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ORIGINAL ARTICLE

Attenuation of Methotrexate-induced Hepatorenal Damage by *Terminalia bellerica* Fruit Extract in Experimental Rats

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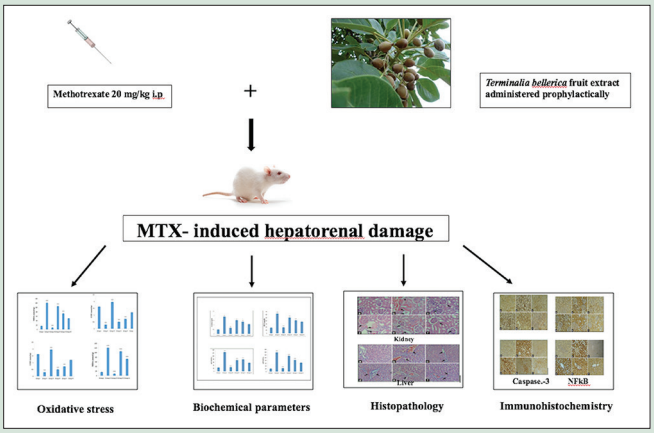
ABSTRACT

Background: Methotrexate (MTX) is used for numerous malignancies and autoimmune disorders. With such widespread use, MTX-induced hepatorenal toxicity is an issue of concern that still needs to be addressed. **Objective:** The aim of the present study is to evaluate the role of *Terminalia bellerica* extract (TBE) in MTX-induced hepatorenal toxicity in Wistar albino rats. **Materials and Methods:** Rats were randomly divided into six groups (n = 6) – received MTX 20 mg/kg intraperitoneally on the 4th day along with pretreatment with different doses of TBE (100 mg/kg, 200 mg/kg, and 400 mg/kg, p.o) given from 1st to 15th day. MTX-induced hepatorenal toxicity was evaluated by biochemical hepatic and renal parameters along with histopathology and immunohistochemistry. **Results:** Hepatorenal toxicity induced by MTX was attributed to increased oxidative stress, biochemical liver, and kidney parameters and upregulation of caspase-3 and nuclear factor kappa B (NFkB). MTX-treated group observed twofold to threefold rise in aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine values–138.49 IU/L, 125.81 IU/L, 63.09 mg/dl, and 1.895 mg/dl, respectively. Groups pretreated with TBE (400 mg/kg) observed a significant decrease (P < 0.001) in oxidative stress and biochemical parameters – AST (63.94 IU/L), ALT (55.98 IU/L), BUN (37.02 mg/dl), and creatinine (1.065 mg/dl). Pretreatment with TBE 400 mg/kg, histopathology of both liver and kidney tissues showed improved architectural damage and immunohistochemistry showed downregulation of increased antigens-caspase-3 and NFkB. **Conclusion:** *T. bellerica* fruit extract (400 mg/kg) showed significant hepatorenal protection by reducing oxidative stress, elevating serum enzymes, and downregulating the tissue expressions of caspase-3 and NFkB.

Key words: Caspase-3, hepatotoxicity, methotrexate, nephrotoxicity, nuclear factor kappa B, *Terminalia bellerica*

SUMMARY

- The aim of the present study is to evaluate the possible beneficial role of *Terminalia bellerica* fruit extract against methotrexate induced hepato-renal damage in Wistar albino rats. MTX induced toxicity was assessed by hepatorenal biochemical parameters (AST, ALT, BUN and creatinine), oxidative stress markers (MDA and GSH), histopathology and immunohistochemistry (caspase-3 and NFkB).
- Results indicated that *T. bellerica* fruit extract might alleviate MTX-induced tissue damage by significantly reducing oxidative stress, decreasing elevated serum enzyme levels and by downregulating the expression of caspase-3 and NFkB in liver and kidney tissues.
- Based on our results, it can be concluded that *T. bellerica* holds the potential to attenuate MTX toxicity and further preclinical and clinical studies are needed to elucidate this effect.



Abbreviations Used: ALT: Alanine transaminase, AST: Aspartate aminotransferase, BUN: Blood urea nitrogen, TBE: Terminalia bellerica fruit extract, GSH: Glutathione, MDA: Malondialdehyde, MTX: Methotrexate, NFkB: Nuclear Factor kappa B.

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INTRODUCTION

Methotrexate (MTX) is a well-known chemotherapeutic and immunosuppressive drug, widely used for leukemias, lymphomas, osteosarcoma, breast cancer, lung cancer, and autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis.[1] With such wide spectrum of uses, MTX-induced hepato- and nephro-toxicity has gained attention of researchers although mechanism of MTX-induced toxicity is still not completely understood.

Unlike other anticancer drugs, MTX is used in varied dose range of 20 mg/m^2–33,000 mg/m^2.[2] Interestingly, MTX causes hepatotoxicity both with high dose and low dose used for prolonged period. Several studies suggest that polyglutamates formed by MTX in high doses (HD-MTX) result in acute and chronic hepatotoxicity.[3] Hepatotoxicity is associated with transaminitis with 2–20-fold increase in serum transaminases, elevated bilirubin levels, fibrosis, and rarely cirrhosis. A study conducted earlier found that the prevalence of fibrosis and cirrhosis associated with MTX was 50% and 26%, respectively.[4]

On the other hand, nephrotoxicity associated with MTX is another grave problem which is still poorly understood. It has been seen that HD-MTX

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is converted to 7-hydroxy MTX and 2, 4-diamino-N10-methylpteroic acid, which are poorly aqueous soluble compounds and hence poorly excreted.[1] Since 90% of the drug is excreted by renal route, this leads to precipitation of MTX metabolites in renal tubules causing renal failure. Consequently, this causes increased plasma concentration of MTX, which further worsens MTX associated myelosuppression, ulcerative stomatitis, and dermatitis.

Nephrotoxicity is presented in the form of elevated levels of creatinine, blood urea nitrogen (BUN), and uric acid that ultimately leads to kidney failure. To control nephrotoxicity, HD-MTX is often administered with hydration, alkalization, and leucovorin. In addition to dialysis, a new recombinant bacterial enzyme, carboxypeptidase-G_2 has also been tried as it converts MTX to inactive metabolites.[5] Both these treatment approaches have a cost limitation. Despite all these measures, nephrotoxicity still continues to occur. This strengthens the need for hepato-nephroprotective therapy that can be taken along with MTX, thereby potentiating its efficacy and minimizing the associated side effects.

Emergence of complementary and alternative medicinal therapies in the form of herbals is believed to be safe and effective because of their natural origin. In Ayurvedic system of medicine, various herbal drugs have been mentioned for their therapeutic role.

One such widely mentioned drug is *Terminalia bellerica*, used both in the combination form as "triphala" or alone which has been known for its anti-inflammatory, antioxidative, antimicrobial, hepatoprotective, nephroprotective, anticancer, and antihyperlipidemic effects.[6]

Acute and subacute toxicity profile of the hydroalcoholic fruit extract of *T. bellerica* showed no signs of toxicity at a dose of 2000 mg/kg p.o and 1000 mg/kg p.o, respectively.[7]

Studies done earlier provide the evidence for the hepato-nephroprotective role of *T. bellerica* extract (TBE) and served as the basis for the hypothesis of the current study. A study conducted by Kuriakose *et al*. showed hepatoprotective potential of *T. bellerica* fruit extract in CCl_4-intoxicated rats.[8] Gallic acid is the active principle present in *T. bellerica* which was responsible for the hepatoprotective potential.[9]

Similarly, dose-dependent nephroprotective activity of TBE was proved earlier in CCl_4-induced renal toxicity model.[9] Hence, the novelty of the present study was to explore the therapeutic potential of TBE in MTX-induced hepatorenal damage.

Therapies targeting drug-induced hepatorenal toxicity are lacking. Therefore, combining hydroalcoholic extract of *T. bellerica* fruits with HD-MTX might reduce the hepatorenal damage induced by MTX.

MATERIALS AND METHODS

Procurement of *Terminalia bellerica* extract

Extract of *T. bellerica* fruit (hydroalcoholic) was procured from Natural Remedies Pvt. Ltd, Bangalore, India, with a Batch No: RD 16180. Hydroalcoholic extract of T. bellerica fruits consist of tannic acid (43.89% w/w), gallic acid (3.58% w/w), and ellagic acid (1.48% w/w) as determined by Natural Remedies Pvt. Ltd, Bangalore.

Experimental animals

Thirty-six Wistar albino rats (200–250 g) were used for the study. Rats were accommodated in clean polypropylene cages at 25°C ± 2°C temperature, 55%–65% relative humidity, and light/dark cycle maintained for 12 h with free access to food and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (IAEC No-950/IAEC/16), All India Institute of Medical Sciences, New Delhi, India.

Chemicals, kits, and antibodies

MTX was purchased from Link Biotech Pvt. Ltd, New Delhi (India) with a batch no AI416G16. Creatinine, BUN, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) kits were purchased from Erba Diagnostics, Himachal Pradesh, India. Immunohistochemistry kits for caspase-3 and nuclear factor kappa B (NFkB) were obtained from Vector Labs, CA, USA. Primary antibodies for caspase-3 and NFkB were procured from Santa Cruz, CA, USA. All other chemicals used were of analytical grade.

Experimental protocol – Methotrexate-induced hepatotoxicity and nephrotoxicity

Before experiments, rats were acclimatized under laboratory conditions. Rats were divided into six groups ($n = 6$ in each group). Experimental rats were administered TBE starting from day 1 till the end of experimental period (14 days). MTX (20 mg/kg intraperitoneally [i.p]) was administered to Groups II, IV, V, and VI on the 4th day of the experiment.

Following were administered to different study groups:

- Group I: Normal control (1 ml/kg/day distilled water; p.o)
- Group II: MTX (20 mg/kg i.p)
- Group III: TBE (*per se*; p.o)
- Group IV: TBE (100 mg/kg/day; p.o) + MTX 20 mg/kg i.p
- Group V: TBE (200 mg/kg/day; p.o) + MTX 20 mg/kg i.p
- Group VI: TBE (400 mg/kg/day; p.o) + MTX 20 mg/kg i.p.

On the day 15th, blood was collected from retro-orbital plexus of the anesthetized rats (pentobarbitone 60 mg/kg i.p). Collected blood was centrifuged at 3000 rpm for 15 min and serum was separated and stored at −20°C for the estimation of liver (AST and ALT) and kidney (creatinine and BUN) biochemical parameters. The rats were sacrificed; both the kidneys and liver were harvested, cleaned from blood by saline, dried on filter paper, and then stored in −80°C for biochemical estimation; and other kidney and liver samples were kept in 10% neutral-buffered formalin for histopathology and immunohistochemistry.[10]

Oxidative stress markers in renal and hepatic tissues

Estimation of reduced glutathione content

The principle of reduced glutathione (GSH) estimation is that the thiol group present in glutathione forms yellow-colored complex with 5,5-dithiobis 2-nitrobenzoic acid (DTNB) at pH 8. The 10% homogenate of kidney and liver tissues was prepared separately in 0.1 M phosphate buffer (pH 7.4), equal volume of 10% tricarboxylic acid was added to each homogenate and then centrifuged at 5000 rpm for 10 min, and the supernatant was collected. Further, upon addition of DTNB, a yellow complex was formed and thiol concentration was measured at an absorbance of 412 nm.[11]

Estimation of malondialdehyde content

In both liver and kidney tissues, malondialdehyde (MDA), a lipid peroxidation marker was analyzed by thiobarbituric acid reactive substances (TBARS) method. For this, 10% homogenate of the rat kidney and liver was prepared separately in 0.1 M phosphate buffer (pH 7.4). MDA present in the tissue reacted with TBARS and formed a colored complex and absorbance was read at 532 nm.[12] The concentration was calculated using standard curve equation.

Estimation of kidney function tests (serum creatinine and blood urea nitrogen)

Serum creatinine was estimated by Jaffe's method with the help of Erba assay kit. Serum sample was mixed with reagent 1 and reagent 2 followed by 3-min incubation period at 37°C after each reagent, and final absorbance was measured at 340 nm.

BUN was estimated by enzymatic method using Erba assay kit. Serum sample was mixed with reagent 1 followed by incubation at 37°C.

Absorbance was taken after 30 s and the next absorbance was taken after 1 min. All the readings were taken with the help of semi autoanalyzer.[13]

Estimation of liver function tests (serum alanine aminotransferase and aspartate aminotransferase)

Serum ALT was estimated by International Federation of Clinical Chemistry (IFCC) method with the help of Erba diagnostic kit. Principle of the test is that ALT reacts with L-alanine, 2-oxoglutarate and converts them into pyruvate, L-glutamate; pyruvate further reacts with lactate dehydrogenase (LDH) and forms L-lactate with nicotinamide adenine dinucleotide. Test samples were added to the working reagents and the absorbance was measured at 340 nm.

Serum AST was measured by IFCC method using Erba diagnostic kit. This test is based on the principle that AST converts L-aspartate, 2-oxoglutarate to oxaloacetate, L-glutamate; further oxaloacetate is converted to malate by malate dehydrogenase; final step is the conversion of sample pyruvate to L-lactate by LDH. Test serum samples were added to the working reagent and the absorbance was measured at 340 nm.[10]

Histopathology estimation

Formalin-preserved kidney and liver tissues were fixed for 24 h in Bouin's fixative. After fixation, tissues were washed with water and dehydrated with ethanol (30%–100%). Tissues were then embedded in paraffin wax at 60°C and paraffin blocks were prepared. Further, sections were stretched on albumin-coated slides and air-dried. Sections were dewaxed with the help of xylene, serially hydrated, and dehydrated with the help of ethanol (30%–100%). Finally, slides were stained with hematoxylin and eosin and observed under a microscope (Eclipse E200; Nikon, Tokyo, Japan).[14]

Table 1: Estimation of hepatic tissue oxidative stress (reduced glutathione and malondialdehyde)

Treatment groups	GSH (umol/g)	MDA (nmol/g)
Group I (control)	1.616±0.012	15.91±2.18
Group II (MTX (20 mg/kg))	0.299±0.086***	121.00±0.45***
Group III (TBE/s)	1.958±0.069	8.41±0.68
Group IV (TBE 100 mg/kg + MTX 20 mg/kg)	0.506±0.005***	105.20±0.93***
Group V (TBE 200 mg/kg + MTX 20 mg/kg)	0.715±0.005***	72.50±0.55***
Group VI (TBE 400 mg/kg + MTX 20 mg/kg)	1.198±0.003***	50.29±0.72***

***P<0.001 was considered statistically significant. All values are represented as mean±SEM. TBE: *Terminalia bellerica* extract; MTX: Methotrexate; GSH: Reduced glutathione; MDA: Malondialdehyde; SEM: Standard error of mean

Table 2: Estimation of renal tissue oxidative stress (reduced glutathione and malondialdehyde)

Treatment groups	GSH (umol/g)	MDA (nmol/g)
Group I (control)	1.450±0.061	23.75±2.93
Group II (MTX (20 mg/kg))	0.200±0.002***	154.83±1.30***
Group III (TBE/s)	1.831±0.048	8.58±0.91
Group IV (TBE 100 mg/kg + MTX 20 mg/kg)	0.43±0.039***	132.33±1.52***
Group V (TBE 200 mg/kg + MTX 20 mg/kg)	0.603±0.005***	106.20±1.23***
Group VI (TBE 400 mg/kg + MTX 20 mg/kg)	1.001±0.003***	57.83±1.10***

***P<0.001 was considered statistically significant. All values are represented as mean±SEM. TBE: *Terminalia bellerica* extract; MTX: Methotrexate; GSH: Reduced glutathione; MDA: Malondialdehyde; SEM: Standard error of mean

Immunohistochemistry estimation

Immunohistochemistry was done for renal and hepatic tissues for the characterization of caspase-3 and NFkB antigens. Both liver and kidney sections were fixed for 24 h in paraformaldehyde (4%), washed, and cryoprotected in sucrose (15%–30%). The 6-mm sections were cut with the help of cryotome. Peroxidase activity in tissues was inhibited by adding 30% hydrogen peroxide dissolved in methanol. Tissue sections were blocked with the help of blocking agent, bovine serum albumin for 1 h at 25°C. Primary monoclonal antibodies specific for caspase-3 (1:200) and NFkB (1:200) were added and incubated for 48 h. Final step was the 2-h incubation of sections with horseradish peroxidase-conjugated secondary antibody (1:2000) (Santa Cruz Biotechnology, CA, USA). Immunological reaction between antigens and antibodies was developed with 3,3'-diaminobenzidine tetrachloride stain. Further, sections were dehydrated with ethanol and visualized under a light microscope (Nikon ECLIPSE E600, Japan).[14]

Statistical analysis

Data were expressed as mean ± standard error of the mean and analyzed by one-way analysis of variance followed by Tukey's *post hoc* test (GraphPad Prism version 5.03, San Diego, CA, USA). P <0.05 was considered statistically significant.

RESULTS

High Performance Liquid Chromatography analysis of *T. bellerica* fruit extract The HPLC analysis of *T. bellerica* fruit extract (TBE) was performed to identify the major constituents present in TBE i.e gallic acid and ellagic acid [Figure 1].

Effect of methotrexate and *Terminalia bellerica* extract on hepatic oxidative stress.

In MTX-treated group, GSH levels were lowest whereas MDA levels were highest in hepatic tissue, as compared to control group with highest GSH and lowest MDA values [Figures 2 and 3]. Pretreatment with TBE significantly increased GSH levels and decreased MDA levels in a dose-dependent manner (P < 0.001) [Table 1].

Effect of methotrexate and *Terminalia bellerica* extract on renal oxidative stress

Treatment with MTX led to increased MDA levels and decreased GSH levels with respect to control group. However, groups where TBE was administered before MTX, there was significant reduction in MDA

Table 3: Estimation of serum creatinine, blood urea nitrogen, aspartate transaminase, and alanine transaminase parameters

Treatment groups	Creatinine (mg/dl)	BUN (mg/dl)	AST (IU/L)	ALT (IU/L)
Group I (control)	0.483±0.083	17.85±0.974	20.58±1.341	25.33±3.55
Group II (MTX (20 mg/kg))	1.895±0.028***	63.09±1.16***	138.49±12.43***	125.81±3.69***
Group III (TBE/s)	0.673±0.085	18.80±1.03	23.57±2.64	27.10±2.76
Group IV (TBE 100 mg/kg + MTX 20 mg/kg)	1.515±0.045***	50.47±1.00***	112.85±3.69***	73.35±2.44***
Group V (TBE 200 mg/kg + MTX 20 mg/kg)	1.340±0.32***	41.66±1.10***	83.68±5.37***	78.08±3.00***
Group VI (TBE 400 mg/kg + MTX 20 mg/kg)	1.065±0.038***	37.02±1.01***	63.94±4.06***	55.98±3.08***

***P<0.001 was considered statistically significant. All values are represented as mean±SEM. TBE: Terminalia bellerica extract; BUN: Blood urea nitrogen; AST: Aspartate transaminase; ALT: Alanine transaminase; MTX: Methotrexate; SEM: Standard error of mean

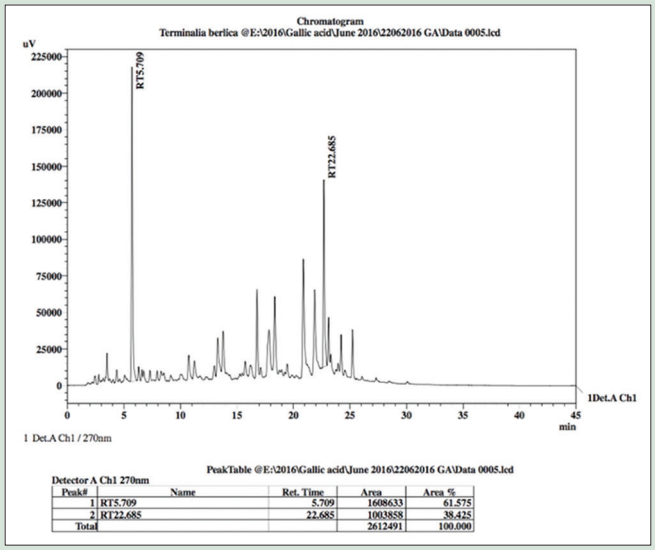

Figure 1: High-performance liquid chromatography chromatogram of *Terminalia bellerica*

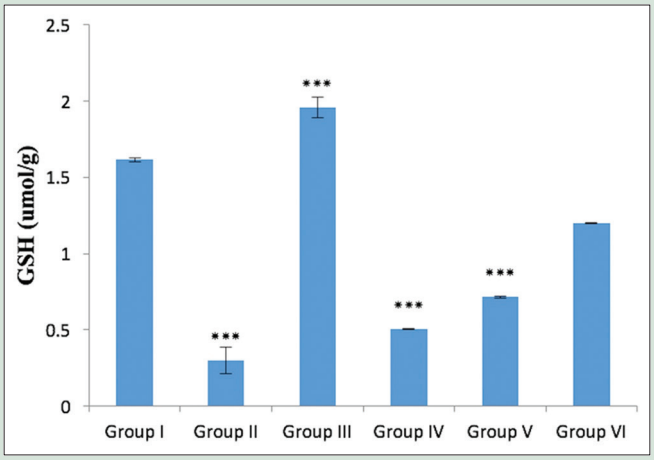

Figure 2: Effect of MTX and TBE on GSH in liver tissue. Group I: Normal control (1 ml/kg/day, p.o); Group II: MTX 20 mg/kg i.p.; Group III: TBE *per se* group; Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p.; Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p.; Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p. ***$P < 0.001$. GSH: Reduced glutathione; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

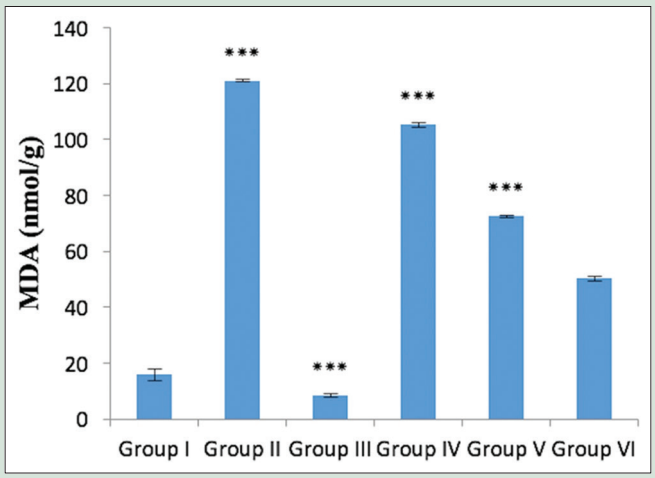

Figure 3: Effect of MTX and TBE on MDA in liver tissue. Group I: Normal control (1 ml/kg/day distilled water, p.o); Group II: MTX 20 mg/kg i.p.; Group III: TBE *per se* group; Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p.; Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p.; Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p.; ***$P < 0.001$. MDA: Malondialdehyde; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

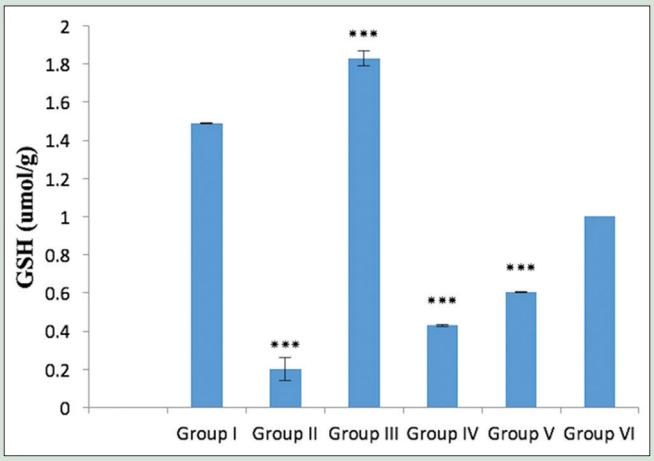

Figure 4: Effect of MTX and TBE on GSH in kidney tissue. Group I: Normal control (1 ml/kg/day, p.o), Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p, ***$P < 0.001$. GSH: Reduced glutathione; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

values and significant elevation in GSH values in a dose-dependent manner ($P < 0.001$) [Table 2 and Figures 4 and 5].

Effect of *Terminalia bellerica* extract on methotrexate-induced hepatotoxicity

MTX (20 mg/kg, i.p) induced hepatotoxicity in rats. This statement was evident by significant rise in the serum levels of AST and ALT with respect to the control group [Figures 6 and 7]. Furthermore, histopathology showed hepatic injury in the form of deranged architecture of cells, congestion, and hemorrhage in MTX-treated group when compared with normal control and TBE per se groups [Figure 8a-c].

Prior treatment with TBE significantly decreased the levels of AST in a dose-dependent way [Table 3]. Maximum decrease in serum enzymes was observed with TBE 400 mg/kg. Similarly, minimal liver injury was seen with TBE 400 mg/kg group when compared with other treatment groups [Figure 8d-f].

Effect of *Terminalia bellerica* extract on methotrexate-induced nephrotoxicity

MTX (20 mg/kg, i.p)-treated rats observed significantly increased creatinine and BUN levels with respect to control group. In addition, histopathology of kidney tissue showed renal injury in MTX-treated group when compared with normal control and TBE per se groups [Figure 9a-c]. Pretreatment with TBE significantly decreased the levels of creatinine ($P < 0.001$) and BUN ($P < 0.001$) in a dose-dependent manner, TBE 400 mg/kg being the most effective dose [Figures 10 and 11]. Moreover, in histopathology findings, improvement in renal damage

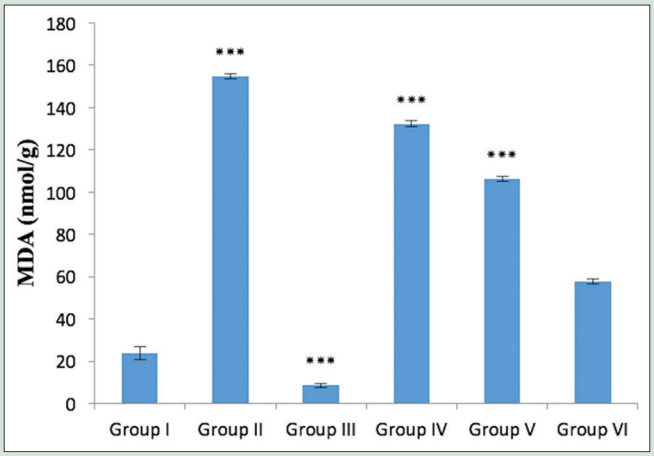

Figure 5: Effect of MTX and TBE on MDA in kidney tissue. Group I: Normal control (1 ml/kg/day, p.o), Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p, ***$P < 0.001$. MDA: Malondialdehyde; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

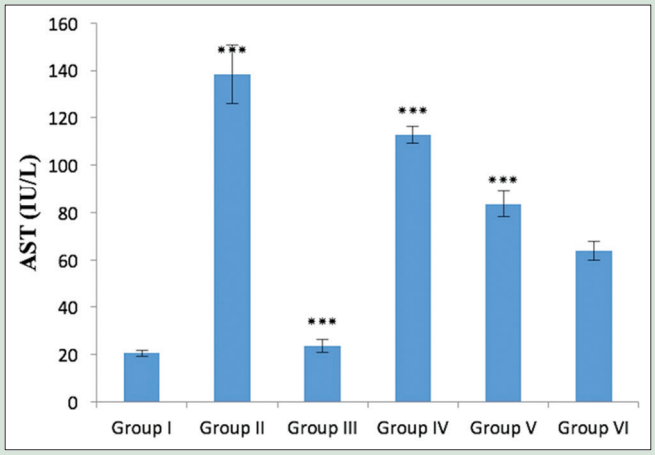

Figure 6: Effect of MTX and TBE on AST. Group I: Normal control (1 ml/kg/day, p.o), Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p. ***$P < 0.001$. AST: Aspartate transaminase; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

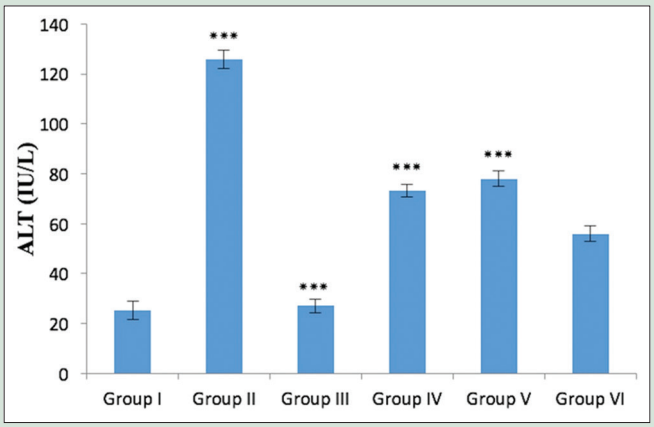

Figure 7: Effect of MTX and TBE on ALT. Group I: Normal control (1 ml/kg/day, p.o), Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p, ***$P < 0.001$. ALT: Alanine transaminase; TBE: *Terminalia bellerica* extract; MTX: Methotrexate

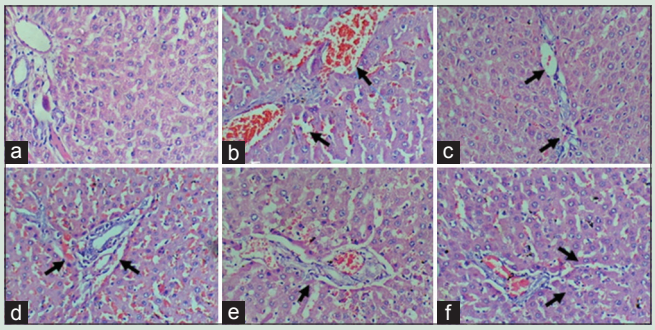

Figure 8: Effect of TBE on histopathology of hepatic tissue in MTX-intoxicated rats. Pictomicrographs of rat liver sections stained with H and E. (a) Control group with normal liver histopathology with polygonal hepatocytes and granular cytoplasm. (b) Methotrexate (20 mg/kg, i.p) group showing sinusoidal dilatation, congestion with hemorrhage and hepatocyte nuclear-cytoplasmic ratio is increased. (c) *Terminalia bellerica per se* group with normal architecture of liver hepatocytes. (d) TBE 100 mg/kg + MTX 20 mg/kg group showed less severe sinusoidal dilatation, disturbed radial arrangement of cords, central vein congestion, and hemorrhage. All the pathological changes were less marked than MTX group. (e) TBE 200 mg/kg + MTX 20 mg/kg group showed improved hepatic histology with moderate disturbance of hepatocytes, sinusoidal dilatation, hemorrhage, vacuolated and atrophied hepatocytes. (f) TBE 400 mg/kg + MTX 20 mg/kg group demonstrated maximum improvement in hepatic histopathology. Minimal architectural loss was seen. Radial arrangement of hepatocytes was seen (×20). MTX: Methotrexate; TBE: *Terminalia bellerica* extract

was seen in the dose-dependent manner in pretreated TBE groups [Figure 9d-f].

Effect of *Terminalia bellerica* extract on the expression of caspase-3 and nuclear factor kappa B in liver tissue

MTX (20 mg/kg, i.p) administration resulted in increased expression of caspase-3 and NFkB in hepatic tissue. Normal control and TBE per se groups showed negative expression for caspase-3 and NFkB [Figures 12a-c and 13a-c]. Prior treatment with TBE decreased the expression of caspase-3 and NFkB in the respective groups. Maximum decrease of caspase-3 and NFkB expression was observed with TBE (400 mg/kg) [Figures 12d-f and 13d-f].

Effect of *Terminalia bellerica* extract on the expression of caspase-3 and nuclear factor kappa B in kidney tissue

Kidney tissue revealed significant upregulation of caspase-3 and NFkB expression upon MTX (20 mg/kg, i.p) administration [Figures 14b and 15b]. Normal control and TBE per se groups showed absence of caspase-3 and NFkB expression [Figures 14a-c and 15a-c].

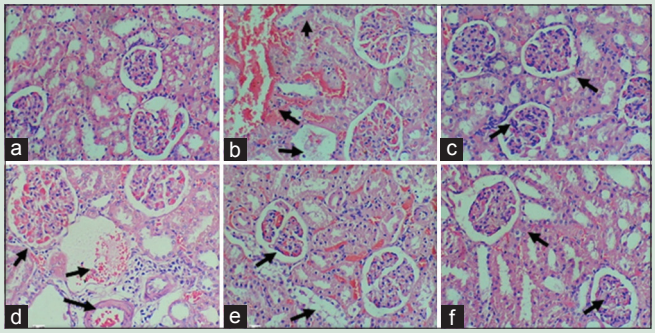

Figure 9: Effect of TBE on histopathology of renal tissue in MTX-intoxicated rats. Pictomicrographs of rat kidney sections stained with H and E. (a) Normal control group showing normal structure of glomerulus, tubular cells, cortex, and medulla. (b) Methotrexate (20mg/kg, i.p) group showing peritubular dilatation with architectural loss of tubules and vascular congestion with clogging of RBCs. Severe hemorrhage was observed in glomerulus. (c) *Terminalia bellerica per se* group showing normal liver histopathology that resembles control group. (d) TBE 100 mg/kg + MTX 20 mg/kg group showing pathological changes with less severe tubular cell vacuolization, hemorrhage, peritubular dilatation, vascular congestion, changes were less marked as compared to MTX 20 mg/kg group. (e) TBE 200 mg/kg + MTX 20 mg/kg group depicts moderate peritubular dilatation, glomerular hemorrhage, less prominent vascular congestion and vacuolization. All the pathological changes were less severe than the above two groups. (f) TBE 400 mg/kg + MTX 20 mg/kg group showed maximum improvement with TBE 400 mg/kg group with no hemorrhage, minimal tubular dilatation and cell infiltration. This group resembled the normal control group histology (×20). TBE: *Terminalia bellerica* extract; MTX: Methotrexate

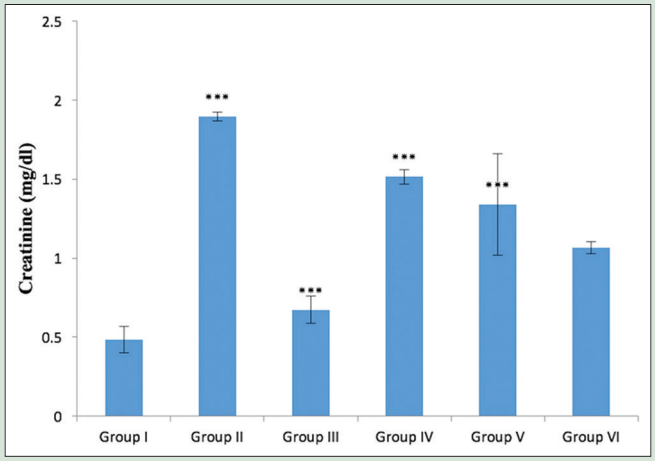

Figure 11: Effect of MTX and TBE on creatinine. Group I: Normal control (1 ml/kg/day, p.o), Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p, ***$P < 0.001$. TBE: *Terminalia bellerica* extract; MTX: Methotrexate

Pretreatment with TBE (100 mg/kg, 200 mg/kg, and 400 mg/kg) attenuated the expressions of caspase-3 and NFkB. Maximum downregulation in both the expressions was observed with TBE (400 mg/kg) [Figures 14d-f and 15d-f].

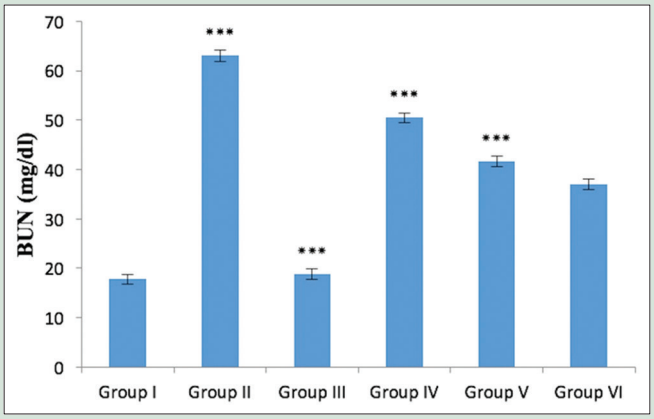

Figure 10: Effect of MTX and TBE on BUN. Group I: Normal control (1 ml/kg/day, p.o); Group II: MTX 20 mg/kg i.p, Group III: TBE *per se* group, Group IV: TBE 100 mg/kg/day, p.o, MTX 20 mg/kg i.p, Group V: TBE 200 mg/kg/day p.o, MTX 20 mg/kg i.p, Group VI: TBE 400 mg/kg/day p.o, MTX 20 mg/kg i.p, *** $P < 0.001$. BUN: Blood urea nitrogen, TBE: *Terminalia bellerica* extract; MTX: Methotrexate

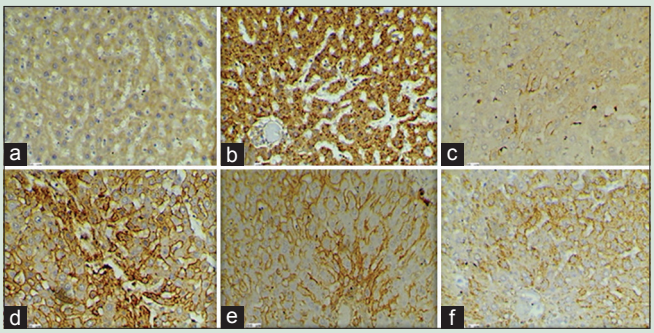

Figure 12: Effect of TBE on the expression of caspase-3 in hepatic tissue in MTX-intoxicated rats. Pictomicrographs of rat liver sections showing the presence and distribution of caspase-3 expression. (a) Normal control group showed no evidence of caspase-3 expression. (b) MTX 20 mg/kg i.p group demonstrated highest expression of caspase-3 in the liver tissue which shows the evidence of apoptotic changes in the hepatocytes. (c) TBE *per se* group showed similarity with normal control group as evident by negative immunoreaction for caspase-3. (d) TBE 100 mg/kg + MTX 20 mg/kg group depicted increased expression of caspase-3 in hepatic lobules, however the intensity was less than MTX *per se* group. (e) TBE 200 mg/kg + MTX 20 mg/kg group showed that the intensity of caspase-3 expression in lobules. However, the intensity was less with respect to the above two groups. (f) TBE 400 mg/kg + MTX 20 mg/kg group showed least intensity of caspase-3. TBE: *Terminalia bellerica* extract; MTX: Methotrexate

DISCUSSION

MTX is most commonly used anticancer and immunosuppressant agent.[15] Cytotoxic effects produced by MTX are nonselective, and it equally affects both normal and cancer cells. Among all the toxicities associated with MTX, hepatotoxicity and nephrotoxicity are most prominent. Unfortunately, to date, no such hepato-nephroprotective therapy has come across that can lessen the troublesome side effects caused by MTX. Although several herbal drugs have been reported to decrease the MTX associated adverse effects, none of these drugs have

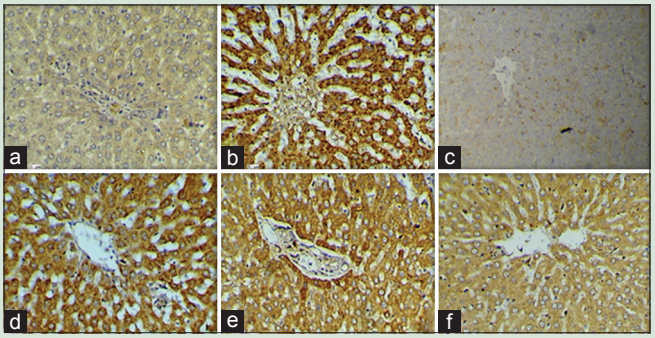

Figure 13: Effect of TBE on the expression of NFkB in hepatic tissue in MTX-intoxicated rats. (a) Normal control group showed no evidence for the presence of NFkB expression. (b) MTX 20 mg/kg i.p group showed highest expression of NFkB as clearly seen by the deep brown color. Maximum inflammatory and apoptotic changes were observed in this group. (c) TBE *per se* group demonstrated negative reaction for NFkB expression. (d) TBE 100 mg/kg + MTX 20 mg/kg group showed high intensity of caspase-3 expression near central vein and lobules, although it was less marked than MTX-treated group. (e) TBE 200 mg/kg + MTX 20 mg/kg group showed moderate intensity of NFkB in hepatic lobules but was less marked than the above two groups. (f) TBE 400 mg/kg + MTX 20 mg/kg group described minimal NFkB expression in the lobules. TBE: *Terminalia bellerica* extract; MTX: Methotrexate; NFkB: Nuclear factor kappa B

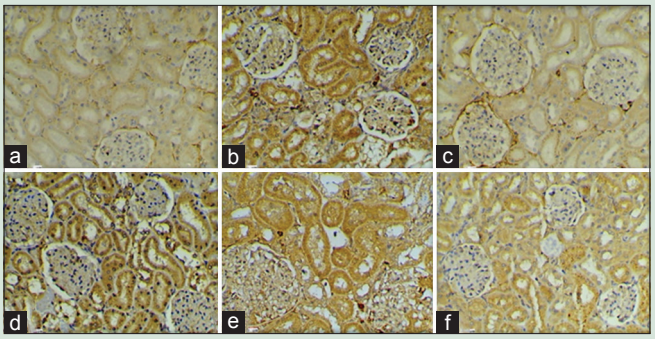

Figure 15: Effect of TBE on the expression of NFkB in renal tissue in MTX-intoxicated rats. (a) Normal control group depicted no expression of NFkB. (b) MTX 20 mg/kg i.p group showed the maximum immunostaining for NFkB in the tubules. (c) TBE *per se* group showed no expression of NFkB. (d) TBE 100 mg/kg + MTX 20 mg/kg group showed maximum immunostaining with NFkB in tubules, but intensity was less than the MTX 20 mg/kg group. (e) TBE 200 mg/kg + MTX 20 mg/kg group showed moderate color intensity of NFkB in tubular region, but it was less than the above two groups. (f) TBE 400 mg/kg + MTX 20 mg/kg group showed minimal presence of NFkB expression. TBE: *Terminalia bellerica* extract; MTX: Methotrexate; NFkB: Nuclear factor kappa B

proved to be effective during clinical trials. Therefore, it is mandatory to find an adjunct having hepatorenal protective properties.

The aim of the present study was to evaluate the effects of hydroalcoholic extract of TBE on MTX-induced hepatotoxicity and nephrotoxicity. However, toxicity induced by MTX in vital organs such as liver and kidney necessitates the need for the discovery of novel hepatorenal protective drug therapy. The current study unfolds the possible protective role of TBE (100 mg/kg, 200 mg/kg, and 400 mg/kg) in MTX-induced hepato- and nephro-toxicity model.

Data obtained from this study showed increased levels of MDA and decreased levels of GSH in liver and kidney organs with HD-MTX.

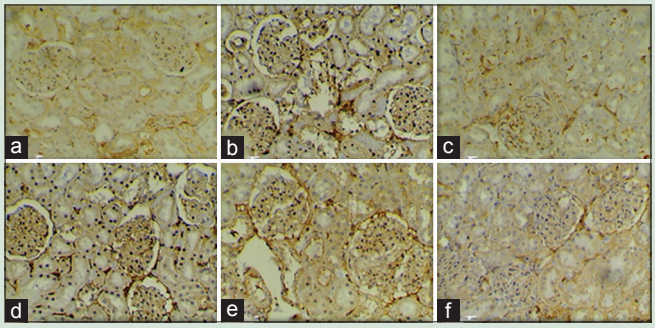

Figure 14: Effect of TBE on the expression of caspase-3 in renal tissue in MTX-intoxicated rats. (a) Normal control group showed negative reaction for caspase-3 in renal tissue. (b) MTX 20 mg/kg i.p showed strong reaction for caspase-3 in renal tubules. (c) TBE *per se* group showed no evidence of caspase-3. (d) TBE 100 mg/kg + MTX 20 mg/kg group showed the presence of tubular caspase-3, but it was less marked than the MTX *per se* group. (e) TBE 200 mg/kg + MTX 20 mg/kg group showed the expression of caspase-3, but it was less compared to the above two groups. (f) TBE 400 mg/kg + MTX 20 mg/kg group showed minimal immunological reaction for caspase-3. TBE: *Terminalia bellerica* extract; MTX: Methotrexate

This points toward one of the possible mechanisms for MTX-induced hepatorenal toxicity, i.e., oxidative stress and lipid peroxidation.[16,17] Pretreatment with TBE showed dose-dependent decrease in MDA and increase in GSH values.

In this study, hepatotoxicity induced by MTX was demonstrated by elevated serum enzyme levels of AST and ALT. Rise in the levels of these enzymes is the marker of hepatic injury.[17] Most common reason behind elevated serum AST and ALT enzymes is the loss of hepatocyte structural integrity.[18] Maximum hepatoprotective effect was shown by TBE (400 mg/kg) as it significantly decreased the elevated levels of these enzymes.

Further, histopathology findings confirmed the MTX-induced hepatic injury, as tissue histology showed architectural loss of hepatocytes in the form of sinusoidal dilatation, derangement of radial cords, and congestion due to hemorrhage. Prior treatment with TBE observed minimal pathological changes with minimal sinusoidal dilatation and congestion in a dose-dependent manner. This study was in concurrence with the previous study carried out by Ali *et al*, who reported protective effects of chrysin in MTX-intoxicated rats.[17]

On the other hand, MTX-induced nephrotoxicity was manifested by increased serum levels of creatinine and BUN. Furthermore, renal histology showed architectural loss of renal tubules with peritubular dilatation along with severe vascular congestion. Analogous study was performed by El-Sheikh *et al* who reported beneficial role of thymoquinone in MTX-induced toxicity rat model.[19]

The results from biochemical tests and histopathology findings showed that TBE at a dose of 400 mg/kg proved to be most beneficial in decreasing creatinine and BUN levels and improving renal architectural features, thereby proving that hydroalcoholic extract of 400 mg/kg TBE was most effective in attenuating MTX-induced nephrotoxicity.

Immunohistochemistry examination of both liver and kidney tissues showed increased expression of caspase-3 and NFkB. Caspase-3 is a proapoptotic factor, primarily responsible for executing apoptosis of cells.[20] Immunohistochemistry results point toward the fact that MTX is responsible for the cellular apoptosis of hepatic and renal tissue. Involvement of caspase-3 in apoptosis after MTX administration has also previously been described.[21]

It has been reported previously that NFkB activation is responsible for the increased expression of interleukins.[22] Therefore, NFkB is an

important target in regulating the expression of other interleukins. Interestingly, MTX-induced increased expression of NFkB was decreased by pretreatment with TBE administered at 100 mg/kg, 200 mg/kg, and 400 mg/kg doses. Decrease in the expression of NFkB was observed in a dose-dependent manner. This finding was in agreement with the study done previously.[19] Hence, downregulation of NFkB by TBE might play an important role in inhibiting drug-induced interleukin expression.

The study suggested that *T. bellerica* fruit extract may confer protection against MTX-induced hepatorenal damage. The plausible mechanisms extracted out from the above-mentioned results were – acts as a protective agent against increased oxidative stress, normalized the elevated hepatorenal biochemical parameters, and conferred protection against apoptosis and inflammation by downregulating caspase-3 and NFkB expression.

CONCLUSION

The present study concluded that pretreatment with *T. bellerica* fruit extract ameliorated the MTX-induced hepatorenal damage. Extract of *T. bellerica* holds the potential to act as an adjuvant therapy that may be coadministered along with MTX to reduce its adverse effects. This combined therapy may be further investigated to explore the feasibility in clinical applications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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