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Research Article

EVALUATION OF HEPATO PROTECTIVE ACTIVITY OF ETHANOLIC ROOT EXTRACT OF *PUNICA GRANATUM*

M.CHINNA ESHWARAIAH[1], MANASA.N[*1] KAVITHA.K[1], DIPANKAR BARDALAI[2]

Anurag Pharmacy College, Ananthagiri (V), Kodad(M), Nalgonda(Dist.),Andhra Pradesh, India. Email: eswarphd@gmail.com

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ABSTRACT

Objective: The present study was aimed to evaluate the protective effect of the ethanolic root extract of *Punica granatum* on carbon tetrachloride induced hepatotoxicity.

Methods: The extract was evaluated for hepatoprotective activity against CCl_4-induced hepatotoxicity using rat liver**.** Hepatic enzymes studied include Alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP). Hepatic injuries involved with possible necrosis which may have contributed to increase level of hepatic enzyme levels. *Punica granatum* root extract was administered orally by dissolving in water. The root extract was screened for toxicity by oral toxicity studies according to OECD guidelines 423. LD_{50} was calculated for selection of dose. Liver was excised and histopathological study was conducted by keeping in 10% formalin. They were stained with haematoxyline and eosin and photographed.

Results: Results showed that treatment with *ethanolic root extract of Punica granatum* normalization of cells and reduced sinusoidal dilation along with mild inflammogens which are compared with Silymarin. This was evident from significant reduction in $P<0.05$, $P<0.01$, $P<0.001$ in serum enzyme levels. In the hepatoprotective and curative period, the highest damages in liver tissue were found in the order Carbon tetrachloride > low dose > high dose> silymarin> normal control. This clearly explained the reason for the Hepato protective activity of pomegranate root extract.

Conclusion: It was concluded from the result that the ethanolic extract of *Punica granatum* root possesses hepatoprotective activity.

Keywords: Carbon tetrachloride, Hepato protective activity, Histopathology, *Punica granatum.*

INTRODUCTION

Liver in normal adult weighs nearly three pounds. It produces and secretes bile into intestinal lumen and assists in digestion of fat. Liver helps in purifying blood. Liver disease is a term for a collection of conditions, diseases and infections that affect the cells, tissues, structures or functions of the liver. Liver identifies xenobiotics and metabolize them and make them suitable for elimination. This involves chemical transformation, decreasing lipid solubility, change the biological activity. Mainly smooth endoplasmic reticulum of liver principally participates in metabolism.

Hepatic damage is the most common disadvantage of maximum NSAIDs. Liver is the largest metabolizing organ in the body which regulates homeostasis of the different body system. Its important functions include protein synthesis, storage and metabolism of fats and carbohydrates, detoxification of drugs and other toxins, excretion of Bilirubin and metabolism of hormones [1]. Hepatoprotective agents are those compounds, which mitigate the liver injury caused by hepatotoxic agents. Hepatoprotective effects of plant drugs and herbal formulations are studied against chemicals (Alcohol, CCl_4, Beta galactosamine, Thioacetamide) and drugs (Paracetamol, Nimesulide, Antitubercular drugs like Isoniazid, Rifampicin etc.) induced hepatotoxicity in rats and mice as they virtually mimic any form of naturally occurring liver disease. Liver damage is associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, Cholesterol, Bilirubin, alkaline phosphatase are elevated [2]. Herbs have been used by human being throughout the history from ancient primitive to modern time. Now herbs are one of the integral sources for research and development of modern therapy. *Punica granatum Linn*. (Lythraceae), commonly known as pomegranate, is a shrub or a small tree, native to the Mediterranean region. The plant possesses an immense therapeutic value [3]. A number of biological activities such as antitumor [4], antibacterial [5], antidiarrhoeal [6], antiulcer [7] have been reported with various extracts/constituents of different parts of this plant. Silymarin was a flavonoid obtained from *Silybum marianum* or milk thistle and was composed of three isomers: silybinin, silydianin and silychristin [8], silybinin being quantitatively the most important [9, 10] showed standard drug silymarin a remarkable protection of serum AST, ALT and ALP levels towards CCl_4 induced hepatotoxicity. Carbon tetrachloride (CCl_4) has been widely used in animal models to investigate chemical toxin-induced liver damage[11]. The most remarkable pathological characteristics of CCl_4-induced hepatotoxicity are fatty liver, cirrhosis and necrosis, which have been thought to result from the formation of reactive intermediates such as trichloromethyl free radicals (CCl_3) metabolized by the mixed function cytochrome p450 in the endoplasmic reticulum . Usually, the extent of hepatic damage is assessed by the increased level of cytoplasmic enzymes (ALT, AST and ALP), thus leads to leakage of large quantities of enzymes into the blood circulation. This was associated by massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. Oxidative stress is considered to play a prominent causative role in many diseases including liver damage. Oxidative stress is the state of imbalance between the level of antioxidant defense system and production of oxygen-derived species. The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage [12]. The alcoholic, fraction of *Punica granatum* has shown decrease in enzymatic activity of SGOT, SGPT, ALP and also reduction in increased liver weight of treated groups and were significantly low ($P<0.001$) compared to that of silymarin which is used as standard. Hence the alcoholic extract of *Punica granatum* roots is having the good potency and this can be considered to be responsible for the hepatoprotective activity.

MATERIALS AND METHODS

Collection of Plant material

Pomegranate root extract (*Punica granatum)* was used in our present work. The pomegranate roots were collected from local area, Kodad. The roots were extracted with ethanol.

Authentification

The plant *Punica granatum* was authentified by Dr. K. Madhav Chetty, Asst. Prof., Department of Botany, Sri Venkateswara University, Tirupathi. The plant *Punica granatum* belonging to the family Lythracea.

Drugs and Chemicals

Silymarin (Symed Pharm. Pvt. Ltd, Hyderabad) used as the standard hepato protective drug and all other laboratory reagents were

obtained from the Aman Scientific chemicals, Vijayawada and are analytical grade.

Extraction

The roots were dried and made into a coarse powder with the help of electric grinder. About 400gm of grinded plant material was subjected to Soxhlet extraction (65^0-75^0C) employing ethanol as solvent. The solvent was evaporated at 40^0 C to obtain the extract. The obtained extract was golden yellow in colour and was stored in refrigerator until use.

Experimental Animals

Rats of either sex weighing 150-200 g of body weight were used in experiment. Animals were kept under standard conditions at 23-25^0C for 12 hr light/dark cycle and given standard pellet diet and water. The animals were accustomed to the laboratory conditions for a week prior to the experimentation. The fresh diet and water has to be supplied daily to the animals. The condition of the animals has to be supervised daily till the completion of the experiment. Before performing the experiment the ethical clearance was obtained from institutional animal ethics committee (IEAC).

Vehicles and preparation of doses

To prepare the dosage forms of *punica granatum root* extract is dissolved in water. Silymarin standard hepatoprotective drug also prepared in water. The dose in required concentration was administered at 1ml/100g body weight of the animal.

Toxicological Evaluation (Acute Oral Toxicity Study)

Acute toxicity studies were performed according to OECD-423 guidelines [13]. The animals were fasted for 12 hrs with free access to water only. The various extract and fractions of *Punica granatum* were administered orally. The initial dose of 5mg/kg of various extract and fractions of *Punica granatum* administered and mortality if any was observed for 24 hrs. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals, then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, the higher (50,100,250, 500, 1000, 2000, 3000mg/kg) doses of various extracts and fraction of *Punica granatum for* further studies. One tenth of LD50 was used as a maximum dose of extracts tested for acute toxicity. The doses were selected for evaluation of hepatoprotective activity was 300mg/kg.

Evaluation of hepatic profiles by CCl_4 induced hepatotoxicity

Experimental design

Group I: Normal (liquid paraffin 3ml/kg, S.C.) for 8days

Group II: Control (CCl4 in liq paraffin 1:2, v/v, 1ml of CCl_4/kg, S.C.) For 8 days

Group III (test 1): PGERE (150mg/Kg, P.O for 8days) + CCl_4

Group IV (test 2): PGERE (300mg/Kg, P.O for 8days) + CCl_4

Group V: Silymarin (100mg/kg, P.O for 8days) + CCl_4

For evaluation of hepatoprotective activity, all animals were randomly divided into five groups of six animals each. Each group of animals were treated with respective vehicles or drugs and extracts for 8 days, after 30minutes post dose administration all groups(except group-1 normal) were received CCl_4 at the dose of 1ml/kg(1:2 v/v of CCl_4 in liquid paraffin).S.C.

Estimation of Hepatic Enzymes

The blood was obtained from all the animals by puncturing retro-orbital plexuses. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation 10000 rpm for 5min at 30^oC and utilized for estimation of various biochemical parameters namely SGPT (Aghape, India), SGOT (Teco, US), ALP (Teco, US), total bilirubin (Bayer, India).

Histopathological studies

On the 9th Day, after scarification of rats by cervical dislocation, liver samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible. A portion of liver tissue in each group was preserved in 10% formaldehyde solution for histopathological studies. Haematoxylin and eosin were used for staining and later the microscopic slides of the liver tissue were photographed at magnification 40X.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Dunnett's multiple comparison tests and one-way analysis of variance (ANOVA) using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between the groups were considered statistically significant at $P<0.05$, $P < 0.01$ and $P<0.001$.

RESULTS

There was significant decrease in hepatic enzyme levels observed in drug treated animals with PGERE (150mg) and PGERE (300mg).Given in table 1, fig 1, 2 and 3. The results of CCl_4 induced hepatotoxicity are shown in the Table 1.Ccl_4 intoxication in normal rats significantly elevated the serum levels of SGPT, SGOT and ALP. Similarly simultaneous administration of test drug and CCl4 for 8 days showed marked decrease in SGOT, SGPT and ALP (fig 1, 2 and 3) values on the 8th day. Treatment with the alcoholic fraction at the dose of 300mg/kg showed significant decrease in the SGOT, SGPT and ALP enzyme levels. The standard drug (Silymarin 100mg/kg) also prevented the elevation of serum enzyme levels. (Table 1) The group-II (control) produce significant increase in hepatic serum enzyme levels ($P<0.05$, $P<0.01$ and $P<0.001$) comparable with normal. The group-III PGERE (test1) (150mg) produce decreased hepatic enzyme levels comparable with control and statistically significant at ($P<0.05$, $P<0.01$ and $P<0.001$), whereas group-IV PGERE (test2) (300mg) also produce significant ($P<0$, 05, $P<0.01$and$P<0.001$) decrease hepatic enzyme levels which is comparable to standard drug silymarin (group-V) ($P<0.05$, $P<0.01$ and $P<0.001$).

Table 1: Effect of *ethanolic root extract of punica granatum (PGERE)* on hepatic enzyme levels

Treated groups	Hepatic enzyme levels on 9th Day		
	SGOT(aspartate aminotransferase/ AST) (U/L)	SGPT(alanine aminotransferase/ALT) (U/L)	ALP (U/L)
Normal	28.00±0.5774	29.50± 0.7638	83.50± 0.9916
Control(CCl4)	82.67±0.881[a]***	90.33±1.382[a] ***	149.5± 0.763 [a] ***
PGERE150mg	53.00±1.461 [b]***	57.50±1.118 [b] ***	116.8± 0.6009[b]***
PGERE 300 mg	36.50±0.7638 [b] ***	38.83±0.4733[b] ***	90.83± 0.6009 [b] **
Silymarin 100mg	34.50±0.5627[b] ***	38.67±0.4944 [b] ***	86.50± 0.7638[b] ***

Values are in Mean ± S.E.M (n=6); [ns] -Non Significant, *p<0.05, **p<0.01, ***p<0.001

[a] Control compared with Normal, [b] All test groups compared with Control using One

Way ANOVA followed by Dunnet's "t" test

Histopathological studies

The results were given in fig 4, 5, 6, 7and 8. Histopathological studies of control rat liver treated with carbon tetrachloride exhibited severe necrosis with disappearance of hepatocytes, areas of inflammation and increased sinusoidal spaces (fig 5). Liver section of the rat treated with 150mg of PGERE and carbon tetrachloride exhibited mild degree of necrosis, normalization of cells and reduced sinusoidal dilation (fig 6). Liver section of the rat treated with 300mg of PGERE and carbon tetrachloride exhibited normalization of cells and reduced sinusoidal dilation along with mild inflammogens (fig 7). Liver section of the rat treated with Silymarin and Ccl_4 exhibited normal hepatocytes (fig 8).

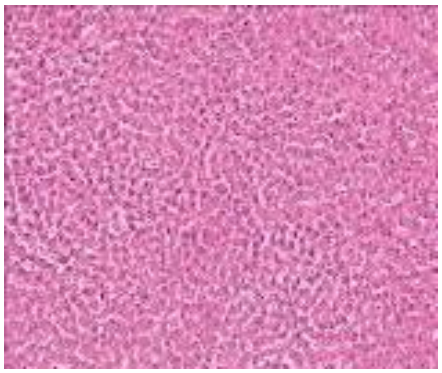

Fig. 4: Normal 40X, Group-I: Treated with liquid paraffin, exhibited normal hepatocytes

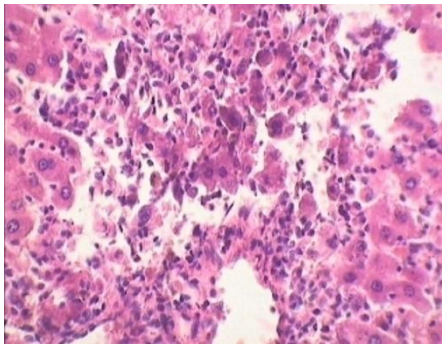

Fig. 5: Control 40X, Group-II: Treated with carbon tetrachloride, exhibited severe necrosis with disappearance of hepatocytes, areas of inflammation and increased sinusoidal spaces

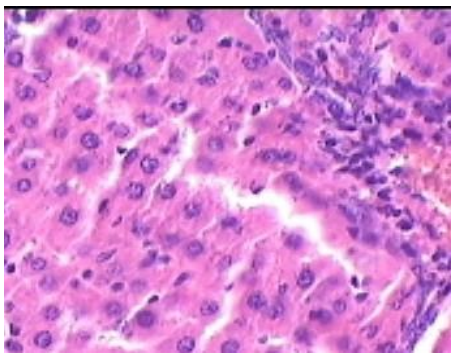

Fig. 6: Low dose 40X, Group III: Treated with 150mg of PGERE and carbon tetrachloride exhibited mild degree of necrosis, normalization of cells and reduced sinusoidal dilation.

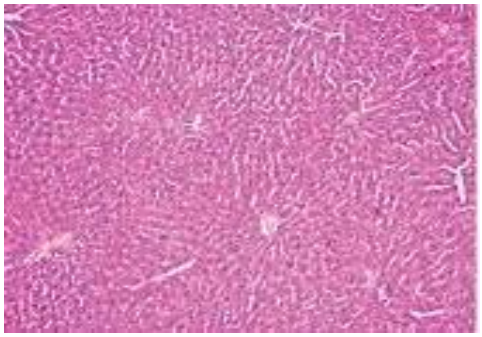

Fig. 7: High dose 40X, Group-IV: Treated with 300mg of PGERE and carbon tetrachloride exhibited normalization of cells and reduced sinusoidal dilation along with mild inflammogens.

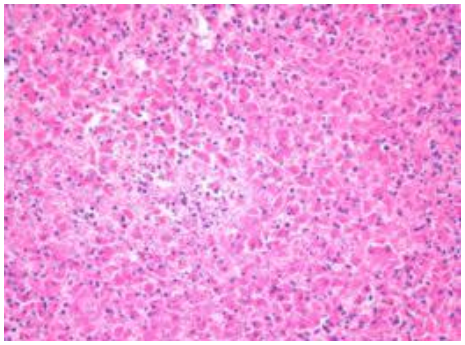

Fig. 8: Standard 40X, Group-V: Treated with Silymarin and CCl4 exhibited normal hepatocytes

DISCUSSION

In the experimental method, the effect of *punica granatum* root extract was comparable with that of standard drug silymarin. *Punica granatum* root extract showed significant hepato protective activity in dose dependent manner and showed decrease in hepatic enzyme levels. From the (Table 1) it was evident that reduced hepatic enzyme levels in treated group animals. It is well established that CCl_4 is metabolized in the liver to the highly reactive trichloromethyl radical and this free radical leads to auto-oxidation of the fatty acid present in the cytoplasmic membrane phospholipids causes functional and morphological changes in the cell membrane[14,15]. From the figures (Fig 1,2 and 3) evidenced by an elevation of the serum marker enzymes namely SGOT, SGPT, and ALP in CCl_4 treated rats. Treatment with the test drug *Punica granatum* (300mg/kg b.wt.) as well as treatment with standard drug silymarin significantly ($P<0.001$) reduced SGOT, SGPT and ALP these levels showing that the *Punica granatum* has hepatoprotective action as shown in (Table 1). Histopathological findings indicated that treatment with *Punica granatum* offered protection to the hepatocytes damage induced by CCl_4 , with mild fatty changes observed in the hepatic parenchymal cells, which corroborated the changes observed in the hepatic enzymes (Fig 6,7 and 8).

CONCLUSION

Ethanolic root extract of *Punica granatum* possesses potent hepato protective activity which is comparable to standard silymarin. It also contained phyto constituents like phenols which are responsible for major pharmacological responses included hepato protective activity. Based on the present study, it can be concluded that ethanolic root extract of *Punica granatum* have potent hepato protective activity and by further studies it can be possible to formulate natural hepato protective drug of ethanolic root extract *of punica granatum.*

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