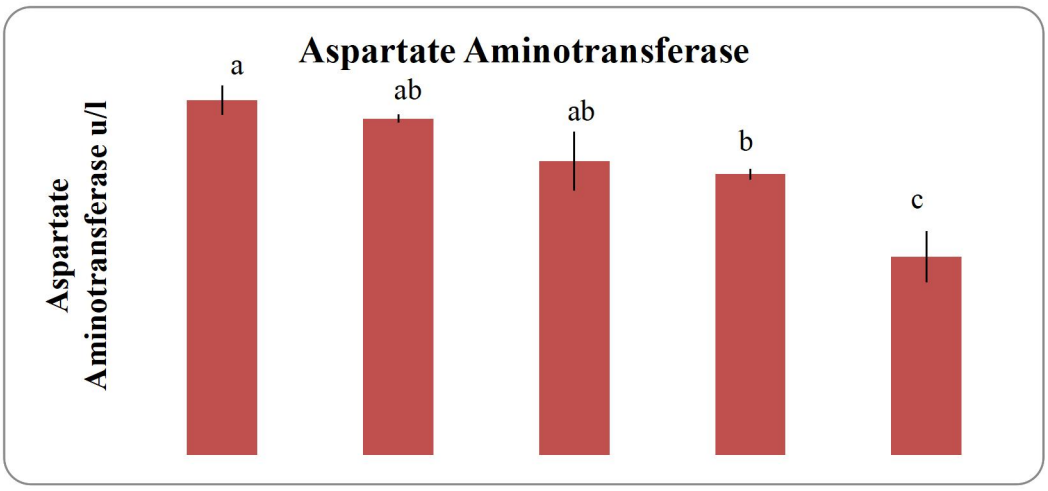

FIG. 7: EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ASPARTATE AMINOTRANSFERASE

G1(Negative control),G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5) and G5 (Methotrexate-MK 10).

3.7. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM TOTAL PROTEINS

Although the overall ANOVA indicated no significant between-group differences, Duncan's post-hoc analysis revealed specific inter-group variations in serum total protein concentrations. The untreated control group (G1) demonstrated significantly lower total protein levels compared to all treatment groups. Conversely, the methotrexate monotherapy group (G3) exhibited elevated total protein concentrations relative to the montelukast control (G2) and both combination therapy groups (G4 and G5)(Table 9).

TABLE. 9 TOTAL PROTEINS CONCENTRATION IN SERUM

Group	Serum Total Protiens g/dl
Group1	4.71± 1.11
Group2	5.31± 0.83

Group3	6.24± 0.79
Goup4	5.27± 0.29
Group5	5.41± 0.88
P-value	0.777

Data were presented as Mean ± S.E.M. Different G1 (Negative Control), G2 (MK+ve Control), G3 (Methotrexate +ve Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

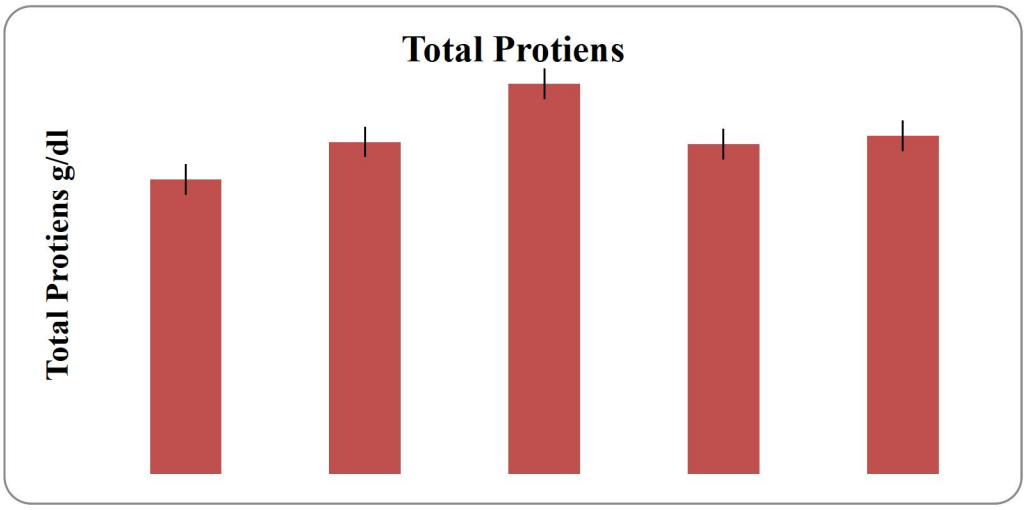

FIG. 8.EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM TOTAL PROTEINS: G1(Negative control),G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

3.8. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ALBUMIN

Results show non-significant changes in the groups however, post hoc Duncan's test shows G3 methotrexate treated group increases albumin concentration in serum as compared to other groups G2, G3, and G4 (Table 11).

TABLE.10 ALBUMIN CONCENTRATION IN SERUM

Group	Serum Albumin g/dl
Group1	1.17± 0.17
Group2	0.87± 0.21
Group3	1.15± 0.15
Goup4	0.72± 0.11
Group5	0.89± 0.10
P-value	0.235

Data were presented as Mean ± S.E.M. Different superscripts [a-b] represent significant differences between the groups in the rows at P<0.05. G1 (Negative Control), G2 (MK+ve Control), G3 (Methotrexate +ve Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

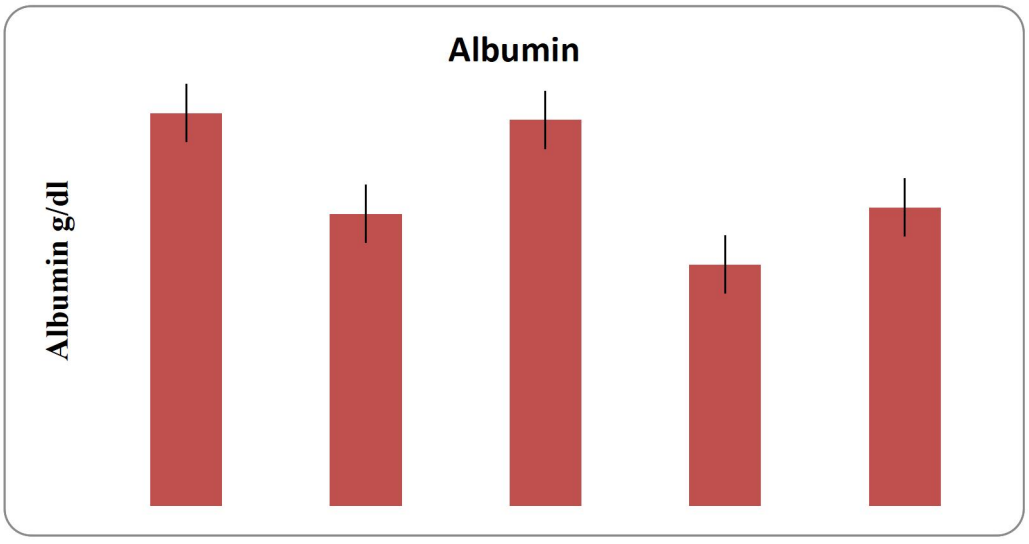

FIG. 9.EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM ALBUMIN

G1(Negative control),G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10

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3.9. EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM GLOBULIN

ANOVA shows nonsignificant results in the groups however post hoc Duncans test revealed that there was a reduction in serum globulin levels in G1, G2,G4, and G5 as compared to G3 Methotrexate treated group (Table 12).

TABLE 11. GLOBULIN CONCENTRATION IN SERUM

Group	Serum Total Globulin g/dl
Group1	3.53± 1.24
Group2	4.43± 0.89
Group3	5.08± 0.86
Goup4	4.54± 0.23
Group5	4.52± 0.96
P-value	0.818

All data are expressed as mean ± S.E.M. Groups within a row that do not share the same superscript letter (a–b) were considered significantly different at $P < 0.05$ G1 (Negative Control), G2 (MK+ve Control), G3 (Methotrexate +ve Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10).

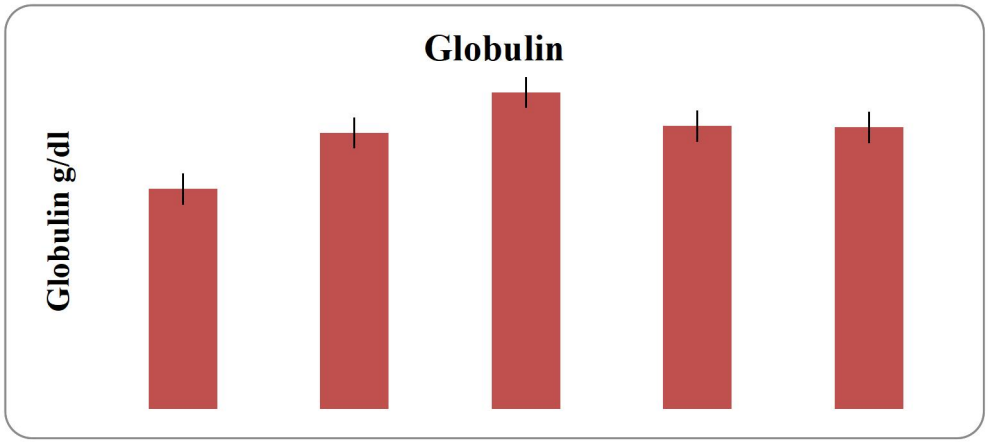

FIG. 10.EFFECT OF METHOTREXATE AND MK TREATMENT ON SERUM GLOBULIN

Figure shows: G1(Negative control), G2 (MK +ve Control), G3 (Methotrexate +VE Control), G4 (Methotrexate-MK 5), and G5 (Methotrexate-MK 10)

4. DISCUSSION

Methotrexate represents a cornerstone therapeutic agent in oncological and autoimmune disease management, yet its clinical utility remains constrained by dose-limiting organ toxicity, particularly hepatic and renal complications. The present investigation evaluated methotrexate-induced hepatotoxicity and the potential ameliorative effects of montelukast co-administration. The experimental protocol involved methotrexate administration on day zero to establish hepatic injury, followed by seven days of montelukast treatment before specimen collection. .Cholesterol serves essential structural and metabolic functions within cellular membranes, though elevated concentrations constitute a recognized cardiovascular risk factor. High-dose methotrexate therapy has been associated with hypercholesterolemia in clinical practice. Our findings demonstrate that montelukast co-administration attenuated methotrexate-induced cholesterol elevation in a dose-dependent manner. Specifically, both combination therapy groups (G4 and G5) exhibited reduced total cholesterol concentrations compared to untreated controls (G1), while the methotrexate monotherapy group (G3) showed significantly elevated cholesterol levels relative to all other cohorts. These observations align with established literature demonstrating methotrexate-associated dyslipidemia, which may result from folate antagonism, elevated homocysteine concentrations, and subsequent cardiovascular risk amplification[16].

High-density lipoproteins (HDLs) are referred to as "good cholesterol." In our study, we observed a beneficial effect when methotrexate (MTX) toxicity decreased the HDL level

in Group G3 compared to Groups G1 and G2. However, in Groups G4 and G5, HDL levels increased. High doses of MTX combined with folate (MK) resulted in higher HDL levels in the serum of Groups G4 and G5. Similar results were reported in a previous study, where cholesterol, triglycerides, and low-density lipoproteins (LDL) increased after treatment with cancerous drugs, while HDL levels decreased significantly. In contrast, our study showed an increase in HDL levels after MTX toxicity and an increase in HDL levels [17].

Triglycerides constitute essential lipid components vital for cellular metabolism and energy storage. The current investigation demonstrated elevated triglyceride concentrations in the methotrexate monotherapy group, while combination therapy with montelukast produced significant reductions in serum triglyceride levels.

Compared to untreated controls, methotrexate monotherapy resulted in significantly elevated triglyceride concentrations, consistent with drug-induced dyslipidemia. This elevation may be attributed to altered hepatic lipid metabolism and impaired very-low-density lipoprotein (VLDL) processing, as VLDL particles serve as the primary transport mechanism for endogenous triglycerides from hepatic synthesis sites to peripheral tissues. The application of gut microbiota along with hepaprotective drugs improved the efficiency of absorption of the active part of the drug, lowers lipid content digested and reduces harmful metabolites to the environment [18, 19].Specifically, and montelukast co-administration effectively attenuated the hypertriglyceridemic effects of methotrexate treatment [20].

Alanine aminotransferase (ALT), also known as glutamate-pyruvate transaminase, is primarily found in the liver. Methotrexate (MTX) can cause hepatotoxicity, leading to liver damage and inflammation. In such cases, liver enzymes, including ALT, are released into the bloodstream. In our study, the ALT levels were lower in Group G5 and G2

compared to other groups. Long-term use of MTX resulted in a decrease in ALT levels. Previous studies have shown that MTX doses of 7.5 mg initially and at the end of treatment increased serum liver enzymes. However, when folate was administered alongside MTX, it progressively reduced hepatic enzymes[21].

Aspartate aminotransferase represents a ubiquitous cytosolic and mitochondrial enzyme with highest concentrations in cardiac muscle, skeletal muscle, and hepatic tissue. The present study demonstrated significant AST elevation in the methotrexate control group (G3), consistent with drug-induced hepatocellular injury. Montelukast treatment groups (G2 and G4) exhibited comparable AST levels, suggesting protective effects against methotrexate-induced enzyme leakage. The untreated control group (G1) maintained physiological AST concentrations, while the high-dose combination therapy group (G5) demonstrated the most pronounced reduction in serum AST activity. These findings corroborate previous clinical observations documenting AST elevation following methotrexate administration, indicative of hepatocellular membrane compromise. The dose-dependent reduction in transaminase levels observed with montelukast co-administration suggests effective hepatoprotection against methotrexate-induced cytotoxicity, with superior efficacy demonstrated in the higher-dose treatment regimen [22].

In our study, significant differences ($p < 0.05$) in bilirubin levels were observed across all groups. Groups G2 and G3 showed distinct values, while Groups G3 and G5 also demonstrated differences compared to other groups. Significant changes were present in all groups. The same study revealed that bilirubin levels increased after methotrexate treatment, which was attributed to liver damage and the development of fatty liver [23]. Bilirubin is a brownish pigment produced as a result of the breakdown

of red blood cells. Elevated bilirubin levels are commonly associated with liver diseases and hemolytic anemia. In our study, the total protein level increased in the methotrexate-treated group, with non-significant differences observed across all groups. Groups G2, G4, and G5 showed similarities, while Groups G2 and G3 had distinct results. When liver damage occurs, protein synthesis decreases, leading to reduced serum protein levels due to the abnormal structural and functional state of the liver. Recent studies on methotrexate have shown that liver oxidative damage results in increased lipid peroxidation and decreased antioxidant enzyme levels, alongside elevated ALT, AST levels and decreased total protein and albumin values, indicating hepatic dysfunction. Thyme extract possesses antioxidant properties and helps protect the liver from damage caused by methotrexate.[24].

Albumin is a protein produced by the liver, and its level in the blood reflects the overall protein content. In our study, non-significant results ($p > 0.05$) were observed for albumin levels. Groups G2 and G3 showed distinct values compared to Groups G4 and G5, while Groups G2, G4, and G5 demonstrated similar results when compared to Group G3. A previous study found that methotrexate injections did not significantly affect total protein and albumin levels [25]. In our study variations in values may be due to low protein diet and insufficient hepatocyte biosynthesis.

Globulin is produced by the liver and the immune system. In our study, the values of globulin showed non-significant results ($p>0.05$). G2, G4,G5 show similar results as compared to G1. Liver insufficient biosynthesis decreases albumin and total protein levels due to liver disorder [23]. Our results show high value of methotrexate monotherapy group (G3) relative to untreated controls (G1). Similarly, all treatment groups demonstrated increased globulin concentrations compared to the control cohort,

potentially reflecting hepatocellular stress responses and oxidative injury pathways associated with drug-induced liver toxicity..

5. CONCLUSION

These findings suggest that montelukast exhibits significant hepatoprotective activity against methotrexate-induced toxicity, effectively attenuating hepatocellular injury and normalizing key biochemical markers including transaminases, bilirubin, and lipid profiles. Montelukast demonstrated particular efficacy in reducing total cholesterol, alanine aminotransferase, and bilirubin levels while restoring physiological albumin concentrations. These preclinical observations support the potential clinical utility of montelukast as an adjuvant therapy to minimize methotrexate-associated hepatotoxicity. Translation of these findings to human clinical trials is warranted to validate therapeutic efficacy and establish optimal dosing protocols

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

STATEMENT OF ANIMAL STUDY

Informed consent was received from the University of Veterinary and Animal Sciences Pakistan for experiments on animal specimens. All research studies were carried out in compliance with the protocols and conjunction with the U.K. Animals (Scientific Procedures) Act, 1986.

AUTHOR CONTRIBUTIONS

Sarwat Noreen; Habib Ur Rahman; Shamaila Kausar; Fahad Said Khan; Muhammad Akram Fethi Ahmet Ozdemir and Gawel Solowski participated in the work's conception and design; all the writers contributed to the work's production, review, and

interpretation; accepted the manuscript; and decided to be responsible for the work's accuracy and honesty.

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