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Hepatoprotective Effect of Pomegranate Juice Extract in Methotrexate Induced Hepatic Dysfunction in Rats

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ABSTRACT

The current study was aimed to assess the progression of hepatic fibrosis and to compare the therapeutic efficacy of pomegranate juice to the known anti-fibrotic drug, enalapril. In this study, a total of 24 rats were used and divided into 4 groups: groups I & II were kept as normal control & MTX controls, whilst groups III and IV were kept as MTX+ pomegranate and MTX+ enalapril, respectively, and the study was carried out for 28 days. Serum samples were collected on the 14th and 28th days for the estimation of AST, ALT, GGT, and TGF-1 levels. Rats were sacrificed at the end of the experiment, and liver samples were collected for further estimation of anti-oxidant levels and histopathology of the liver. The study results showed significantly elevated the activity of AST, ALT and GGT, TGF- β1 in MTX received rats group (II) compared to control group (I) along with elevated levels of TBARS and significant diminished antioxidant enzymes GSH, GPx, SOD and GST. However, group III receiving pomegranate juice revealed substantial improvement in all parameters and results were validated through the histopathology of the liver. The presence of phytoconstituents ellagic acid, gallic acid, anthocyanins and catechins, could abrogate MTX-induced hepatic fibrosis.

HIGHLIGHTS

- MTX causes changes in the liver due to release of free radicals.
- MTX is an anticancerous agents used to treat cancer, inflammation like rheumatoid arthritis.
- Pomegranate is a easily available in the market which has anti-inflammatory, antioxidant rich fruit and could able to ameliorates adverse effect caused by MTX.

Keywords: Methotrexate, Pomegranate juice, Hepatic dysfunction, TGF-beta, Oxidative stress

Methotrexate (MTX) is a folic acid analogue used for the treatment of various cancers and its actions are mediated through competitive inhibition of Dihydrofolate Reductase (DHFR) enzyme thereby interfering with the nucleic acid synthesis in the growing cancer cells. It is also used in the treatment of different types of cancers, psoriasis, autoimmune disorders and medical termination of pregnancy (Vanisthasree *et al*., 2011; Bedoui *et al*., 2019). The MTX causes renal toxicity, intestinal mucositis, and testicular toxicity, several studies have also documented that hepatotoxicity is a significant adverse effect of the MTX (Abo-Haded *et al*., 2017). The precise mechanism of MTX involved in the development of hepatotoxicity is still elusive but numerous studies suggested that oxidative

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stress could be responsible for the underlying cause of hepatotoxicity (Ali *et al*., 2014). Excessive production of free radicals through oxidation and which assail the polyunsaturated fatty acids of a biological membrane, resulted in lipid peroxidation and damage of protein and DNA, eventually causing liver damage (Anilkumar *et al*., 2013; Arundhathi *et at.,* 2015; Namratha *et al*., 2021). Chronic usage of MTX has been linked to causing fatty liver, moderate steatosis, fibrosis, and cirrhosis in many patients receiving MTX drug as well as cause choline insufficiency (Bessone *et al*., 2018). Chemicals often cause subclinical injury to the liver, which manifests only as abnormal elevation of liver enzymes (ALT, AST and GGT). Antioxidants are effective for preventing liver fibrogenesis by elevating antioxidant enzymes in the body (Asadi-Samani *et al*., 2015; Allawadhi *et al*., 2021). The progression of fibrosis is a deposition of extracellular matrix (ECM) that is formed through an overactive hepatic stealate cell occurs in the majority of chronic liver diseases and results in the formation of scar tissue and ends with the development of cirrhosis, which is characterised by alteration in the blood circulation in the liver and liver architecture (Gopi *et al*., 2010). Liver cirrhosis is the leading cause of morbidity and mortality in individuals with liver disease, as well as predisposing them to liver failure and primary liver cancer (Popov and Schuppan, 2009).

Amelioration of MTX-induced toxicity has been the prime concern during therapeutic intervention with MTX. Both folate and folinic acid (leucovarin) have been shown to reduce MTX toxicity. However, folic acid supplements could reduce MTX efficiency in the treatment of cancer(Cabrera *et al*., 2019). Since MTX induces toxicity through systemic oxidative stress, a search for drugs or agents with antioxidant properties has been extensively carried out (Ozogul *et al*., 2013), most of which proved partially helpful in preventing MTX toxicity and some associated with additional toxic effects. Henceforth, there is a need to explore alternative therapy for MTX-induced hepatotoxicity to replace the currently used drugs. Numerous medicinal plant extracts and phytochemicals are being used for hepatic disorders in ethno- medicinal practice and the traditional system of medicine (TSM) of India (Kumar *et al*., 2012; Priyanka *et al*., 2020).

Pomegranate juice contains a large number of polyphenolic substances like anthocyanins, hydrolysable tannins – ellagitannins & gallotannins and condensed tannins – proanthocyanidin which exhibited evidence in the culminating of oxidative stress mediators and its anti-oxidant ability added due to the presence of phenolic compounds that scavenge free radical (Husain *et al*., 2018). Based on the above facts, the present study was designed to evaluate the efficacy of pomegranate juice was taken up with the following objectives.

The present study was planned on male Wistar albino rats of uniform age (about 3 months). The rats were procured from Jeeva life science Pvt. Limited, Hyderabad.

MATERIALS AND METHODS

Drugs

1. Methotrexate (Ipca Laboratories PVT. Ltd. Mumbai, M.H.)
2. Enalapril Tablets (Dr.REDDY's Laboratories Ltd., Khol, Baddi, H.P)
3. Pomegranate Fruits (Local Market)

Chemicals and Kits

Analytical grade chemicals were used in the biochemical analysis in the current study. All the chemicals (for preparation of reagents and buffers) were procured from Qualigens Pvt. Ltd. Mumbai, Himedia Pvt. Ltd., and SRL Pvt. Ltd., Mumbai. Kits for AST, ALT, GGT were procured from ERBA diagnostics Ltd, Surat, India ELISA kit for TGF-β1 was procured from KRISHGEN bio systems, Mumbai.

Animals

A total of 24 male *Wistar albino* rats (200-250 grams), 3 months old were used and acclimatized for 1 week before initiation of study. The uniform weights of rats were randomly divided into four groups consisting of 6 rats per group and kept in polypropylene cages and maintained with 12 hour dark/light cycle at college animal house. All the rats were provided with feed and water *ad libitum* throughout the experiment. The study protocol was prior approval by the Institutional Animal Ethics Committee (I/2018-31/IAEC/CVSc., Hyd, Dated 16/07/2018). All

the groupswere maintained for 28 days with the following treatment schedule.

Experimental plan

Group 1:	Normal control (@100 µl Normal Saline IP daily)
Group 2:	Methotrexate @ 100 µg/kg body weight (BW) once daily IP route
Group 3:	Methotrexate @ 100 µg/kg BW once daily IP for 28 days + Pomegranate juice @ 1 ml/Rat once daily orally
Group 4:	Methotrexate @ 100 µg/kg BW I P + Enalapril @ 5 mg/kg body weight once daily orally

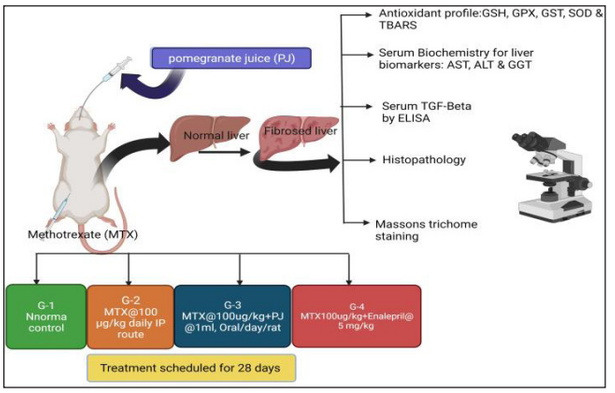


Fig. 1: Depiction of research methodology

Blood collection

Prior to the blood collection all the rats were off fed for 12hrs and blood samples were collected through retro-orbital plexus in to blood vacutainers. Sera samples were separated by centrifugation @400 RPM for 5 minutes for the estimation of AST, ALT, GGT and TGF-β1. On the 28th day, rats were euthanized by using carbon dioxide exposure and rats were sacrificed, liver tissues were collected and homogenized for the assay of GSH, GPX, GST, SOD and TBARS. Piece of liver tissues were collected for the histopathological examination for further validation of the results.

Antioxidant defense profile

The concentrations of Reduced glutathione (GSH) in the liver homogenate were done by the procedure earlier described (Moron *et al*., 1979), Superoxide dismutase (SOD) (Madesh *et al*., 1998) Thiobarbituric acid reacting substances (TBARS) (Balasubramanian *et al*., 1988) Glutathione peroxidase (GPx) (Paglia *et al*., 1967) and Glutathione S-transferase (GST) (Habig *et al*., 1974). The concentration of protein in the liver samples was estimated by using BCA (Bicinchoninic acid) protein assay (Hill and Straka, 1988).

Histopathological studies

Pieces of liver were collected and fixed the liver tissue in the 10% NBF (Neutral buffered saline) for the liver histopathology. The fixed tissues were processed and stained with Masson's trichrome stain as described by the authors (Singh and Sulochana, 1996).

STATISTICAL ANALYSIS

The present study data were analyzed by one-way ANOVA using SPSS version 23. The obtained differences in the mean values were tested through Duncans multiple comparison test and the values were tested at a 5% significance level ($p<0.05$).

RESULTS

Aspartate transaminase (AST)

The activity of AST (IU/L) was estimated on days 14 & 28. Group II (133.20 ± 3, 149.80 ± 3.4, respectively) had shown significant ($p<0.05$) elevated levels of AST between the groups and also between day 14 & day 28. Group III (105.10 ± 1.4, 114.20 ± 2.1, respectively) showed a decrease in AST levels which were significantly ($p<0.S05$) lower than group II alone but numerically more than group IV (100.70 ± 5.4, 107.00 ± 2.3, respectively) (depicted in graph 1).

Alanine transaminase (ALT)

The activity of ALT (IU/L) in group II (87.00 ± 1.9, 101.10 ± 1.6, respectively) had a significant ($p<0.05$) rise at different time intervals. As the treatment progressed to day 28, there was a further rise in the level of ALT. In groups III & IV (69.50 ± 0.7, 79.90 ± 1.3, 67.40 ± 1.6, 79.80 ± 1.4, respectively) there was a significant ($p<0.05$) decrease as compared to group II on day 14 & day 28. However, groups III & IV seem to have the same levels during the study period (depicted in graph 2).

Gamma – glutamyltransferase (GGT)

The activity of GGT (IU/L) in group II (6.65 ± 1.16, 9.5 ± 1.15 respectively) had revealed elevated levels of GGT which was significantly ($p<0.05$) higher between the groups and also between day 14 & day 28. Group III (5.65 ± 1.16, 8.13 ± 1.10 respectively) showed a decrease in GGT levels which were significantly ($p<0.05$) lower than group II alone but higher than group IV (5.16 ± 1.06, 6.26 ± 1.14 respectively) (depicted in graph 3).

Cytokine TGF-β1

TGF-β1 (pg/ml) was estimated on day 14 and day 28. Group II (18.67 ± 3.43) had significantly ($p<0.05$) higher values on day 14 compared to groups I, III & IV (10.07 ± 1.04, 15.34 ± 1.34, 13.66 ± 0.95 respectively) and as it progressed to day 28, there was further significant ($p<0.05$) increase in the level of TGF-β1. Group III & IV had TGF-β1 levels comparable to the group I, but significantly ($p<0.05$) higher than group I. There was significant variation between group III (15.34 ± 1.34, 18.03 ± 2.3 respectively) & group IV (13.66 ± 0.95, 16.27 ± 1.54 respectively) on day 14 & day 28 (depicted in graph 4).

Antioxidant profile

Antioxidant profile GSH, SOD, TBARS, GPx and GST was assessed in the liver homogenate

Reduced glutathione (GSH)

GSH (n moles/mg protein) concentration in group II (2.36 ± 0.11) dipped to a significantly ($p<0.05$) low level when compared with groups I, III & IV. Group III & IV (8.22 ± 0.09, 8.50 ± 0.16 respectively) showed statistically similar concentrations of GSH, which was statistically lower than group I (9.56 ± 0.11) (values are depicted in graph 5).

Super oxide dismutase (SOD)

SOD activity was decreased significantly ($p<0.05$) in group II (7.38 ± 0.06) when compared to other groups in the study. Group III & IV (11.16 ± 0.08, 11.41 ±0.13 respectively) had similar values without a statistically significant difference but were significantly ($p<0.05$) lesser than group I (12.24 ± 0.06) (values are depicted in graph 6).

Thiobarbituric acid reactive substances (TBARS)

TBARS (n moles of MDA/mg protein) in group II (54.30 ± 5.13) was increased significantly ($p<0.05$) compared to other groups. Groups, I, III & IV (35.30 ± 3.07, 36.10 ± 2.02, 36.03 ± 2.09 respectively) had similar values but were significantly ($p<0.05$) lower than group II (values are depicted in graph 7).

Glutathione peroxidase (GPx)

GPx (U/mg protein) activity in group II (18.28 ± 0.09) was significantly ($p<0.05$) lower than other counterparts in the study. Group III & IV (21.32 ± 0.07, 21.61 ± 0.14 respectively) had statistically similar values of GPx but lower than group I (22.30 ± 0.10), such a difference between group I & III was statistically significant ($p<0.05$) (values are depicted in graph 8).

Glutathione S transferase (GST)

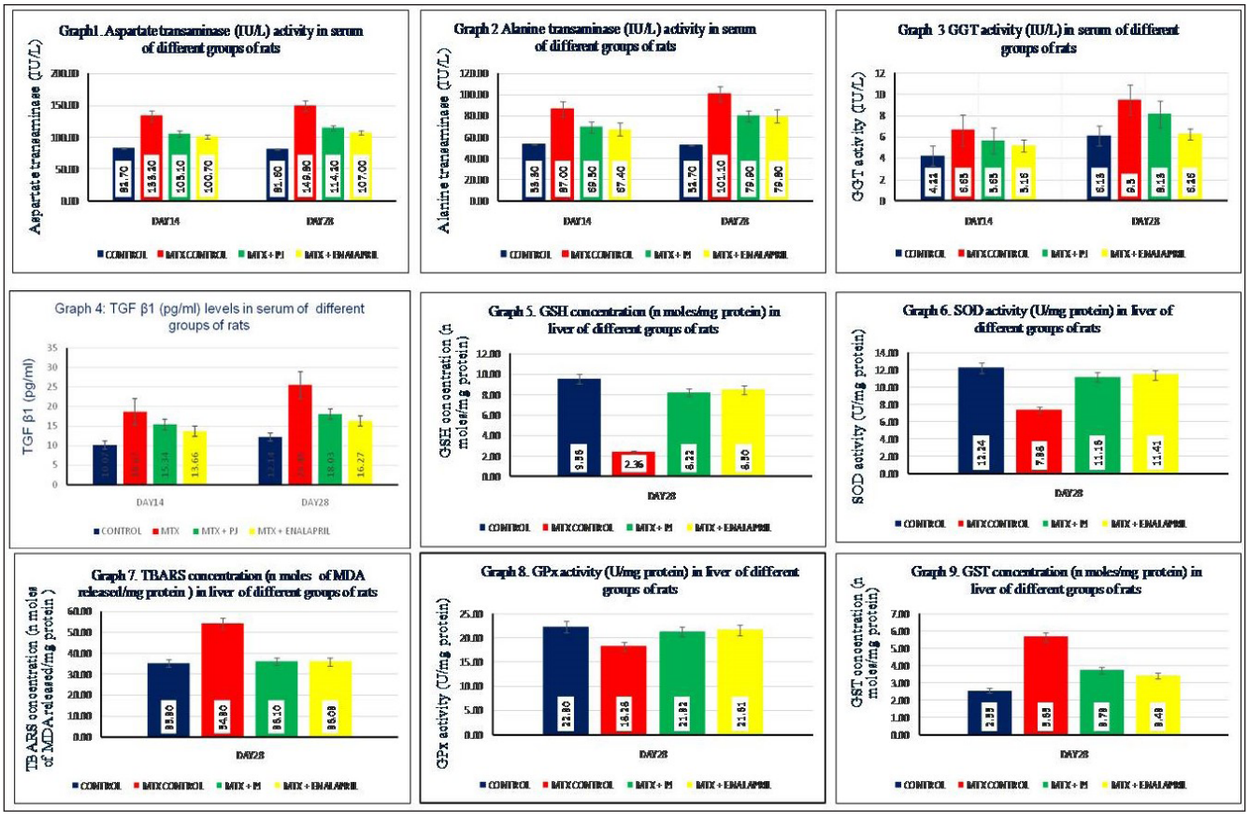
GST (n moles/mg protein) activity in group II (2.25 ± 0.13) was significantly ($p<0.05$) lower than other counterparts in the study. Group III & IV (3.43 ± 0.06, 3.93 ± 0.15 respectively) had statistically similar values of GST but lower than group I (4.55 ± 0.13), such a difference between group I & III was statistically significant ($p<0.05$) (values are depicted in graph 9).

Histopathology of liver

Histopathological examination of the liver in toxic control (group II) revealed moderate degeneration with necrosis, centrilobular and periportal fibrosis (Fig. 2) whereas group III (Fig. 3) and IV (Fig. 4) showed mild periportal fibrosis, degenerative changes with some normal architecture of hepatocytes compared to group II. The sections of group I (Fig.1) did not reveal any significant pathological lesions and the architecture was normal.

DISCUSSION

Methotrexate is being used for the treatment of various cancer and limits its therapeutic use by causing



Graph 1 to 3 represented liver biomarkers (AST, ALT and GGT (IU/L respectively), **Graph 4** TGF –β (pg/g) and **Graph 5 to 9** represents the antioxidant profile (GSH, SOD, TBARS, GPx and GST respectively) of liver tissue homogenates in different groups of rats

hepatotoxicity, including steatosis, cholestasis, fibrosis, and cirrhosis. The mechanism of MTX-induced hepatotoxicity is excessive accumulation inside the cell in the form of polyglutamated. After administration of MTX reaches the liver where it gets metabolized through the oxidation process from 7- hydroxymethotrexate and within the hepatocyte as polyglutamate form. Excessive accumulation of polyglutamate form and diminishing folate levels within in hepatocyte after long-term drug administration which caused hepatocyte damage. MTX treatment causes oxidative stress by the formation of excessive free radical generation and affects lipids. Proteins and DNA to form lipid peroxidation, protein carbonylation, and alkylation of DNA (Mani *et al*., 2021).

In the present study, the ameliorating and protective effect of pomegranate juice on MTX-induced hepatotoxicity was evaluated by estimation of AST, ALT and GGT levels in the serum. MTX a known hepatotoxicant has increased the levels of AST, ALT and GGT which are very much in tune with its hepatocellular and bile duct necrosis. It was observed that concurrent treatment of pomegranate juice with MTX, improved the levels of transaminases when compared to those of the MTX group. However, the enzyme levels did not reach the normal levels as that of the control group at any interval of observation. Supplementation with pomegranate juice in group III has decreased AST, ALT & GGT indicated by the recovery of three enzymes. Pomegranate juice with its phytochemical constituents flavonoids, alkaloids, terpenes, tannins, sugars, anthocyanins, phenolic compounds, ascorbic acid, proteins, polyphenols, ellagic acid, gallic acid, and tert gallic acid which has anti-oxidant properties might have protected the hepatic tissue from methotrexate-induced injury.

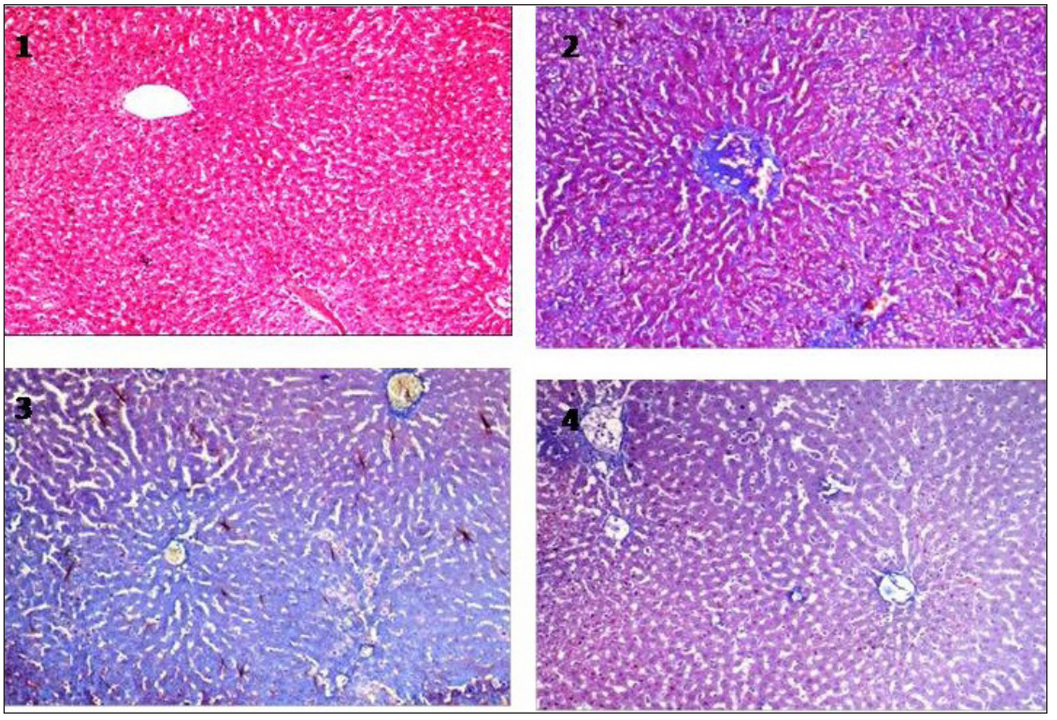


Fig. 1: Group I liver showing normal architecture. Masson's Trichrome 100X. **(Fig. 2)** Group II liver showing fibrosis around central vein, necrosis, fatty changes and sinusoidal haemorrhages. Masson's Trichrome 100X. **(Fig. 3)** Group III liver showing mild perivascular fibrosis, altered hepatic cords and shrunken hepatocytes. Masson's Trichrome 100X. **(Fig. 4)** Group IV liver showing mild proliferation of fibrous tissue, hepatocytes and hepatic cords appears to be normal. Focal area reconstructed. Mild haemorrhages are noticed. Masson's Trichrome 100X

The results are in accordance with previous authors (Celik *et al*., 2009) who administered pomegranate flowers extract against trichloroacetic acid exposure in rats, mirtazapine (Ozogul *et al*., 2013) and quercetin against MTX-induced haemotoxicity (Aparna *et al*., 2021). Further, administered pomegranate peel & seeds extracts on the liver fibrosis model by using carbon tetrachloride in rats (Wei *et al*., 2015). Protective effect of milk thistle on methotrexate-induced hepatic fibrosis in rats (Ghaffari *et al*., 2011).

MTX after converting to its metabolite 7-hydroxy methotrexate inhibits oxygen uptake in cells and reduces trans-plasma membrane redox activity including an increase in glycogenolysis. The net result would be a decrease in energy which can impact mitochondria in generating ROS. Apart from the above, reduction of NADPH dehydrogenase and Glucose-6-phosphate dehydrogenase reduces NADPH synthesis which causes the prevention of glutathione reductase activity and glutathione activity (Anilkumar *et al*., 2010; Tummala Srinivas *et al*., 2021). This fall in anti-oxidant defence has been observed in our study in the form of a decrease in SOD, GSH, GPx, GST and an increase in TBARS. This is in agreement with the previous studies (Saeed *et al*., 2018) on feeding with pomegranate juice. Pomegranate juice is a good source of polyphenol to the tune of 3 times as that of red wine and green tea. Polyphenol includes flavonoids & tannins, both condensed and hydrolysable, these are responsible for 90% of the antioxidant activity of polyphenols as reported by (Shukla *et al*., 2008). Polyphenolic compounds like punicalagins, punicalins, anthocyanins, gallagic acid, and ellagic acid are responsible for in vivo anti-oxidant effects as investigated by (Johanningsmeier *et al*., 2011). Pomegranate fruit extract is rich in flavonoid content which possesses an anti-oxidant character as evidenced by decreased MDA and increased GSH, SOD, GPx, and GST. Anthocyanins such as delphinidin, cyanidin, and

pelargonidin might have contributed to the anti-oxidant activity.

The expression of a biomarker of fibrogenesis namely, transforming growth factor (TGF)- β1 peptide, was studied in serum biochemistry. In the present study, the TGF-β1 level in the serum was significantly increased in groups treated with MTX when compared to the control group. The increased level of TGF-β1 could be attributed to liver injury and released TGF-β1 may cause necrotic hepatocytes, activated hepatic stellate cells and macrophages (Wu *et al*., 2018). Enalapril treated group IV showed decreased TGF-β1 levels attributed to the protection of hepatocytes against oxidative stress and suppression of HSCs activation by angiotensin II type 1 receptor expressed on the surface of hepatic stellate cells (HSCs), (Ahmad and Ahmad 2012). *In vitro* and *in vivo* studies on hepatic fibrosis suggest that the angiotensin II type 1 receptor antagonists suppress proliferation, collagen synthesis and expression of pro-fibrogenic cytokines (TGF-β1 and CTGF) in activated HSCs (Friedman, 2004). Two mechanisms of action have been suggested: first, the angiotensin II type 1 receptor antagonist inhibits activated HSCs by blocking angiotensin II type 1 receptor expressed on the surface of HSCs; second, it suppresses the activation of HSCs as a result of the decrease in TGF-β1 (Ahmad and Ahmad, 2012). Similarly, pomegranate juice treated group III also revealed a significant decrease in TGF-β1 levels, which might be due to improved anti-oxidant defences (increase SOD, GST, GSH and GPx) and decreased MDA levels, thus protecting hepatocytes from oxidative damage and subsequent prevention of HSC activation, which ultimately resulted in the decreased levels of TGF-β1. Similar protective effects with pomegranate were found with carbon tetrachloride (Pan *et al*. 2018) and in ethanol-related fibrosis (Zhuge *et al*., 2006) and oxymatrine protected thioacetamide-induced hepatic fibrosis (Wu, Pan *et al*. 2018).

However, pomegranate juice when compared with standard antifibrotic drug enalapril was falling short in preventing hepatic fibrosis. Based upon the above findings and histopathological examination it can be concluded that pomegranate juice with poly probiotics can arrest hepatic fibrosis generated by the use of MTX. Pomegranate juice can be used as a beverage supplement during MTX therapy to counter the adverse effect of the drug. Further studies on the pharmacokinetics of MTX, when co-administered with probiotics, need to be carried out.

CONCLUSION

In conclusion, the results of the current study suggested that Pomegranate juice extract has exerted potent hepatoprotective activity based on biochemical, antioxidants and histopathological parameters. Pomegranate juice extract is effectively scavenging free radicals generated during the metabolism of Methotrexate and preventing binding of their toxic reactive metabolites with intracellular macromolecules, proteins, lipids and nucleic acids, which were manifested in this study by reducing the liver toxic markers, restoration of antioxidant enzymes, decreased TGF-β1 protein expression and histopathology. The overall beneficial effects of pomegranate juice extract could be endorsed to their antioxidant potential as evident from oxidant-antioxidant markers in this study coupled with the hepatoprotective properties.

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