

AMELIORATION EFFECT OF POMEGRANATE JUICE AGAINST METHOTREXATE INDUCED HEPATIC FIBROSIS IN RATS

By

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B.V.Sc. & A.H

RVM/2016- 58

MASTER OF VETERINARY SCIENCE



**DEPARTMENT OF VETERINARY PHARMACOLOGY & TOXICOLOGY
COLLEGE OF VETERINARY SCIENCE**

RAJENDRANAGAR, HYDERABAD-500 030.

JUNE, 2019

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**THESIS SUBMITTED TO THE
P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

MASTER OF VETERINARY SCIENCE



**DEPARTMENT OF VETERINARY PHARMACOLOGY & TOXICOLOGY
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RAJENDRANAGAR, HYDERABAD-500 030.

JUNE, 2019

CERTIFICATE

Mr. S.PAVAN KALYAN has satisfactorily prosecuted the course of research and that the thesis entitled "**AMELIORATION EFFECT OF POMEGRANATE JUICE AGAINST METHOTREXATE INDUCED HEPATIC FIBROSIS IN RATS**" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

Date: 25-06-2019

Place: Hyderabad



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Dr. B. KALA KUMAR
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CERTIFICATE

This is to certify that the thesis entitled "AMELIORATION EFFECT OF POMEGRANATE JUICE AGAINST METHOTREXATE INDUCED HEPATIC FIBROSIS IN RATS" submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** of **P.V. Narsimha Rao Telangana Veterinary University**, is a record of the bonafide research work carried out by **Mr. S. PAVAN KALYAN** under my guidance and supervision. The student's Advisory Committee has approved the subject of the thesis.

No part of the thesis has been submitted for any other degree or diploma. The author of the thesis has duly acknowledged all the assistance and help received during the course of investigation.


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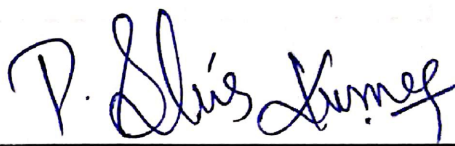
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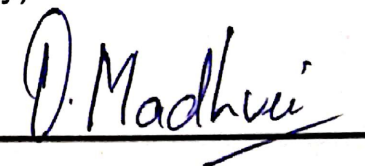
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DECLARATION

I, Mr. S.PAVAN KALYAN, hereby declare that the thesis entitled **entitled "AMELIORATION EFFECT OF POMEGRANATE JUICE AGAINST METHOTREXATE INDUCED HEPATIC FIBROSIS IN RATS"** submitted to P.V. NARSIMHARAO TELANGANA VETERINARY UNIVERSITY for the Degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date: 25-06-2019

Place: Hyderabad


(SHIVARCHAKAM PAVAN KALYAN)

ACKNOWLEDGEMENTS

*“gururbrahmaa ururvishnu gururdevoo maheswarah
gurussaakshaat parabrahmaa tasmai sri gurave namah”*

*I express my deep-rooted indebtedness and reverence towards **Dr. B. Kala Kumar**, Professor, Head, Dept of Pharmacology and Toxicology, College of Veterinary Science, Hyderabad, for his meticulous guidance and patience. I am grateful to him for his constant fomenting punctilious and impeccable advices all the time. Above all, his affectionate of dealing with the things throughout the course of my study has helped me to consummate the research work with a grand success. His scientific acumen, critical judgments and trust in my abilities has guided and motivated me throughout the course of this investigation and compilation of manuscript. He is continuous source of inspiration. I considered myself truly fortunate to work under his expertise guidance. I sincerely thank him from the inner core of my heart. His mentorship was paramount in providing a well-rounded experience in consistent to my long-term career goals.*

*With respectful regards and immense pleasure, I express my profound sense of gratitude and abysmal thanks to **Dr. P. Shiva Kumar**, Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Hyderabad. His cooperation, critical judgment and trust has guided and motivated me throughout the course of this investigation. I sincerely thank him from the inner core of my heart.*

*I feel inadequacy of words to express my deep sense of gratitude to **Dr. D. Madhuri**, Professor, University Head, Veterinary Pathology, College of Veterinary Science, Korutla, for her help and kind cooperation during my course of work. And also for her valuable suggestions and timely help during the research and presentation of this thesis.*

*I express my heartfelt gratitude to **Dr. A. Gopala Reddy**, Professor and University Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Hyderabad, for his consistent and invaluable inspiration, prolific and introspective guidance with constructive criticism, encouragement, and active persuasion throughout the course of my study. His dedication to research,*

meticulous planning, scholarly guidance and unreserved help served as beacon light throughout the course study and research work.

*I extend my honorific thanks to **Dr. B. Anil Kumar**, Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Hyderabad and consider fortunate enough to have his advice, constructive suggestions, untiring help, inspiring guidance and generous willingness to render all possible help and guidance throughout the M.V.Sc. programme.*

*I wish to express my indebtedness and gratitude to **Dr. M. Usha Rani**, Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Hyderabad for her encouragement, guidance, valuable and constructive suggestions during every phase of my study.*

*I am extremely grateful to **Dr. M. Lakshman**, Professor, Department of Veterinary Pathology, College of Veterinary Science, Hyderabad and **Dr. Y. Ravi kumar** Assistant Professor, Department of Veterinary Pathology, College of Veterinary Science, Hyderabad for their timely help in carrying out the research work and his precious guidance in successful completion of my research.*

*I would like to place on record my appreciation and love towards my colleagues, seniors and juniors **Drs. Anudeep, Dhanya, Dipali, Sushma, Swapna, Vanitha, Manvitha, Priyanka, Saipratheek, Vikram, Ramesh, Ashok**, for their endless support in successful completion of my research work.*

*I am very much thankful to my friends **Pavan, Srujan, Sammaiah, Ravi sudhakar, Hala** for giving cheerful environment filled with constant support, help and affection.*

*I am very much thankful to **Dr. L. Atchamamba and library staff** for their help during my PG program.*

*I would like to extend my thanks to **Smt. Rama and Sri. Balanarsimha**, Non-teaching staff, Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science, Rajendranagar, Hyderabad, for their help. I acknowledge help, guidance, teachings offered by All Teachers of Veterinary College, Hyderabad.*

I extend my heartfelt thanks to **The College of Veterinary Science, Rajendranagar, PV Narsimha Rao Telangana Veterinary University** for providing the facilities to fulfill my research work.

Words are not enough to express gratitude and regards from heart to my beloved parents **Mr. S. Veeraiah** and **Mrs. S. Padma**, who inspired me to make things always better and for their inbounding love, unflinching support and unconditional blessings. It is only due to their affectionate encouragement, this manuscript has taken its form. I am extremely grateful to my brothers **Shivaprasad sharma, Veda vyas** and sister **Chaithanya** for their incomparable love, support and affection which enabled me to reach up to this step.


(Shivarchakam Pavan Kalyan)

Name of the Author : **SHIVARCHAKAM PAVAN KALYAN**
Title of the Thesis : **AMELIORATION EFFECT OF POMEGRANATE JUICE AGAINST METHOTREXATE INDUCED HEPATIC FIBROSIS IN RATS**
Degree to which : **MASTER OF VETERINARY SCIENCE**
It is submitted
Faculty : **VETERINARY SCIENCE**
Discipline : **VETERINARY PHARMACOLOGY**
Major Advisor : **Dr. B. KALA KUMAR**
University : **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY**
Year of submission : **2019**

ABSTRACT

Methotrexate (MTX) is folic acid analogue widely used in treatment of various cancers. One of the important adverse effects of MTX is hepatotoxicity that followed by hepatic fibrosis. Hence, present study was conducted to assess hepatic fibrosis and compare efficacy of pomegranate juice with enalapril as a standard anti-fibrotic agent. A total 24 rats divided in to 4 groups group I and group II served as sham and MTX control where as group III and group IV were kept as MTX+ pomegranate and MTX+ enalapril and study was conducted for a period of 28 days.

Serum samples were collected at 14th and 28th day for estimation of hepatic biomarkers (AST, ALT & GGT), Lipid profile (total cholesterol, HDL, LDL and triglycerides), protein profile (albumin & globulin) and profibrogenic cytokine TGF- β 1. Blood samples were drawn on 14th and 28th day for hematology (TEC, TLC, Hb & PCV). At the end of the experiment rats were sacrificed and liver samples were collected for estimation of anti-oxidant profile (GSH, SOD, GST, GPx and TBARS)

and histopathological examination with Masson's Trichrome stain. The results of present study revealed that the levels of AST, ALT, GGT in group II was significantly increased when compared to control group whereas treatment groups (groups III and IV) showed significant decrease at different time intervals. The concentrations of total cholesterol, LDL, triglycerides, TGF- β 1 were significantly increased whereas concentration of HDL, albumin, globulin, TC, TLC, Hb & PCV decreased in group II compared to control groups at different time intervals. Oxidant markers TBARS were significantly increased and concentrations of GSH, GST, SOD & GPx significantly decreased in MTX control group, whereas pomegranate juice treated group showed significant improvement in all the parameters as similar to the enalapril treated groups. Pomegranate juice contains poly phenolics and exerted anti-oxidant action due to presence of anthocyanins, gallic acid, ellagic acid, caffeic acid, quercetin, catechins and rutin and counter the results obtained with MTX. Hence, it can be concluded in the light of the study that pomegranate juice can serve as ameliorating agent in MTX induced hepatic fibrosis.

INTRODUCTION

CHAPTER I

INTRODUCTION

Fibrosis is an excessive wound healing response that occurs in most forms of chronic liver disease and results in the deposition of scar tissue, i.e., excess extracellular matrix (ECM). With ongoing liver damage, fibrosis may progress to cirrhosis, which is characterized by a distortion of the liver vasculature and architecture, and which is the major determinant of morbidity and mortality in patients with liver disease, predisposing to liver failure and primary liver cancer (Popov and Schuppan, 2009).

Methotrexate (MTX) is a folate antagonist 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 treatment of different types of cancers, psoriasis, autoimmune disorders and medical termination of pregnancy (Ali *et al.*, 2014). The MTX causes renal toxicity, intestinal mucositis, testicular toxicity, several studies have also documented that hepatotoxicity is major adverse effect of the MTX (Horie *et al.*, 1999; Asvadi *et al.*, 2011; Abo-Haded *et al.*, 2017). The exact mechanism of MTX induced hepatotoxicity is still elusive but several studies suggested that oxidative stress could be responsible for underlying cause of hepatotoxicity (Ali *et al.*, 2014). Free radicals formed as a result of oxidation, attack the unsaturated fatty acids of bio membrane, which results in lipid peroxidation and destruction of protein and DNA, leading to various liver injuries. Chronic usage of MTX has been

shown to cause fatty liver, fibrosis, mild steatosis and cirrhosis in patients receiving this drug and cause choline deficiency in the patients (Vonen and Morland, 1984). 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.

Amelioration of MTX induced toxicity has been the prime concern during therapeutic intervention with MTX. Both folate and folinic acid (leucovarin) have shown to reduce MTX toxicity. However, folic acid supplement could reduce MTX efficiency in the treatment of cancer.

Since MTX induces toxicity through systemic oxidative stress, a search for drugs or agents with antioxidant property has been extensively carried out (Ozogula *et al.*, 2013), most of which proved partially helpful in preventing MTX toxicity and some associated with additional adverse effects. Therefore, there is a need to search for alternative drugs for the treatment of MTX induced hepatotoxicity in order to replace the currently used drugs. Numerous medicinal plants and their formulations are being used for liver disorders in ethno-medicinal practice and in traditional system of medicine in India.

Pomegranate contains large amount of polyphenolic constituents - anthocyanins, hydrolysable tannins – ellagitannins & gallotannins and condensed tannins - proanthocyanidin have been found and reported in diverse parts of the fruit. Pomegranate had exhibited evidences regarding the reduction of oxidative stress mediators thorough its anti-oxidant ability contributed by its phenolic compounds for its free radical scavenging properties (Husain *et al.*, 2018). Hence, the present study was designed to evaluate the efficacy of pomegranate juice was taken up with the following objectives.

1. To assess the toxic potential of methotrexate on liver in rats.
2. To evaluate the ameliorative effect of pomegranate juice against methotrexate induced hepatic fibrosis in rats.
3. To compare the efficacy and potency of pomegranate juice with a standard anti-fibrotic agent enalapril.

REVIEW OF LITERATURE

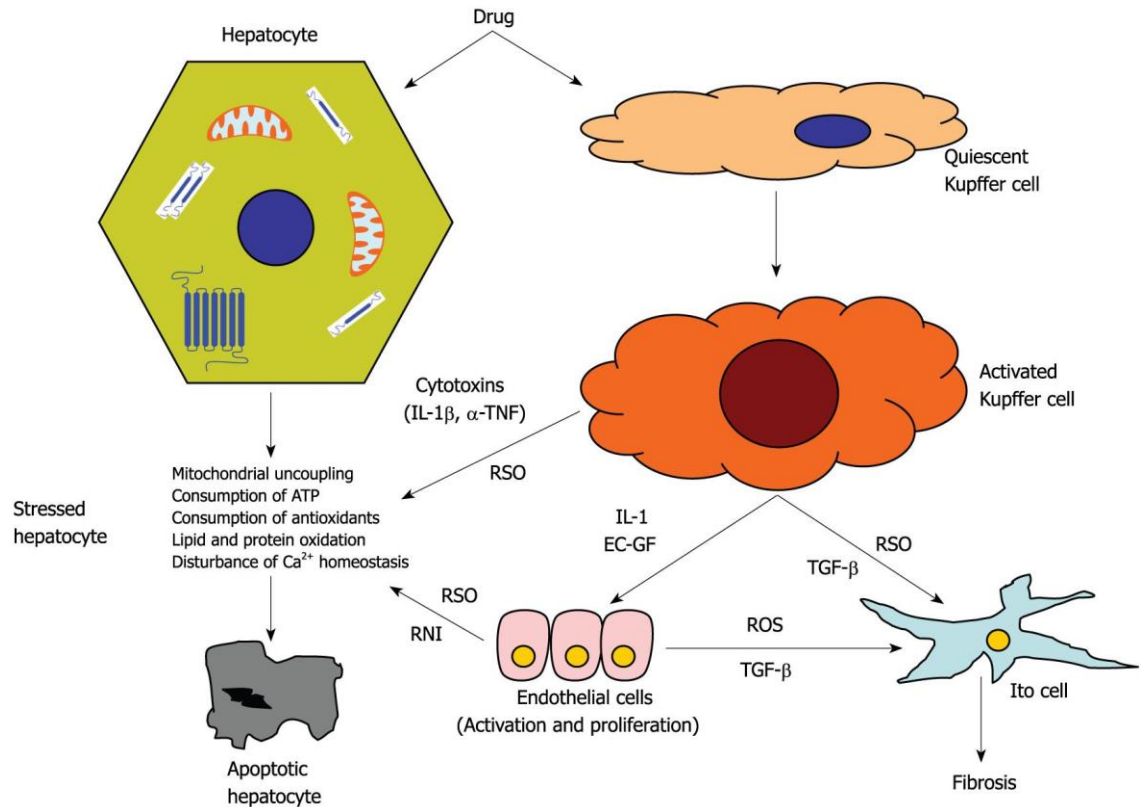
Hepatic fibrosis is the main complication of chronic liver disease, it is usually initiated by hepatocyte damage, leading to recruitment of inflammatory cells and platelets, activation of kupffer cells and subsequent release of cytokines and growth factors (Kullak and Meier, 2000). As a result of fibrosis the normal liver structure is distorted by scar tissue which may lead to overt cirrhosis. The chronic liver injury may result from a large number of etiologies including alcohol consumption, hepatotropic viral infections, metabolic disorders, drug effects and chronic bile duct obstruction.

2.1 Methotrexate (MTX)

MTX is a chemotherapeutic agent widely used in the treatment of several types of cancers and various inflammatory diseases such as rheumatoid arthritis and psoriasis (Bayram *et al.*, 2005; Sener *et al.*, 2006).

Methotrexate (4-amino-10 methyl folic acid) is an antimetabolite and an analogue of folic acid. The drug enters the cells via an active transport system for reduced folates and due to a relatively irreversible binding; it inhibits the enzyme dihydrofolate reductase which catalyses the reductive process of folic acid into tetrahydrofolic acid. The inhibited formation of tetrahydrofolates results in an interference with DNA synthesis, repair and cell replication. The affinity of dihydrofolate reductase for methotrexate is far greater than its affinity for folic or dihydrofolic acid and therefore, even very large amounts of folic acid given simultaneously will not reverse the effects of methotrexate (Goodshell, 1999). The drug also seems to cause an increase in intracellular deoxyadenosine

triphosphate, which is thought to inhibit ribonucleotide reduction and polynucleotide ligase, an enzyme concerned in DNA synthesis and repair (Ortiz *et al.*, 1998).



(Grattagliano *et al.*, 2009)

Li and Kaminskas (1984) reported that methotrexate binds to dihydrofolate reductase (DHFR) and completely inhibits the activity of this enzyme leading to methotrexate (MTX) induced apoptosis in liver, spleen and thymus gland. It has been suggested that the continuous inhibition of DHFR activity might cause an imbalance in the deoxyribonucleotide triphosphate (dNTP) pool due to the storage of thymidylate and purine nucleotides and as a consequence leads to DNA lesions.

As per the report of Merkle *et al.* (2000) and Kobayashi *et al.* (2002), methotrexate induces apoptosis in cultured bovine pulmonary artery endothelial cells, intestinal epithelial cells and hepatocytes of rat.

Kobayashi *et al.* (2002) suggested that the inhibition of N-terminal kinase activator protein pathway and concurrent up-regulation of p53 and p21 were involved in hepatocyte apoptosis.

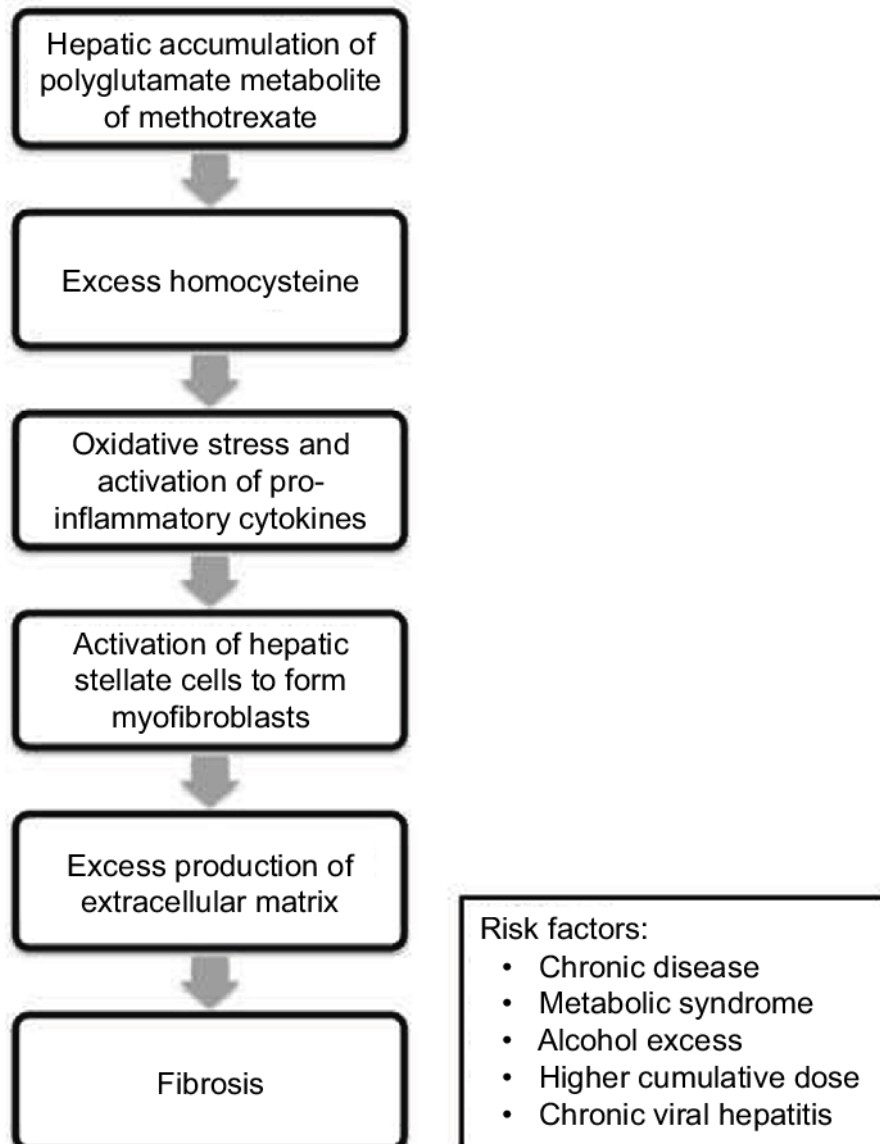
The study of Jahovic *et al.* (2003) and Cetinkaya *et al.* (2006) showed that the association of the efficacy of MTX in high doses causes hepatotoxicity. The mechanism of MTX toxicity suggested due to decrease in the levels of cellular glutathione (GSH) and increased in oxygen radicals. (Jahovic *et al.*, 2003; Cetinkaya *et al.*, 2006; Sener *et al.*, 2006 and Cetin *et al.*, 2008).

Celtikci *et al.* (2009) demonstrated that cytosolic nicotinamide adenosine diphosphate (NADP) dependent dehydrogenases were inhibited by MTX, and reported that the MTX decreases the availability of NADPH in cells. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of GSH, an important cytosolic antioxidant that protects cells against reactive oxygen species (ROS). Thus, the significant reduction in GSH levels induced by MTX therapy leads to suppression of the antioxidant enzyme defense system, sensitizing the cells to ROS. The antifolate methotrexate is a widely used chemotherapeutic drug which inhibits DNA synthesis and induces apoptosis.

Aydin (2011) studied the effect of quercetin on MTX induced hepatotoxicity and reported that administration of MTX at 10 mg/kg/day, i.m. for consecutive 3 days impaired antioxidant mechanisms and caused substantial increases in the levels of liver injury markers in serum.

Ghaffari *et al.* (2011) concluded that milk thistle can effectively prevent methotrexate induced liver dysfunction and fibrosis in rats by using serum biochemistry and semi-quantitative scoring system.

2.1.1 Methotrexate Toxicity



(Cheng *et al.*, 2018)

2.1.1.1 Body weight

Kumari *et al.* (2016) reported that there was decrease in body weights in Methotrexate group when compared to Amlycure treated group.

2.1.1.2 Effect on Haematology

Patel *et al.* (2014) studied the sub-acute toxicopathological effects of methotrexate in *Wistar* rats and the haematological study revealed that there was significant reduction ($p < 0.05$) in RBC, Hb, PCV, WBC, neutrophil, lymphocytes of rats that received MTX at 0.25 mg/kg by oral route for 28 days.

A significant increase ($p < 0.05$) was also observed in MCV, MCH of these rats as compared to control rats.

2.1.1.3 Effect on oxidative stress

Jahovic *et al.* (2003) demonstrated in a rat model that MTX administration resulted in increased levels of malondialdehyde and increased TBARS (MDA) levels which could be due to lipid peroxidation induced by MTX itself or as a result of a possible increase in ROS induced by MTX. TBARS is the breakdown product of the most important chain reactions which leads to the oxidation of polyunsaturated fatty acids and believed to serve as a reliable marker of oxidative stress-mediated lipid peroxidation. MTX causes increased malondialdehyde (MDA) and lipidperoxidation (LPO) activities and collagen content and reduced the GSH levels in the ileum, liver and kidney (Ozguner *et al.*, 2005). They observed a significant reduction in GSH levels and also reduction in effectiveness of the antioxidant enzyme defence system, sensitizing the cells to ROS.

Notable reduction in the levels of superoxide dismutase (SOD), catalase (CAT) in liver was observed by Hemeida and Mohafez (2008), following injection of single dose of MTX (20mg/kg, I/P) in rats. The liver SOD and CAT activities were decreased after MTX treatment by about 50 and 52 per cent respectively compared to the normal control group.

Tunali *et al.* (2010) investigated that possible protective effect of resveratrol on some liver and blood parameters in MTX induced toxicity in rats and observed significantly higher ($p < 0.001$) levels of MDA in liver tissue in rats administered with a single intraperitoneal injection of MTX at 20mg/kg when compared with those of control group.

Coleshowers *et al.* (2010) investigated the status of antioxidant enzymes during oxidative stress in liver homogenates of rats subjected to oral methotrexate administration at a dose of 13.4 mg/kg at weekly intervals for 6 consecutive weeks and revealed that thiobarbituric acid reactive substance (TBARS) levels and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were subsequently determined spectrophotometrically on liver homogenates of all animals. They also found that methotrexate caused a significant increase in TBARS in the methotrexate administered groups when compared to the control group and the levels of superoxide dismutase, catalase, and glutathione reductase were significantly decreased in the methotrexate administered rats when compared to the control group.

Vardi *et al.* (2013) documented that the role of oxidative stress on the hepatic damage caused by MTX administration in rats. They observed a significant decrease ($p < 0.05$) in the levels of SOD, CAT and GPx in liver tissue and a significant increase in the levels of MDA when compared to those of normal untreated rats.

Asvadi *et al.* (2011) studied the protective effect of pentoxifylline in renal toxicity after methotrexate administration and revealed that, significant reduction ($p < 0.05$) in the levels of SOD and glutathione peroxidase (GPx) activity in renal tissue of rats after MTX treatment at 20mg/kg intraperitoneally compared to control rats. Similar results were also obtained by Aydin (2011) in mice were treated with MTX at 5 mg/kg for three consecutive days.

Experimental study was conducted by Kose *et al.* (2012) to assess the beneficial effects of montelukast against methotrexate induced liver toxicity in rats and found that a single dose of MTX administered at the rate of 20 mg/kg

i.p, caused significant increase in the levels of malondialdehyde in liver when compared to those of control animals.

Ali *et al.* (2014) measured the anti-oxidant activities of catalase, superoxide dismutase and glutathione peroxidase following a single intraperitoneal injection of MTX at 20 mg/kg in rats and found a significant decrease ($p < 0.01$) in the levels of CAT, SOD and GPx activity when compared to normal control group.

Study of Wei *et al.* (2015) observed that, MDA levels were significantly higher in CCl₄ received group in rats than in the control group. In the Extract of pomegranate peel (EPP) and Extract of pomegranate seed (EPS) treated groups, hepatic MDA levels were markedly decreased compared with that of the CCl₄ group. SOD and GPx were lower in the CCl₄ group than in the control group. EPP and EPS treatment groups efficiently increased the SOD and GPx activity than in the CCl₄ group.

2.1.1.4 Effect on serum enzymes

Enzymes are complex protein substances found in the serum and tissues in varying concentrations. Alterations in serum enzyme levels may be due to increased enzyme formation as in cases of malignant tissue growth, increased escape into serum following necrosis of cells or decreased escape from the body as a result of obstruction of excretory pathways, like obstruction of the biliary system.

Elevation in serum ALT and AST values characterize the hepatic injury. Elevated levels of serum ALT and AST in rats, following injection of single dose of MTX (20 mg/kg I.P) was reported by Hemeida and Mohafez (2008) who observed that administration of MTX increased the serum activities of ALT and

AST by 51 per cent and 45 per cent respectively compared to normal control group. Similar reports of rise in various serum enzyme levels following MTX administration in rats were reported by Bajin-Katic *et al.* (2004); Perianayagasamy *et al.* (2010); Vardi *et al.* (2010) and Mashhadi *et al.* (2011).

Tunali *et al.* (2010) investigated the possible protective effect of resveratrol on some liver and blood parameters in MTX induced toxicity in rats and they observed a significantly higher ($p < 0.001$) serum AST and ALT levels in rats administered a single intraperitoneal injection of MTX at 20 mg/kg when compared to control group.

The study of Vardi *et al.* (2010) on role of oxidative stress in connection with hepatic damage caused by MTX administration in rats and observed that significant increase ($p < 0.05$) in the levels of AST and ALT enzymes in serum when compared to those of normal untreated rats.

Ozogula *et al.* (2013) studied the effect of mirtazapine on methotrexate induced toxicity in rat liver and reported a significant increase ($p < 0.01$) in the levels of AST and ALT, compared to normal control, following a single intraperitoneal injection of MTX at 5 mg/kg in rats.

Ali *et al.* (2014) recorded a significant increase ($p < 0.001$) in the levels of hepatic toxicity serum markers like ALT and AST, following a single intraperitoneal injection of MTX at 20 mg/kg in rats when compared to normal control group.

A study of El-Azim (2014) on antioxidant effect of olive leaf extract with MTX induced hepatic injury in rats observed a significant rise in the serum AST and ALT values following a single dose of MTX at 20 mg/kg when compared to those of normal rats.

Patel *et al.* (2014) studied on subacute toxicopathological effects of MTX and reported significantly higher levels of AST, ALT, ALP, creatinine and BUN in rats administered with MTX at different dose levels.

Alsaadi *et al.* (2013) reported that increase in serum levels of GOT, GPT, ALP, total cholesterol and triglyceride, however HDL showed a significant decrease in mice treated with methotrexate when compared to control group.

Uraz *et al.* (2008) studied the role of Ursodeoxycholic acid in prevention of methotrexate induced liver toxicity and reported rise in serum ALT, ALP, GGT and tissue ROM levels were higher and GSH levels was lower in methotrexate group when compared to other groups.

Wu *et al.* (2018) concluded that oxymatrine was effective in protecting rats from thioacetamide induced hepatic fibrosis by regulating TGF- β 1 expression.

Niu *et al.* (2016) reported that TGF- β 1/Smad3 signalling was an important target for acute liver injury by carbon tetrachloride.

Wei *et al.* (2015) observed significant higher levels of ALT, AST, and total bilirubin in the serum of the rats in the CCl₄ group compared to control group. Extract of pomegranate peel (EPP) and extract of pomegranate seed (EPS) treatment efficiently reduced ALT and AST levels. The levels of TGF- β 1 in CCl₄ group were elevated significantly compared to that of control group. EPP and EPS significantly decreased the TGF- β 1 level in serum compared to that of the CCl₄ groups.

Alsaadi *et al.* (2013) reported that significant increased in serum levels of total cholesterol and triglyceride but HDL levels were significantly decreased in mice treated with MTX in comparison to control group.

Kumari *et al.* (2016) studied in rats that Methotrexate hepatotoxicity was characterized by marked elevation in circulating level of AST, ALT, cholesterol, LDL and decreased level of albumin, total protein and HDL. Amlycure DS significantly decreased the AST, ALT, LDL and increased the level of albumin, total protein and HDL as compared to methotrexate treated group.

2.1.1.5 Methotrexate induced Pathology

Baskerville and Cox (1978) studied the histopathological effects of MTX on mouse liver and reported ballooning degeneration, vacuolation of centrilobular hepatocytes, necrosis of a few hepatocytes, hyperchromicity and irregularity in nuclear size with significantly enlarged nucleus compared to those of control animals.

Hemeida and Mohafez (2008) induced hepatotoxicity in rats by injection of a single dose of MTX (20 mg/kg, I.P) and observed changes that were well characterized histopathologically. They observed an increase in the liver/body weight ratio and the microscopic changes included marked pleomorphism and presence of apoptotic bodies, which appeared as acidophilic bodies. Additionally, they were also observed cholestasis, steatosis, fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation, lobular disarray as well as mild dilatation of centrilobular sinusoids.

Chelab and Majeed (2009) studied the histopathological changes in the kidneys of mice treated with different doses of MTX and observed lesions like infiltration of lymphocytes and congestion of glomeruli, dilated tubules in the cortical areas and degeneration of cortical tubules.

Damage to liver parenchyma in rats was observed by Tunali *et al.* (2010) following a single intraperitoneal injection of MTX at 20 mg/kg which included

degeneration of hepatocytes, dilatation and vascular congestion in sinusoids, increased number of activated Kupffer cells and infiltration of inflammatory cells around portal area and central vein.

Vardi *et al.* (2010) observed presence of foci of apoptotic cells, shrinkage and inflection of nuclear contour along with chromatin condensation in MTX treated rats following a single injection of MTX at the rate of 20 mg/kg.

El-Boghdady (2011) observed severe intestinal damage caused by MTX administration with sloughing of lining mucosa, thickening of the mucosal folds with inflammatory and necrotic cells, increase in mucosal goblet cell activity and oedema of submucosal and mucosal layers

In a study on the beneficial effects of montelukast against methotrexate induced liver toxicity in rats, Kose *et al.* (2012) observed that a single dose of MTX (20 mg/kg i.p.) caused eosinophilic staining and swelling of hepatocytes and degenerative changes.

Ozogula *et al.* (2013) observed apoptotic bodies with condensed cytoplasm, peripheral, and pyknotic nuclei in the liver cells, focal necrosis and intense inflammation in the interstitial areas, following a single intraperitoneal injection of MTX at 5 mg/kg in rats.

Study of Acipayam *et al.* (2014) on MTX induced intestinal epithelial damage in rats, found that the rats treated with MTX showed greater villus atrophy, necrosis, decreased number of epithelial cells and an atrophic appearance of basal crypts with pronounced cellular infiltration.

Ali *et al.* (2014) recorded the toxic effects of MTX on liver in rats following administration of a single intraperitoneal injection at 20 mg/kg and observed abnormal and distorted hepatocytes with presence of fatty infiltration.

Patel *et al.* (2014) studied the subacute toxicopathological studies of methotrexate in *Wistar* rats to assess its effects on haematology, serum biochemistry and histopathology. They observed dose dependent pathological changes characterized by degeneration, necrosis, congestion and haemorrhages and found that liver, kidney, lungs and testes were the main target organs of MTX.

de Almeida *et al.* (2014) studied experimentally the MTX induced small intestinal mucositis in mice and recorded mild goblet cell depletion and villus atrophy, epithelial damage and apoptosis in small intestine.

El-Azim (2014) studied the microscopical changes in liver of rats after following MTX administration and found that in MTX treated group liver sections displayed numerous areas of inflammatory or necrotic cells along with lymphocytic infiltration. He also observed widening of sinusoidal spaces and presence of activated Kupffer cells, mild fatty changes in hepatocytes and sinusoidal lining cells, pyknosis of nuclei and hepatic apoptosis.

Taifoor (2014) studied histopathological effect of MTX in mice by administered single and multiple doses and found that single dose of MTX at 0.05 mg/kg did not produce any histopathological effects in liver while lesions like lymphocyte infiltration, necrosis, liver tissue fatty degeneration and congestion and dilatation of the hepatic vein were observed following six weeks of MTX administration.

2.1.2 Drugs used against methotrexate toxicity

The adverse effects caused by the continuous or intermittent use of MTX are well known. Though folic acid is being prescribed routinely along with MTX, it has failed to reduce the adverse effects caused by MTX. Many drugs are

continuously being tried to prevent/reduce the adverse effects of MTX (Al-Motabagani, 2006).

Kanauchi *et al.* (1998) studied the protective effects of dietary germinated barley foodstuff (GBF), in methotrexate induced enteritis model in rats that were injected intraperitoneally with MTX at the rate of 10 mg/kg body weight. They found that GBF effectively prevented diarrhoea and mucosal damages caused by MTX administration and concluded that GBF has a potential as therapeutic diet in decreasing the adverse effects of anticancer chemotherapy by MTX.

Horie *et al.* (1999) studied the protective effect of aged garlic extract (AGE) in MTX induced damage and observed that it effectively ameliorated the small intestinal crypt epithelial damage induced by MTX.

Li *et al.* (2005) studied the anti-apoptotic action of aged garlic extract (AGE) against methotrexate induced injury in epithelial cells (IEC-6), an *in-vitro* intestinal model and found that AGE inhibited fragmentation of DNA and significantly reduced activation of caspase-3 induced by MTX.

Hemeida and Mohafez, (2008) used curcumin a main component of the turmeric pigment of *Curcuma longa* to attenuate the MTX- induced hepatic oxidative damage in rats. They found that the administration of curcumin at 100 mg/kg, I.P once daily for five consecutive days after MTX challenge mitigated the injurious effects of MTX and ameliorated its effects on all the biochemical parameters.

Tunali *et al.* (2010) investigated the possible protective effects of resveratrol, a phytoalexin found in wide variety of dietary sources including grapes, plums, peanuts and red wine, on some liver and blood parameters in

MTX induced toxicity in rats. They also showed that resveratrol, 10 mg/kg orally for five days protected against MTX-induced hepatic injury and concluded that resveratrol has therapeutic potential in alleviating the systemic side effects of MTX.

Asvadi *et al.* (2011) studied the protective effect of pentoxifylline (PTX), an anti-inflammatory and anti-oxidant agent, in MTX induced renal toxicity and hypothesized that PTX may afford renal protection by down regulating TNF- α as well as by improving cellular anti-oxidant activity.

Aydin (2011) in his studies on the effect of quercetin (QE) on the microsomal drug metabolizing and antioxidant systems in methotrexate (MTX) treated mice, reported pre-treatment with quercetin at 50 mg/kg p.o for 10 days inhibited MTX induced liver injury and oxidative stress by decreasing microsomal lipid peroxidation, increasing cellular glutathione content and maintaining the levels of antioxidant enzymes close to control values.

El-Boghdady (2011) investigated the protective effects of ellagic acid (EA) and pumpkin seed oil (PSO) against MTX induced small intestinal damage in rats found that administration of EA and PSO decreased the intestinal injury and concluded that EA and PSO could be used as a promising drugs in the prevention of undesired side effects of MTX.

Ozogula *et al.* (2013) studied protective effect of mirtazapine, an antidepressant drug, against MTX induced oxidative stress in rats and showed that mirtazapine significantly prevented methotrexate induced oxidative stress and they have concluded that the desired dose of MTX can safely be used with mirtazapine in the treatment of cancer and non-cancerous diseases.

Acipayam *et al.* (2014) studied the protective effects of hesperidin (HES), a flavanone glycoside which found in citrus fruits, on MTX induced intestinal epithelial damage in rats and found that HES was effective in reducing the intensity of MTX induced small intestinal damage.

Effects of chrysin, a flavonoid present in bee propolis and honey, against MTX induced hepatotoxicity in rats was studied by Ali *et al.* (2014). They observed that pre-treatment with chrysin at 20 mg/kg and 40 mg/kg orally for 20 days prior to administration of a single intraperitoneal injection of MTX at 20 mg/kg prevented hepatotoxicity by ameliorating oxidative stress, histopathological alterations, apoptosis and improved the therapeutic index of MTX if co-administered.

de Almeida *et al.* (2014) studied the protective effects of Camellia sinensis (green tea) on MTX induced small intestine mucositis in mice and found that green tea has a potential anti-inflammatory effect and the results corroborates with the trend of green tea being promoted as health drink.

2.2 Enalapril:

Enalapril was an important drug in the class of the angiotensin converting enzyme (ACE) inhibitor. These ACE inhibitors have been used in the treatment of several human diseases including hypertension, congestive heart failure, coronary artery diseases and diabetic nephropathy. Therapeutic effects of enalapril is mostly by modulation of the renin-angiotensin system (RAS) (Brown and Vaughan, 1998).

Lee *et al.* (2011) reported that serum and dialysate TGF β 1 levels were significantly lower for the high dose enalapril group compared to the peritoneal fibrosis (PF) group when compared with low dose of enalapril group, serum and

dialysate TGF beta1 levels were lower in the high dose enalapril group in rats. The serum and dialysate TGF beta1 levels were not statistically significant in the low dose enalapril group compared with the PF group.

Karimian *et al.*, (2008) suggested that the antifibrotic effect of enalapril was mostly related to the inhibition of angiotensin converting enzyme in the liver of bile duct ligated rats.

Ghazi–Khansari *et al.*, (2007) studied that the antifibrotic effect of captopril and enalapril on paraquat induced lung fibrosis in rats by inhibition of angiotensin converting enzyme.

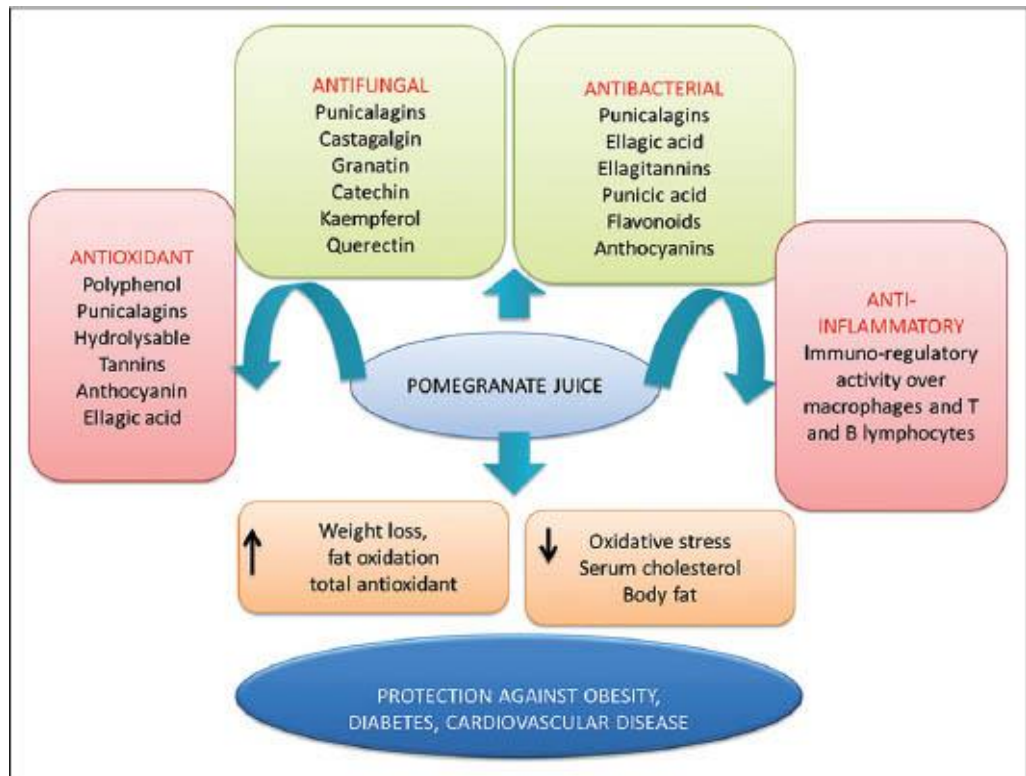
2.3 Pomegranate (*punica granatum*)

Pomegranate is a fruit of the *punicaceae* family. It is used as functional food and source of nutraceuticals being enriched in antioxidant, anticarcinogenic, anti-obesity, anti-tumor and anti-inflammatory medicinal properties (Saeed *et al.*, 2018).

phytochemicals in *punica granatum*

Pomegranate juice rich in anthocyanins, gallic acid, ellagic acid, caffeic acid, quercetin, catechins, and rutin; the seed oil is rich in fatty acids, punicalic acid, ellagic acid and sterols; the pericarp (peel, rind) is rich in catechin, fatty acids, punicalagins, rutin, quercetin, flavones, gallic acid, flavonones, flavnols, and anthocyanidins; leaves are rich in tannins (punicalin and punicafolin), and flavone glycosides like apigenin and luteolin. Ursolic acid, gallic acid, triterpenoids like asiatic acid and maslinic are found in flower while ellagitannins (punicalin and punicalagin) and numerous piperidine alkaloids are formed in roots and bark (Saeed *et al.*, 2018).

Abbasi et al. (2015) reported that pomegranate methanolic extract increased the catalase and GPx in liver content to control oxidative stress induced by methotrexate.



(Lalwani et al., 2014)

Wei et al. (2015) studied the extract of pomegranate peel & pomegranate seeds have preventive effects against carbon tetrachloride induced liver fibrosis. They also reported their antioxidant activity, decreased the serum levels of TGF- β_1 and inhibition of collagen synthesis.

Zou et al. (2014) reported protective effect of punicalagin on non-alcoholic fatty liver disease (NAFLD) by promoting mitochondrial function, eliminating oxidative stress & inflammation.

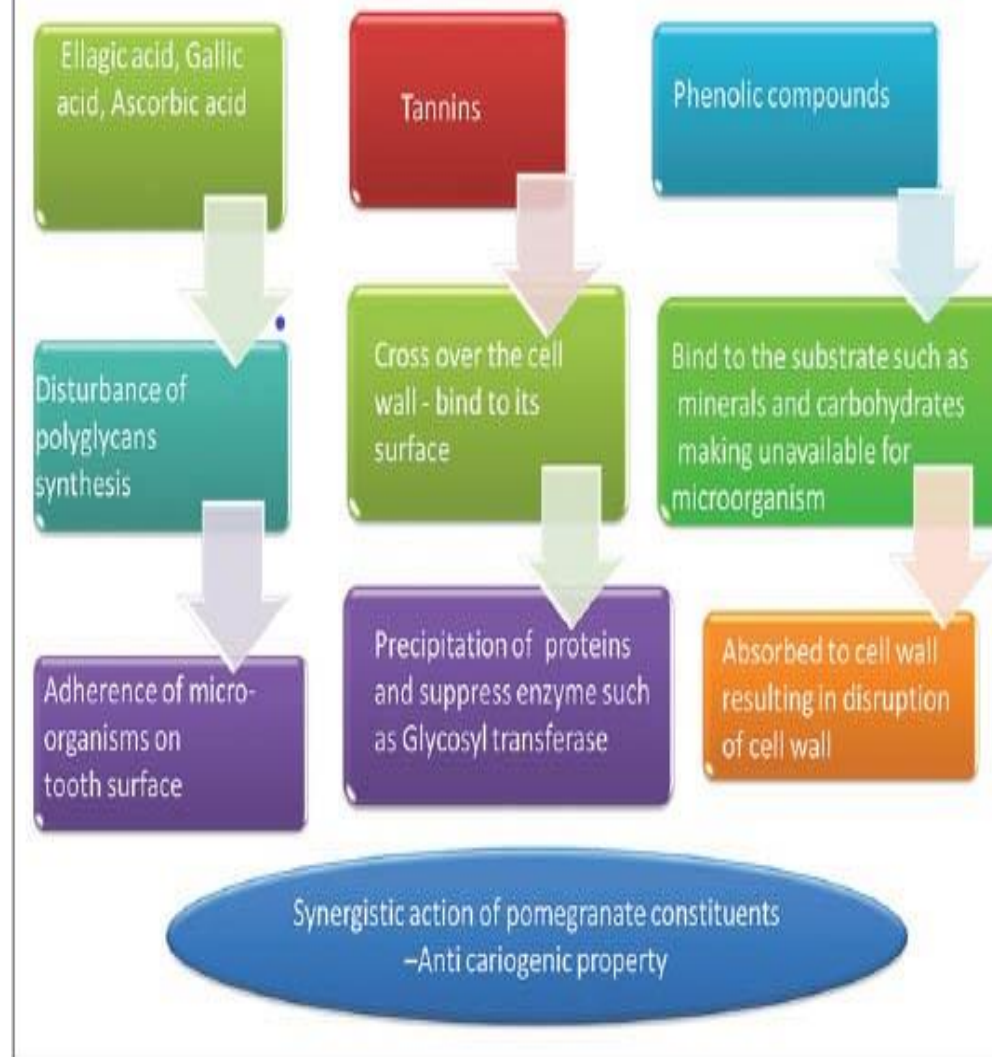
Lee *et al.* (2011) concluded that the enalapril has protective effect against chlorhexidine digluconate induced liver peritoneal fibrosis in rats by decreasing TGF- β_1 production.

Celik *et al.* (2009) investigated the protective anti-oxidant properties of *Punica granatum* against tri chloro acetic acid (TCA) induced liver fibrosis.

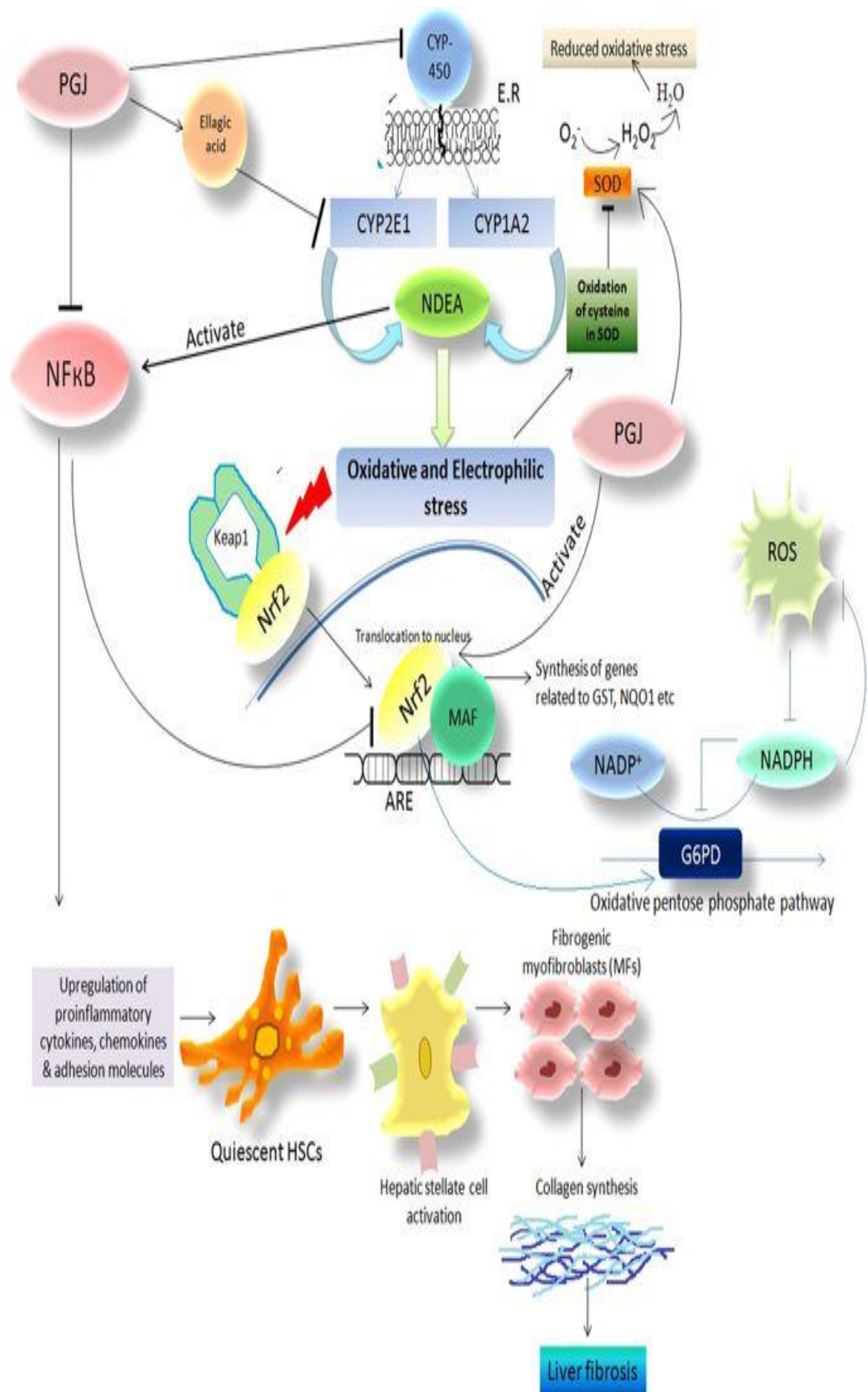
Toklu *et al.* (2007) concluded that the methanolic pomegranate peel extract provided protection against hepatic fibrosis induced biliary obstruction as shown by biochemical, histology studies conducted on the serum and liver respectively.

Kaur *et al.* (2006) studied the pomegranate flower extract protection against ferric nitrilotriacetate (NTA) induced liver injury by inhibition in the modulation of liver markers.

Mechanism of action - Punica granatum juice



(Lalwani et al., 2014)



(Husain *et al.*, 2018)

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The experimental study was conducted in male *Wistar* albino rats of uniform age (about 3 months). The rats were procured from Jeeva life science Pvt. Limited, Hyderabad.

3.1 MATERIALS

3.1.1 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)

3.1.2 Chemicals and Kits

All chemicals used in the biochemical analysis were of analytical grade.

1. All the chemicals (for preparation of reagents and buffers) were procured from Qualigens Pvt. Ltd. Mumbai, Himedia Pvt. Ltd., and SRL Pvt. Ltd., Mumbai.
2. Kits for AST, ALT, GGT and HDL, LDL, Triglycerides, Albumin, Globulin were procured from ERBA diagnostics Ltd, Surat, India and Coral diagnostics, Goa.
3. ELISA kit for TGF- β 1 was procured from KRISHGEN bio systems, Mumbai.

3.1.3 Instruments

- a) UV/Vis spectrophotometer (Tech Comp UV 7500; Tech Comp Ltd. Kowloon Bay, Hongkong)
- b) Digital Electronic balance (ATCO)
- c) Digital pH meter (Toshniwal)
- d) Hot water bath
- e) Vortex mixture
- f) Micropipettes – Various measures – (Eppendorf)
- g) Hot air oven (Deccan Tekno Corporation)
- h) Tissue homogenizer with speed regulator (York Scientific's Delhi)
- i) Laboratory freezing centrifuge – R8C (Remi)
- j) Ultracentrifuge
- k) Thermo scientific ultra-low deep freezer (-80⁰ C).
- l) Serum auto analyzer (Erba Mannheim Chem 7)
- m) iMark™ Microplate Absorbance Reader (Bio-rad)
- n) Automatic whole blood analyser (Humacount, Medsource Ozone Biomedicals Pvt. Ltd)

3.1.4 Feed

Rat feed in the form of pellets (NIN feed standard) was procured from National Institute of Nutrition, Hyderabad, with the following formulation and specifications:

Carbohydrate	- 56.04 %
Protein	- 22.18 %
Fat/Total oil	- 4.98 %

Dietary fiber	- 3.86 %
Moisture	- 9.12 %
Salt	- 0.13 %
Ash	- 3.69 %
Aflatoxin	- Not present
<i>Salmonella</i>	- Not present
<i>E.coli</i>	- Not present
<i>Clostridium</i>	- Not present

3.1.5 Stains

Stains and chemicals for the histopathological study of liver were obtained from Qualigens Pvt. Ltd., Mumbai.

3.2 METHODS

3.2.1 Animals

A total of 24 male *Wistar albino* rats of 3 months old were procured and acclimatized for at least 1 week before the study. The rats were randomly divided into 4 groups consisting of 6 rats in each 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 experimental protocol was approved by the Institutional Animal Ethics Committee (I/2018-31/IAEC/CVSc., Hyd, Dated 16/07/2018). All the groups were maintained as per the following treatment schedule for 28 days.

3.2.2 Experimental design

Group 1: Normal control

Group 2: Methotrexate @ 100 µg/kg body weight once daily intra peritoneally for 28 days.

Group 3: Methotrexate @ 100 µg/kg body weight once daily intra peritoneally for 28 days + Pomegranate juice @ 1ml/Rat once daily orally for 28 days.

Group 4: Methotrexate @ 100 µg/kg body weight (intra peritoneally) + Enalapril @ 5 mg/kg body weight once daily orally for 28 days.

3.2.3 Blood collection

Blood collection was carried out on day 14 (24 h after the last dose of Methotrexate) and 28 (24h after the last treatment dose) in heparinized vials from the rats in each group for hematological parameters.

Feed was withdrawn 12h before the blood collection and blood was collected through retro-orbital plexus after ether anesthesia. Sera samples were separated from the blood for the estimation of AST, ALT, GGT, Albumin, Globulin, Total cholesterol, HDL, LDL, Triglycerides and TGF-β1.

On the 28th day, after blood collection rats were euthanized by carbon dioxide exposure and the liver tissues were collected and homogenized for the assay of GSH, GP_x, GST, SOD and TBARS.

Histopathological examination of liver tissues was conducted to draw possible conclusions at the end of the experiment.

3.2.4 Body weights

Body weights were recorded on 14th and 28th day of experiment.

3.2.5 Biochemical Profile

Serum was separated from the blood and used for biochemical analysis at the end for the following parameters by using diagnostic kits:

1. Alanine transaminase
2. Aspartate transaminase
3. Gamma-glutamyltransferase
4. Total cholesterol
5. HDL
6. LDL
7. Triglycerides
8. Albumin
9. Globulin
10. TGF- β 1

3.2.6 Antioxidant defense profile

3.2.6.1 Reduced glutathione (GSH) (Moron *et al.*, 1979)

Reagents:

- 25% TCA

Trichloroacetic acid: 25 g

Distilled water: 100 ml

- 0.6 mM DTNB (5- 5' dithiobis-2-nitrobenzoic acid) in 0.2 M Sodium phosphate

Sodium phosphate: 2.839 g

Distilled water: 100 ml adjusted to the desired pH and then added

DTNB: 23.7 mg

➤ 0.2 M Phosphate buffer (pH 8.0)

Solution A: 0.2 M KH_2PO_4 : 1.36 g KH_2PO_4 in 50 ml of distilled water

Solution B: 0.2 M NaOH : 0.4 g NaOH in 50 ml of distilled water

Solution A was taken into a beaker and titrated with solution B till the desired pH was obtained and the volume was made up to 100 ml with distilled water and stored at 4° C till usage.

Principle:

The method was based on the reaction of reduced glutathione (GSH) with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) to give a compound that absorbs light at 412 nm.

Procedure:

100 µl of 25% trichloroacetic acid was added to 400 µl of homogenate, centrifuged, supernatant collected and was used as the sample. To 2.0 ml of 0.6 mM DTNB in 0.2 M sodium phosphate (pH 8), added 0.1 ml of the sample and 0.9 ml of 0.2 M phosphate buffer and absorbance was read at 412 nm against a reagent blank. The standards (0.05-5 mg/ml) were also treated in the same way.

Units of activity:

The values were expressed as n moles of GSH/ mg of protein

3.2.6.2 Superoxide dismutase (SOD) (Madesh and Balasubramanian, 1998)

Pinciple:

The reaction involves generation of superoxide by pyrogallol autooxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-yl) 2, 5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazan formed. The color evolved was stable for many hours and was expressed as SOD Units (one unit of SOD is the amount in mg of protein required to inhibit the MTT reduction by 50 %).

Reagents:

- 1) Pyrogallol (100 μ M): 6.3 mg of pyrogallol was dissolved in 5 ml of distilled water. One ml from this solution was diluted to 100 ml with distilled water.
- 2) MTT (1.25 mM): 2.58 mg MTT was dissolved in 5 ml of distilled water.
- 3) Phosphate buffer saline (PBS): PBS was prepared by dissolving NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g) and Na_2HPO_4 (0.94 g) in 800 ml of distilled water. The pH was adjusted to 7.4 and the volume was made up to 1 liter with distilled water.

Procedure:

The reagents were added in the sample, control and the blank as shown below. The absorbance was read at 570 nm against distilled water (blank).

	Sample	Control	Blank (Duplicate)
PBS	0.65 ml	0.65 ml	0. 65 ml
MTT	30 μ l	30 μ l	30 μ l
Homogenate	10 μ l	-	-

Pyrogallol	75 µl	75 µl	75 µl
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The sample, control, and blank were incubated for 5 min at room temperature

DMSO	0.75	0.75	0.75
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Homogenate	-	10 µl	-
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Calculation

Superoxide dismutase was expressed as SOD units/ mg of protein

$$Y \% = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

$$\text{SOD (U/mg of protein)} = \frac{\text{mg of protein in 0.01 ml homogenate}}{Y \%} \times 50 \times \text{DF}$$

3.2.6.3 Thiobarbituric acid reacting substances (TBARS)

(Balasubramanian *et al.*, 1988)

Principle:

Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing light maximally at 535 nm.

Materials:

Trichloroacetic acid (Extra pure; Qualigens, Mumbai) Thiobarbituric acid (Analytical grade; Hi-media) TRIS (hydroxymethyl) aminomethane (Excelar grade; Qualigens, Mumbai).

Method:

3.2.6.3.1 1 g of the tissue sample with 10 ml of 0.2M Tris-HCl buffer (pH 7.2) was taken in a tissue homogenizer to get a 10% homogenate.

3.2.6.3.2 500 µl of supernatant from the homogenate, 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were taken in a tightly stoppered tube.

3.2.6.3.3 The tube was heated to boiling temperature for 45 min.

3.2.6.3.4 After cooling the tube, the contents were centrifuged.

3.2.6.3.5 The supernatant was read at 532 nm against blank.

3.2.6.3.6 The concentration of test samples was obtained using the molar extinction coefficient of MDA.

Units of activity:

Units of activity = n moles of MDA/mg of tissue.

Calculation:

$$\begin{array}{l} \text{n moles} \\ \text{(MDA)} \end{array} = \frac{\text{The 2X volume of sample was taken X absorbance of the sample}}{1.56 \times 10^5 \times \text{mg of tissue}} \times 10^9$$

3.2.6.4 Glutathione peroxidase (GP_x) (Paglia and Valentine, 1967)

Reagents:

1.1 M phosphate buffer (pH 7.4)

1.2 Nm NADPH (1.67 mg in 10 ml)

3 units (50 mM) of reduced glutathione (153 mg in 10 ml 0.25 Mm H₂O₂)

Procedure:

S.No	Reagents	Test tube (ml)
1	Phosphate buffer (0.1 M)	2.00
2	Homogenate	0.10
3	Reduced glutathione	0.10
4	H ₂ O ₂	0.10

Tubes were incubated at 25°C for 5 min, following which 0.1 ml NADPH was added and the enzyme activity was monitored at 60-sec intervals for 5 min at 320 nm wavelength.

Calculation:

GP_x Activity = $\Delta A_{320} \times 3781$ units.

(where ΔA_{320} is change in the absorbance per min at 320 nm).

3.2.6.5 Glutathione S-transferase (Habig *et al.*, 1974)

Reagents

➤ 0.3 M Phosphate buffer, pH 6.5

Solution A: 0.3 M KH₂PO₄: 2.04 g KH₂PO₄ in 50 ml of distilled water

Solution B: 0.3 M NaOH : 1.2 g NaOH in 50 ml of distilled water

Solution A was taken into a beaker and titrated with solution B till desired pH was obtained and made up to 100 ml with distilled water and stored at 4° C till usage

➤ 30 mM Glutathione

Glutathione reduced : 0.921 g

Distilled water : 100 ml

➤ 30 mM 1-Chloro-2,4-dinitrobenzene (CDNB prepared in 95% alcohol)

CDNB : 0.606 g

Ethanol : 100 ml

Principle

Glutathione-S-transferase activity was measured by following the increase in absorbance at 340 nm, using 1-chloro-2,4-dinitrobenzene as the substrate

Procedure

1. 10% liver homogenate was centrifuged at 1500 rpm for 60 min. The supernatant was used as enzyme source.
2. 1 ml of phosphate buffer, 0.1 ml of CDNB and 0.1 ml of homogenate was taken into a cuvette and the volume was adjusted to 2.9 ml with glass distilled water.
3. The mixture was incubated for 5 min at 37° C.
4. The reaction was started by addition of 0.1 ml of glutathione and absorbance was read for 5 min at 340 nm
5. Reaction mixture without homogenate was used as blank.

Calculation

$$\begin{array}{lcl} \mu \text{ moles of CDNB-GSH conjugate} & & \text{Absorbance} \times 3 \times 1000 \\ \text{formed/min/mg protein} & = & \frac{\text{Absorbance} \times 3 \times 1000}{9.6 \times 5 \times \text{protein in mg}} \end{array}$$

9.6 is the difference in the milli molar extinction coefficient between CDNB- GSH conjugate and CDNB.

Units of activity

Units of activity = μ moles/min/mg of protein

3.2.7 Estimation of protein (Lowry *et al.*, 1951)

Principle:

Proteins react with copper at an alkaline pH to form the copper-protein complex. This complex, when treated with a phosphomolybdic-phosphotungstic reagent (Folin- ciocalteu), forms a blue color which was measured at 660nm.

Procedure:

100 μ l of homogenate was made up to 1.0 ml with distilled water. To this, 5 ml of freshly prepared alkaline copper sulfate solution (a mixture of 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1.0 ml of copper sulfate in 1% potassium sodium tartrate) was added and kept for 10 min at room temperature. 0.5 ml of Folin-ciocalteu reagent was added and allowed to stand at dark for 30 min. The resultant blue color was read at 660 nm. Bovine serum albumin was used as a standard.

3.3 Histopathological studies:

Tissue pieces of liver were collected from the rats that were sacrificed at the end and fixed in 10% neutral buffered formalin (NBF) for histopathological studies. The fixed tissues were processed and stained with Masson's trichrome stain as described by Singh and Sulochana (1997).

3.4 Statistical analysis

The data were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) latest version 23. Differences between means were tested using Duncan's multiple comparison tests and significance was set at $p < 0.05$.

RESULTS

4.1 Average body weight

Average body weights (g) were estimated on day 14 and day 28 and the results are depicted in table 4.1; Fig. 4.1 The average body weights of rats in all the groups initially did not vary significantly and same was the case on day 14 but on day 28 rats in group II (229 ± 2.3) had significantly ($p < 0.05$) decreased body weight when compared with group I (275.5 ± 9.0) and other treatment groups (group III & IV 253.6 ± 8.3 , 263.6 ± 8.2 , respectively). Group III & IV had numerically lower values compare to group I (275.5 ± 9.0) but difference was not significant.

4.2 Serum biochemistry

4.2.1 Liver biomarkers

4.2.1.1 Aspartate transaminase (AST)

The activity of AST (IU/L) was estimated on day 14 & day 28. The results are depicted in table 4.2; Fig. 4.2. Group II (133.20 ± 3 , 149.80 ± 3.4 , respectively) had shown elevated levels of AST which was significantly ($p < 0.05$) high between the groups and also between day 14 & day 28. Group III (105.10 ± 1.4 , 114.20 ± 2.1 , respectively) showed decrease in AST levels which were significantly ($p < 0.05$) lower than group II alone but numerically more than group IV (100.70 ± 5.4 , 107.00 ± 2.3 , respectively).

4.2.1.2 Alanine transaminase (ALT)

The activity of ALT (IU/L) was estimated on day 14 & day 28. The results are depicted in table 4.3; Fig. 4.3. Group II (87.00 ± 1.9 , 101.10 ± 1.6 , respectively) had a significant ($p < 0.05$) rise in ALT both on day 14 & day 28. As the treatment progressed to day 28, there was further rise in the level of ALT. In group III & IV (69.50 ± 0.7 , 79.90 ± 1.3 , 67.40 ± 1.6 , 79.80 ± 1.4 , respectively) there was significant ($p < 0.05$) decrease as compared to group II on day 14 & day 28. However, group III & IV seem to have same levels during the study period.

4.2.1.3 Gamma – glutamyltransferase (GGT)

The activity of GGT (IU/L) was estimated on day 14 & day 28. The results are depicted in table 4.4 Fig. 4.4. Group II (6.65 ± 1.16 , 9.5 ± 1.15 respectively) had shown 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 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).

4.2.2 Lipid profile

4.2.2.1 Total cholesterol

The concentration of Total cholesterol (mg/dl) was estimated on day 14 & day 28. The results are depicted in table 4.5; Fig. 4.5. Group II (98.06 ± 7.9) had shown significant ($p < 0.05$) increase in total cholesterol level compare to group I, III & IV (67.11 ± 9.2 , 78.84 ± 8.8 , 77.98 ± 7.4 respectively) on day 14 and there was further significant ($p < 0.05$) rise on day 28 when compared to control & treatment

groups. In group III on day 14 there was a significant ($p<0.05$) reduction in total cholesterol when compared to group II but still significantly ($p<0.05$) high in comparison to group I. In group IV had similar levels as that of group III on day 14 but significantly ($p<0.05$) differed with group I on day 14 where as in group III (112.7 ± 9.4) total cholesterol levels were equal to group I (112.32 ± 8.0) & comparable to group IV (118.96 ± 7.0) on day 28.

4.2.2.2 High density lipoprotein (HDL)

The concentration of HDL (mg/dl) was estimated on day 14 & day 28. The results are depicted in table 4.6; Fig. 4.6. Group II (20.4 ± 0.61) had significantly ($p<0.05$) decreased level of HDL on day 14 and there was further decrease (14.8 ± 0.84) on day 28 as compared to other groups on both days. Groups III & IV (25.1 ± 0.67 , 19.4 ± 0.82 , 25.2 ± 0.71 , 21.8 ± 0.78 , respectively) had significantly ($p<0.05$) increased HDL levels on day 14 & 28, however in group IV day 14 levels were comparable to group III but on day 28, there was rise in HDL level as compare to group III.

4.2.2.3 Low density lipoprotein (LDL)

The concentration of LDL (mg/dl) was estimated on day 14 & day 28. The results are depicted in table 4.7; Fig. 4.7. Group II (84.0 ± 1.21 , 97.2 ± 0.9 respectively) had shown a significant ($p<0.05$) rise in LDL concentration as compared to group I, III & IV (70.8 ± 1.51 , 70.3 ± 1.1 , 75.8 ± 1.67 , 79.0 ± 1.3 , 73.5 ± 1.24 , 77.1 ± 1.1 respectively) on day 14 and day 28. as the drug administration progressed to day 28 there was further significant ($p<0.05$) rise in LDL. Group IV had significantly ($p<0.05$) decreased LDL levels compare to group II and

significantly ($p < 0.05$) better than group III & comparable to group I (70.8 ± 1.51) on day 14 but on day 28 it was significantly ($p < 0.05$) falling less than group I (70.3 ± 1.1).

4.2.2.4 Triglycerides

The concentration of Triglycerides (mg/dl) was estimated on day 14 & day 28. The results are depicted in table 4.8; Fig. 4.8. Group II (71.98 ± 2.9) had shown significant ($p < 0.05$) increase in triglyceride levels compared to group I, III & IV (40.7 ± 1.3 , 52.43 ± 1.8 , 51.71 ± 1.4 respectively) on day 14. In group III & IV triglyceride levels on day 14 were significantly less than group II but significantly more than group I, the same results were seen on day 28. The triglyceride levels in group III & IV (60.63 ± 1.86 , 60.86 ± 1.44 respectively) were significantly ($p < 0.05$) less than group II (81.18 ± 1.75) but they were significantly higher than group I (50.9 ± 1.0) even on day 28.

4.2.3 Protein profile

4.2.3.1 Albumin

Albumin (g/dl) concentration was estimated on day 14 & 28. The results are presented in table 4.9; Fig.4.9. Group II (2.67 ± 0.23) had significantly ($p < 0.05$) lower values on day 14 compare to group I, III & IV (4.31 ± 0.09 , 3.18 ± 0.07 , 3.81 ± 0.03 respectively) and as it progressed to day 28 there was further significant ($p < 0.05$) fall in the level of albumin. Group III & IV had albumin levels comparable to group I but significantly ($p < 0.05$) less than group I. There was significant ($p < 0.05$) variation between group III (3.18 ± 0.07 , 2.3 ± 0.08 respectively) & group IV (3.81 ± 0.03 , 2.56 ± 0.07 respectively) on day 14 & day 28.

4.2.3.2 Globulin

Globulin (g/dl) concentration was estimated on day 14 & 28. The results are presented in table 4.10; Fig.4.10. Group II (2.78 ± 0.11) had significantly ($p < 0.05$) lower values on day 14 compared to group I, III & IV (3.33 ± 0.29 , 2.87 ± 0.09 , 2.91 ± 0.12 respectively). As it progressed to day 28, there was further significant ($p < 0.05$) decrease in the level of globulins in group II. On day 28, there was no significant ($p < 0.05$) difference between group III & IV (2.93 ± 0.15 , 3.03 ± 0.10 respectively) but significantly ($p < 0.05$) higher than the group II (2.36 ± 0.13) and significantly lower than the group I (3.20 ± 0.22).

4.2.4 Cytokine TGF- β 1

TGF- β 1 (pg/ml) was estimated on day 14 and day 28. The results are depicted in table 4.11; Fig.4.11. Group II (18.67 ± 3.43) had significantly ($p < 0.05$) higher values on day 14 compared to group 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- β 1. Group III & IV had TGF- β 1 levels comparable to 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.

4.3 Hematology

4.3.1 Total erythrocyte count (TEC)

Total erythrocyte count ($10^6/\text{mm}^3$) was estimated on day 14 & 28. The results are presented in table 4.12; Fig.4.12. Group II had significantly ($p < 0.05$) decrease in TEC on day 14 and there was a further decrease in TEC on day 28 as

compared to group I, III & IV. Group III (7.36 ± 0.08) on day 14, had significantly improved TEC levels than group II (7.08 ± 0.22) but less than group IV (7.63 ± 0.10) & group I (8.20 ± 0.90). On day 28, group III (6.49 ± 0.14) had significantly ($p < 0.05$) higher levels than group II (5.35 ± 0.13) but there was significant ($p < 0.05$) decrease in comparison to group I (8.71 ± 0.09) & group IV (7.19 ± 0.05). Overall the performance of group IV seems to be significantly ($p < 0.05$) closer to group I on both days i.e. day 14 & 28.

4.3.2. Total leucocyte count (TLC)

Total leucocyte count ($10^3/\text{mm}^3$) was estimated on day 14 & 28. The results are presented in table 4.13; Fig.4.13. The TLC in group II (5.47 ± 0.71 , 2.67 ± 0.32) was lower on day 14 & 28 compared to group I, III & IV (9.78 ± 0.05 , 9.07 ± 0.06 , 7.34 ± 0.14 , 6.07 ± 0.11 , 8.45 ± 0.09 , 7.15 ± 0.07 respectively). In group III, there was increase in TLC towards normal but significantly ($p < 0.05$) lower as compared to group IV & I. However, the values of group IV are very much close to group I, but statistically lower than group I.

4.3.3. Hemoglobin (Hb)

Hemoglobin concentration (g/dl) was estimated on day 14 & 28. The results are presented in table 4.14; Fig.4.14. Hb concentration in group II decreased when compared to control & other treatment groups. In group I Hb concentration (15.35 ± 0.14 , 15.37 ± 0.13) was within the normal range on day 14 & day 28, whereas in group III Hb concentration (13.01 ± 0.20 , 13.1 ± 0.02) was significantly ($p < 0.05$) higher than group II (10.96 ± 0.19 , 10.2 ± 0.14) but lesser than group I & group IV.

Overall group IV (14.52 ± 0.15 , 14.26 ± 0.02) values close to group I but statistically lower than group I (15.35 ± 0.14 , 15.37 ± 0.13).

4.3.4. Pack cell volume (PCV)

PCV (%) was estimated on day 14 & 28. The results are presented in table 4.15; Fig.4.15. The PCV % in group II (32.88 ± 0.58 , 30.6 ± 0.44) was lower on day 14 & 28 compared to group I, III & IV (46.07 ± 0.42 , 46.67 ± 0.41 , 39.05 ± 0.61 , 39.30 ± 0.8 , 43.57 ± 0.46 , 42.15 ± 0.05 , respectively). In group III, there was significant ($p < 0.05$) rise in PCV towards normal but significant ($p < 0.05$) fall when compared to group IV & I. However, the values of group IV were very much close to group I but statistically lower than group I.

4.4 Anti-oxidant profile

4.4.1 Reduced glutathione (GSH)

GSH (n moles/mg protein) was estimated on day 28. The results were presented in table 4.16; Fig.4.16. GSH concentration in group II (2.36 ± 0.11) dipped to a significantly ($p < 0.05$) low level on day 28 when compared with group 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).

4.4.2 Super oxide dismutase (SOD)

SOD was estimated on day 28. The results are presented in table 4.17; Fig.4.17. 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 , $11.41 \pm$

0.13 respectively) had similar values without statistically significant difference but were significantly ($p < 0.05$) lesser than group I (12.24 ± 0.06).

4.4.3 TBARS

TBARS (n moles of MDA/mg protein) were estimated on day 28. The results are presented in table 4.18; Fig.4.18. In group II (54.30 ± 5.13) TBARS increased significantly ($p < 0.05$) compared to other groups. Group 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.

4.4.4 Glutathione peroxidase (GPx)

GPx (U/mg protein) was estimated on day 28. The results are presented in table 4.19; Fig.4.19. GPx activity in group II (18.28 ± 0.09) was significantly ($p < 0.05$) lower than other counter parts 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 ($p < 0.05$).

4.4.5. Glutathione S transferase (GST)

GST (n moles/mg protein) was estimated on day 28. The results are presented in table 4.20; Fig.4.20. GST activity in group II (2.25 ± 0.13) was significantly ($p < 0.05$) lower than other counter parts 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 ($p < 0.05$).

4.6 Histopathology of liver

Histopathological examination of liver in toxic control (group II) revealed moderate degeneration with necrosis, centrilobular and periportal fibrosis (Fig.22) whereas group III (Fig.23) and IV (Fig.24) showed mild periportal fibrosis, degenerative changes with some normal architecture of hepatocytes compared to group II. The sections of group I (Fig.21) did not reveal any significant pathological lesions and the architecture was normal.

DISCUSSION

CHAPTER V

DISCUSSION

The use of anticancer drugs is limited due to their acute toxic effects on some organs such as liver, kidney, testis and heart. It has been reported that methotrexate induced liver damage may occur at a high dose or by chronic administration of methotrexate. Methotrexate may lead to liver hepatotoxicity, including steatosis, cholestasis, fibrosis, and cirrhosis. The mechanism of MTX hepatotoxicity can be related to its accumulation inside the cell in a polyglutamated form. The conversion of MTX to its major extracellular metabolite, 7-hydroxymethotrexate, takes place in the liver, where it is oxidised by a soluble enzymatic system. Inside cells, MTX is stored in a polyglutamate form. Long-term drug administration can cause accumulation of MTX polyglutamates and decrease folate levels. The presence of higher levels of polyglutamates cause a longer intracellular presence of the drug, and this has been suggested as a mechanism for MTX hepatotoxicity (Tunali *et al.*,2010; Kose *et al.*,2012).

MTX treatment causes oxidative tissue damage by increased lipid peroxidation mediated by oxygen free radicals, believed to be an important cause of destruction and damage to cell and organelle membrane disturbing the hepatic cytoarchitecture and its function.

Pomegranate is a fruit of Punicaceae family. Sci.name: *Punica granatum*. Pomegranate contain phytochemicals such as tannins (ellagitannin, punicalagin, pedunculagin), flavonoids, and phenols. Pomegranate powder suspension can

enhance humoral & cellular immunity. Plant extract has anti-oxidant, anti-inflammatory, hepatoprotective, cardio protective activity. Pomegranate juice possesses strong antioxidant property which is due to ellagitannin & other hydrolysable tannins and thus reduces the oxidative stress, free radicals and lipid peroxidation.

A total of 24 male wistar rats were divided into 4 groups of 6 rats in each (control, MTX, MTX+PJ and MTX+enalapril groups). Blood was collected on day 14 & day 28 in heparinized and non heparinized vials from the rats in each group for estimation of hematology and biochemical parameters. The rats were euthanized on 28th day after blood collection and the liver samples were collected and homogenized for the assay of antioxidants (GSH, SOD, GPx, TBARS & GST). Histological examination was also conducted on the liver tissue.

In the present study, there was no significant difference in the body weights between the groups on day 0 & day 14 but on day 28 significant decrease in the body weights in group II was observed as compared to other groups, however, the rats of other treatment groups significantly gained weights when comparison was made between day 0, day 14 & day 28 within the group. Similarly, Kumari *et al.* (2016) reported that MTX decreased the body weight of rats but amylocure treatment significantly increased body weight as compared to MTX group. In the present study, decreased body weights in MTX group is attributed to oxidative stress and oxygen free radicals generated in MTX induced hepatotoxicity and altered metabolism in liver. These observations may be supported by the other findings of the study, in which there were significant alterations in the markers of oxidative stress (increased TBARS) and antioxidant defences (reduced concentration of GSH and reduced activity of

GPx, SOD and GST) in liver homogenate and increased serum globulins, AST, ALT & GGT levels. Weight gain observed in the animals treated with pomegranate juice and enalapril (group III and group IV respectively) may be due to restoration of antioxidant defences and recovery of hepatic histoarchitecture as evident from the findings of this study suggesting hepatic regeneration and protection.

MTX is known to cause damage of the bone marrow hence as an indirect measurement haematological assessment was done and a decrease in RBC, WBC, Hb and PCV was noted. Similar findings were reported by Sener *et al.* (2006) and Patel *et al.* (2014). Patel *et al.* (2014) in the discussion on toxicopathology of MTX have opined that drop in RBC count is attributed to ulcers in GI tract and a decrease in PCV values due to cytotoxicity of MTX on haemopoietic system. Dose dependent decrease in Hb and PCV was also observed by Ohbayasi *et al.* (2010) and similar observation of decrease in WBC was found by Rofe *et al.* (1994) and Bussanich *et al.* (1985). In our study pomegranate juice might have nullified the cytotoxic effect of MTX on bone marrow, thus there was an increase in the levels of haematological parameters towards control values.

Aminotransferases, alanine and aspartate play a major role in transferring α amino nitrogen of aspartate & alanine to ketoglutaric acid culminating in the formation of glutamate. Both these enzymes have a major role in gluconeogenesis & urea formation. Any increase in these enzymes is indicative of hepatic injury, inflammation and necrosis. Mostly, the injury can be of hepatic cells and could include cholestatic injury too, in which there would be a rise in GGT as both forms of injury coexist. Also, ALT is concentrated in periportal zone whereas AST is concentrated in centrilobular zone thus a

corresponding rise of the enzymes could lead to an indication of the respective zones. The involvement of centrilobular area could explain the metabolism of MTX to 7-OH MTX in the presence of metabolic enzymes in this region.

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 & 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 MTX group. However, the enzyme levels did not reach the normal levels as that of 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.

The results are in agreement with Celik *et al.* (2009) who administered pomegranate flowers infusion against trichloroacetic acid exposed in rats, Ozogula *et al.* (2013) with mirtazapine and Uraz *et al.* (2008) with Ursodeoxycholic acid in prevention of methotrexate induced liver toxicity. Further, Wei *et al.* (2015) administered pomegranate peel & seeds extracts on liver fibrosis induced by carbon tetrachloride in rats, Kaur *et al.* (2006) administered pomegranate flower extract against ferric nitrilotriacetate (Fe-NTA) induced hepatotoxicity in mice. Toklu *et al.* (2007) used pomegranate peel extract to prevent liver fibrosis in biliary obstructed rats and Ghaffari *et al.*

(2011) found protective effect of milk thistle on methotrexate induced hepatic fibrosis in rats.

Albumin is synthesized in the liver and secreted into the blood stream. Albumin has a free cysteine residue which has a role in its action as an anti-oxidant. This free cysteine, scavenges free radicals and could be an important anti-oxidant in the circulation (Kaneko *et al.*, 2008). Albumin is a negative acute phase protein because its level dips in the inflammation, this is in agreement with our study where albumin levels have gone down with methotrexate in group II but the levels were restored in group III & IV and can be attributed to the presence of gallic acid & epigallocatechin gallate present in the beverage of pomegranate which might have protected the liver.

Globulins are proteins synthesized in the liver but differ in their functions such as transport and inflammatory proteins, clotting factors and immunoglobulins. Globulin concentration decreased in groups treated with MTX because liver in hepatic injury is likely to decrease globulin concentration and when treated with pomegranate juice and enalapril, globulin concentration improved a little on day 14 but the improvement was significant on day 28. Our findings concur with Patel *et al.* (2014).

Cholesterol is a precursor of corticosteroids and sex steroids while the energy source being triglycerides. Cholesterol and triglycerides are both of exogenous (diet) and endogenous (synthesis) origin. Lipids in the form of cholesterol and triglycerides, are transported from the liver to the adipose tissue by very low density lipoproteins (VLDL). Loss of triglycerides by endothelial lipoprotein lipases in fat and muscle from VLDL result in the formation of low density lipoproteins (LDL). High density lipoprotein (HDL) is synthesized by the

liver and transports cholesterol. Any abnormality in the synthesis of these lipoproteins is an indication of the malfunction of the liver. As MTX is known hepatotoxicant it decreased the concentration of HDL but has increased the level of total cholesterol, LDL, triglycerides in the serum and LDL undergoes increased peroxidation in the body. However, simultaneous administration of MTX with pomegranate juice & enalapril a reduction in the LDL concentration on 14th & 28th day was observed. The same observation holds good for other parameters in the lipid profile. Addition of pomegranate juice and enalapril might have reduced hepatic cell injury induced by MTX. The presence of flavonoids, gallic acid, gallactinin and other substances in the pomegranate juice might have prevented hepatic injury and decreased the elevated levels of lipid markers. Enalapril a known anti-fibrotic agent could have prevented the development of hepatic fibrosis and thus paved the way for a decrease in lipid markers. Our results are in synchrony with Kumari *et al.* (2016) and Georgiadis *et al.* (2006). However, Belinsky *et al.* (2007), reported upregulation of genes involved in the fatty acid synthesis by MTX and Van Ede *et al.* (2002) found folic acid deficiency with MTX and an increased cholesterol, LDL, triglycerides and a decrease in HDL.

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 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 an inhibition of glutathione reductase activity and inhibition of glutathione. This fall in anti-oxidant defence has been observed in our study in the form of decrease in

SOD, GSH, GPx, GST and increase in TBARS. This is in agreement with Toklu *et al.* (2007), Shishavan *et al.* (2017), Hozayen *et al.*, (2017), Khalifa *et al.*, (2017) and 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 anti-oxidant 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 effect as investigated by Johanningsmeier and Harris (2011). Pomegranate fruit extract is rich in flavonoid content which possesses anti-oxidant character as evidenced through decreased MDA and increased GSH, SOD, GPx, GST. Anthocyanins such as delphinidin, cyanidin, pelargonidin might have contributed to the anti-oxidant activity (Saeed *et al.*, 2018).

Similar results were observed by Toklu *et al.* (2007) with pomegranate peel extract in bile duct ligation (BDL) induced hepatic fibrosis. Shishavan *et al.* (2017) reported that MTX induced changes in hepatic anti-oxidant enzymes were relieved by methanolic pomegranate peel extract and flower extract abrogated Fe-NTA induced hepatic oxidation in mice (Kaur *et al.*, 2006). Pomegranate flower infusion against trichloroacetic acid exposure in rats (Celik *et al.*, 2009) and on liver fibrosis induced by carbon tetrachloride in the rats through peel & seed extract (Wei *et al.*, 2015).

The expression of biomarker of fibrogenesis namely, transforming growth factor (TGF)- β 1 peptide, was studied in serum biochemistry. In the present study, the TGF- β 1 level in the serum was significantly increased in groups treated with MTX when compared to control group. The increased level of TGF-

$\beta 1$ could be attributed to liver injury and released TGF- $\beta 1$ may cause necrotic hepatocytosis, activated hepatic stellate cells and macrophages (Wu *et al.*, 2018; Niu *et al.*, 2016). Enalapril treated group IV showed decreased TGF- $\beta 1$ levels attributed to protection of hepatocytes against oxidative stress and suppression of HSCs activation by angiotensin II type 1 receptors 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- $\beta 1$ and CTGF) in activated HSCs (Kurikawa *et al.*, 2003; 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 receptors expressed on the surface of HSCs; second, it suppresses the activation of HSCs as a result of the decrease in TGF- $\beta 1$ (Ahmad and Ahmad 2012). Similarly, pomegranate juice treated group III also revealed significant decrease in TGF- $\beta 1$ 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 decrease levels of TGF- $\beta 1$. Similar protective effect with pomegranate were found with carbon tetrachloride (Wei *et al.*, 2015; Niu *et al.*, 2016; and Oliveira *et al.*, 2012) and in ethanol related fibrosis (Zhuge and Cederbaum (2006) and oxymatrine protected thioacetamide induced hepatic fibrosis (Wu *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 phytochemicals 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 phytochemicals needs to be carried out.

SUMMARY

CHAPTER VI

SUMMARY

Pomegranate juice extract was studied for its therapeutic efficacy against experimentally induced hepatic fibrosis by Methotrexate. A total of twenty four male *Wistar* albino rats were procured for the study. The rats were randomly divided into four groups consisting of six in each. All the animals were acclimatized and maintained with basal diet and *ad libitum* of water throughout the experiment. Methotrexate 100 µg/kg was administered to all the groups (except group I) from day 1st to 28th and group II was considered as toxic control. Groups III and IV were administered orally with Pomegranate juice extract @1ml/rat and Enalapril @5mg/kg, respectively from day 1st to 28th and was considered as amelioration groups. The experimental study was carried out for the period of 28 days. The results of the study are summarized as follows:

- 6.1 The body weights (g) in toxic control group II were significantly ($p<0.05$) lowered when compared to group I on 28th day. Group III and IV showed significant ($p<0.05$) reversal of body weights gain was observed when compared to group II on 28th day.
- 6.2 The values of TEC, TLC, Hb, and PCV in toxic control group II were significantly ($p<0.05$) lowered when compared to group I at respective time intervals from 14th and 28th day. Group III and IV showed significant ($p<0.05$) improvement of all the haematological parameters on 14th and 28th day.
- 6.3 The serum total cholesterol, triglycerides, and LDL were significantly ($p<0.05$) higher, while HDL was significantly ($p<0.05$) lower in toxic control

group II when compared to group I and treatment groups III and IV showed significant improvement on 14th and 28th day.

6.3 The activity of AST, ALT and GGT in serum revealed a significant ($p<0.05$) rise in group II when compared to normal control group treatment while groups III and IV showed significant ($p<0.05$) improvement on 14th and 28th day.

6.4 The concentration of serum albumin and globulin were significantly ($p<0.05$) lower in toxic control group II when compared to group I whereas treatment groups III and IV showed significant ($p<0.05$) increased in their concentrations on 14th and 28th day.

6.5 The concentration of TBARS in liver homogenate revealed a significant ($p<0.05$) rise in group II when compared to group I. The groups III and IV showed a significant ($p<0.05$) decrease in the concentration of TBARS at the end of 28th day.

6.6 The concentration/activity of GSH, SOD, GPx and GST in liver homogenate revealed a significant ($p<0.05$) decrease in group II when compared to group I. The treatment groups III and IV showed a significant ($p<0.05$) improvement in their concentrations at the end of 28th day of study.

6.7 The expression of TGF- β 1 in the serum revealed a significant ($p<0.05$) rise in group II when compared to group I. The groups III and IV showed a significant ($p<0.05$) decrease in the concentration of TGF- β 1 at the end of 28th day.

6.8 The histopathology of liver in Methotrexate toxic group II revealed moderate degeneration with necrosis, centrilobular and periportal fibrosis. Enalapril and Pomegranate juice extract treated groups showed mild fibrotic and mild necrotic changes.

In conclusion, the results of the present investigation suggest that Pomegranate juice extract has potent hepatoprotective activity based on haematological, biochemical, antioxidants and histopathological parameters in this study. Pomegranate juice extract is effective in scavenging free radicals released during metabolism of Methotrexate and protecting binding of their toxic reactive metabolites with cellular macromolecules like proteins, lipids and nucleic acids, which were evident in this study by reduced the hepatotoxic markers, restoration of antioxidant enzymes, decreased TGF- β 1 protein expression. The overall beneficial effects of pomegranate juice extract could be attributed to antioxidant potential as evident from oxidant-antioxidant markers in this study coupled to hepatoprotective property.

LITERATURE CITED

LITERATURE CITED

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