

Original Article

HEPATOPROTECTIVE EFFECTS OF *PUNICA GRANATUM* FRUIT AGAINST D-GALACTOSAMINE INDUCED HEPATOTOXICITY IN RATS: *IN VITRO* AND *IN VIVO* STUDIES

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ABSTRACT

Objective: Hepatoprotective activity of *Punica granatum* Linn. (Family: Punicaceae) was evaluated by *in vitro* and *in vivo* model. HepG2 cell lines were used for *in vitro* study and D-Galactosamine (D-GalN) induced hepatic damage model for *in vivo* evaluation.

Methods: Hepatoprotective potential was assessed by measuring serum level of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, total bilirubin, and albumin. Enzymatic antioxidant parameters like TBARs (thiobarbituric acid reactive substances), GSH (glutathione), SOD (superoxide dismutase) and CAT (catalase) also evaluated in this study.

Results: Rats treated with D-GalN showed a significant increase in serum levels of SGOT (serum glutamate oxaloacetate transaminase), SGPT (serum glutamate pyruvate transaminase), ALP (alkaline phosphatase), bilirubin and TBARs, reflecting liver damage. The *in vitro* study indicates a beneficial effect of aqueous extract of *P. granatum* in comparison with methanolic extract, on D-GalN induced toxicity to HepG2 cells. On the basis of *in vitro* study, aqueous extract was selected for *in vivo* evaluation. The aqueous extract of *P. granatum* significantly reduced the elevated serum biomarkers, indicating the recovery of hepatocellular injury. It was also observed that D-GalN induced a significant decrease in GSH, SOD, CAT, protein, and albumin level were increased on the treatment of the rats with aqueous extract of *P. granatum*. The findings were also confirmed by histopathological studies. Periportal area and extensive hepatocyte damage and haemorrhage are seen in D-GalN treated group. The portal triad with less degree of inflammatory cell infiltration around the bile duct is seen in *P. granatum* (500 mg/kg)+(D-GalN) treated group.

Conclusion: Results of this study revealed that *P. granatum* fruit could afford a significant protection for the alleviation of hepatic toxicity. Possible mechanism may involve its action against oxidative stress.

Keywords: *In vitro*, Hepatocellular injury, *Punica granatum*, HepG2, D-galactosamine

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INTRODUCTION

The liver is one of the biggest organs in the human body and the chief site for strong metabolism and excretion. So it has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is concerned with almost all the biochemical pathways to growth, fight against the disease, nutrient supply, energy provision, and reproduction [1]. The main functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile, and storage of vitamins. Therefore, to maintain a healthy liver is an essential factor for overall health and well being. But it is continuously and variedly uncovered to environmental toxins, and abused by poor drug habits, alcohol, and prescribed and over the counter drugs which can eventually lead to different liver ailments such as hepatitis, cirrhosis, and alcoholic liver ailment [2, 3]. Thus liver diseases are some of the lethal diseases in the world today. They pose a serious challenge to international public health.

Liver injury can be caused by different agents, such as viruses, chemicals, alcohol, and auto-immune diseases [4]. D-Galactosamine (D-GalN) is a well-established hepatotoxicant, it induces a diffuse type of liver injury closely resembling human viral hepatitis [5] and acute self-limiting hepatitis with necrosis, inflammation and regeneration, resembling drug-induced diseases in humans [6]. The toxicity of D-GalN is mainly related to the depletion of uridine pools that are associated with limited ribonucleic acid (RNA) and protein synthesis, thus altering hepatocellular function [7].

Modern drugs have little to offer for the alleviation of hepatic diseases and it is mainly the plant based preparations which are

employed for their treatment of liver disorders. World Health Organization (WHO) estimated that around 80% of the world's population depends on medicinal plants as their primary health care source. The WHO has reported around 21,000 plants are used for medicinal purpose. Of which 2500 species are in India, among these 150 species are used commercially on a fairly large scale [8].

Punica granatum Linn. (Commonly known as Pomegranate) belongs to the Punicaceae family [9]. It is an extensively used medicinal fruit of indigenous systems of medicine and commercial horticultural product which is usually very well adapted to the Mediterranean climate [10]. Pomegranate fruits are consumed fresh or processed as juice, jellies and syrup for industrial production [11-13]. The edible part of the fruit contains extensive amounts of tannins [14], flavonoids [15], acids, sugars, vitamins, polysaccharides and important minerals [16, 17]. It is proved to have good antioxidant value [18], antibacterial action and anti-inflammatory activity [19] and high power for cancer prevention [20].

In Ayurvedic medicine, the pomegranate is considered "a pharmacy unto itself" and is used as an antiparasitic agent [21], a "blood tonic," [22] and to heal aphthae, diarrhea, and ulcers [23]. *P. granatum* also serves as a remedy for diabetes in the Unani system of medicine practised in the Middle East and India [24].

But still, no scientific investigation has so far been reported in the literature regarding its action on the liver. Therefore, the present investigation has been designed to study the possible mechanism of fruit extract of *P. granatum* for hepatoprotective activity. In this study, we report the activity of *P. granatum* fruit in D-GalN induced toxicity in HepG2 cells and in a rat model.

MATERIALS AND METHODS

Plant collection and identification

The fresh fruit of *P. granatum* was collected from Delhi, India and was authenticated at Department of Botany, Jamia Hamdard, New Delhi, India, with a voucher specimen (PG/FP-367) which was deposited in the herbarium of Jamia Hamdard New Delhi.

Chemicals

All analytical grade chemicals were purchased from SD Fine Chemicals Ltd., Mumbai, India. MEM (minimum essential medium), MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich (USA). HepG2 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Silymarin was purchased from Microlabs; Holar, TN, India. D-GalN was procured from SRL, Mumbai, India.

Preparation of extracts of *P. granatum*

The fruit of *P. granatum* was dried in hot air oven at 40-50 °C for a week. The dried fruit was powdered using mixer grinder. The accurately weighed powdered sample (10 g) was extracted in 100 ml of extraction solvents water and methanol separately. These were kept overnight with occasional shaking and sonicated for 30 min at 25±3 °C in an ultra sonicator water bath followed by filtration and drying using rotary evaporator at 40 °C.

Determination of total phenolics and flavonoids contents

Total phenolic content was determined by Folin-Ciocalteu reagent similar to the method reported by Amir et al., (2011) [25]. The aluminium chloride colorimetric method was used for flavonoids determination as reported [26].

In vitro assay

In vitro hepatoprotective activity was assessed by the ability of an aqueous and methanolic extract of *P. granatum* to preclude the oxidative stress induced by D-GalN in HepG2 cells. HepG2 cells were plated at a density of 1×10^4 cells per well in a 96 well plate along with 100 µl of MEM supplemented with 10% FBS (fetal bovine serum) in each well. Various concentrations of aqueous and methanolic extract (10, 20, and 30 µg/ml) were exposed to HepG2 cells along with D-GalN (50 mmol) for 8 h and the viability of cells was assessed by MTT assay. The hepatoprotective effect of the test sample was assessed by the cell viability assay and expressed as percent protection. Silymarin was used as the reference drug at a concentration of 100 µg/ml [27].

Animals

Wistar albino rats (150-200 g) of either sex were issued from Central Animal House Facility of Hamdard University and acclimatized for a week under standard housing conditions maintained at a room temperature of 24±1 °C; relative humidity 45-55% with 12:12 h light and dark cycle. The institutional animal ethics committee of Hamdard University, New Delhi, India, approved the experimental protocol in accordance with the guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with registration number 173/CPCSEA.

Acute toxicity

Acute toxicity study of aqueous extract was determined as per the OECD guideline No. 423 [28]. Albino rats were divided into test group comprising of six animals in each group. The test was performed using an increasing oral dose of a herbal extract from 100-3000 mg/kg body weight [29]. The rats were observed continuously for 1 h and then half hourly for 4 h for any gross behavioural change and general motor activities like writhing, convulsion, response to tail pinching, gnawing, pupil size, faecal output, feeding behaviour, etc., and further up to 72 h for any mortality. The extract does not cause any significant behavioral changes and no mortality was observed. The extract was devoid of any toxicity in rats when given in dose up to 3000 mg/kg by the oral route.

Hepatoprotective activity

Albino Rats were randomly divided into five groups of six animals each. Group, I served as normal control and received normal saline (1 ml/kg, p. o.) for 21 d. Group II served as toxic control and received normal saline

(1 ml/kg, p. o.) for 21 d. Groups III was prophylactically treated with silymarin (25 mg/kg, p. o.) for 21 d. Groups IV served as per se group and treated with a higher dose (500 mg/kg, p. o.) of aqueous extract of *P. granatum* for 21 d. Groups V and VI were prophylactically treated with aqueous extract of *P. granatum* (250 mg/kg and 500 mg/kg, p. o.) for 21 d, respectively. Groups II, III, V and VI also received D-GalN (400 mg/kg, i. p.) on 21st day [30]. After 24 h of D-GalN administration blood was collected from tail vein under light ether anaesthesia. Immediately, after blood withdrawal, all the groups were sacrificed. Liver samples were also collected for histological and biochemical estimations. The blood samples were allowed to clot for 30-40 min. Serum was separated by centrifugation at 3000 rpm for 15 min and was used for estimation of biochemical parameters. Liver samples collected were washed with chilled normal saline and 10% (w/v) liver homogenates were prepared.

Analysis of biochemical parameter in serum

The activities of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) [31], albumin [32], total bilirubin [33], and alkaline phosphatase (ALP) [34] were assayed.

Analysis of biochemical parameter in hepatic tissue

Lipid peroxidation (LPO) was determined by measuring thiobarbituric acid reactive substances (TBARS) [35]. Glutathione (GSH) level was determined by using Ellman's reagent method [36]. Superoxide dismutase (SOD) [37], catalase (CAT) [38] was analyzed in homogenate hepatic tissue.

Histological studies

Livers were quickly removed and preserved in neutral buffered formalin. Histological liver sections were prepared [39], stained and evaluated by a pathologist blind to grouping details.

Data analysis

Results are expressed as mean±SD. Total variation present in a set of data was estimated by one-way analysis of variance (ANOVA) followed by Dunnet's *post-hoc* test. $P < 0.05$ and $P < 0.01$ were considered to be significant.

RESULTS

The phenolic and flavonoid content in *P. granatum* was found to be 1.86% w/w and 1.84% w/w, respectively. At the dose of 10, 20, and 30 µg/ml of aqueous extract and methanolic extract were used for testing the protective effect on D-GalN induced oxidative damage in the HepG2 hepatic cell line. The results clearly demonstrated that hepatoprotective activity of aqueous extract of *P. granatum* was significant at 20 and 30 µg/ml concentrations ($p < 0.001$) but not at 10 µg/ml of aqueous extract of *P. granatum*. Maximum protection in D-GalN induced oxidative damage was observed at 30 µg/ml as shown in fig. 1. There is no significant observation in methanolic extract of the drug. The protective effect of aqueous extract can be explained with its ability to quench free radicals generated by D-GalN; therefore aqueous extract was selected for *in vivo* study.

Table 1 shows the dose dependent preventive effect of aqueous extract of *P. granatum* against D-GalN induced toxicity. Acute exposure to D-GalN caused a significant increase in the activity of SGOT, SGPT, ALP and bilirubin ($P < 0.01$) into circulation. Treatment with aqueous extract of *P. granatum* at different doses (250 and 500 mg/kg) caused a significant reduction in the elevated levels of serum enzymes in a dose-dependent manner ($P < 0.05$, $P < 0.01$). The aqueous extract of *P. granatum* significantly decreased the elevated levels of serum albumin ($P < 0.05$, $P < 0.01$) towards normal. Aqueous extract of *P. granatum* therapy showed a better response at restoring these serum variables at the higher dose vs lower dose. Table 2 depicts the effect of D-GalN and therapeutic agent on LPO and GSH in the liver. D-GalN induced acute intoxication significantly increased TBARS level ($P < 0.01$) and significantly decreased GSH content, SOD and CAT in hepatic tissue ($P < 0.01$). Therapy with aqueous extract of *P. granatum* significantly lowered TBARS level ($P < 0.01$) and alleviated GSH content, SOD and CAT as seen by statistical analysis ($P < 0.01$). The observations of comparative histopathological studies of liver from each of the groups of rats are

given in fig. 2. Treatment with *P. granatum* was found to be effective in restoring these enzymatic parameters in dose dependent manner.

The 500 mg/kg dose showed maximum recovery in these enzyme activities in experimental animals.

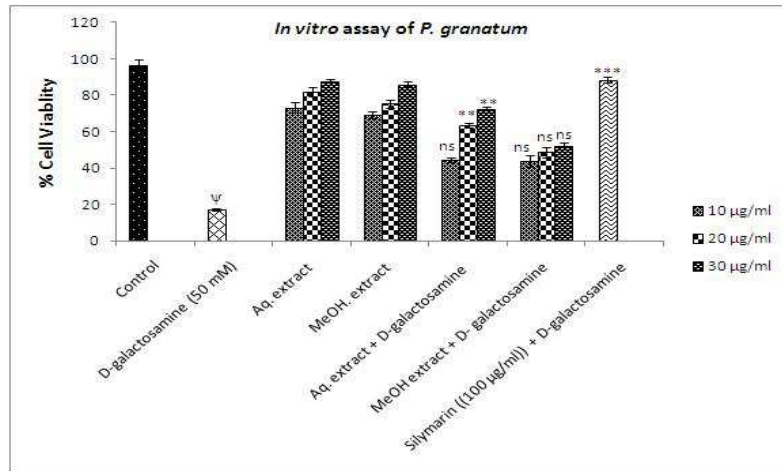


Fig. 1: *In vitro* hepatoprotective activity of an aqueous and methanolic extract of *P. granatum* fruit on D-galactosamine induced hepatic damage in HepG2 cell line, Aq. Extract (Aqueous extract), MeOH extract (Methanolic extract), µg/ml (micrograms per millilitre), mmol (mili mole), Data were presented as mean±SD, No. of experiment (n) =6

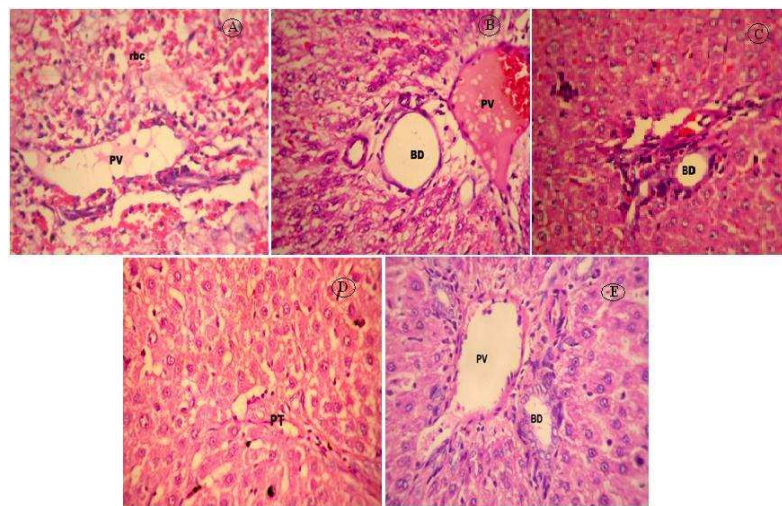


Fig. 2(A): Magnified view of liver from normal control group showing a portal triad with normal structures, PV = Portal Vein, BD = Bile Duct (HE x 400). (B): Magnified view of liver from Toxic Control group showing the periportal area and extensive hepatocyte damage and haemorrhage. PV = Portal Vein (HE x 400). (C): Magnified view of liver from Standard Drug group showing a portal triad and the periportal zone. No evidence of hepatocyte damage is seen (HE x 400). (D): Magnified view of Liver from *P. granatum* (250 mg/kg)+(D-GalN) group showing inflammatory cells in the portal triad (HE x 400). (E) Magnified view of Liver from *P. granatum* (500 mg/kg)+(D-GalN) group showing the portal triad with a moderate degree of inflammatory cell infiltration around the bile duct. PV = Portal Vein, BD = Bile Duct (HE x 400), Data were presented as mean±SD, No. of animals (n) =6

Table 1: Effects of aqueous extract of *Punica granatum* on biochemical parameters in serum. Data were presented as mean±SD, No. of animals (n) =6

Groups	Parameters				
	SGOT (IU/l)	SGPT (IU/l)	Albumin (g/dL)	Total bilirubin (mg/dL)	ALP (KA unit)
Control	42.95±5.36	49.32±5.75	5.85±0.064	0.31±0.04	26.54±5.96
Toxic Control (GalN)	136.55±11.50 [†]	177.70±13.33 [†]	3.46±0.074 [†]	2.34±0.07 [†]	101.71±8.73 [†]
Positive Control (Silymarin)	48.32±6.30 ^{**}	59.64±6.19 ^{**}	5.75±0.070 ^{**}	0.47±0.05 ^{**}	31.73±4.39 ^{**}
<i>P. granatum</i> (500 mg/kg) (per se)	43.86±4.58	51.65±5.91	5.81±0.059	0.35±0.03	28.73±5.17
<i>P. granatum</i> (250 mg/kg)+(GalN)	121.10±8.26 [*]	161.47±10.23 [*]	3.71±0.35	2.05±0.13 [*]	85.97±5.40 [*]
<i>P. granatum</i> (500 mg/kg)+(GalN)	69.79±8.10 ^{**}	91.72±7.58 ^{**}	5.28±0.045 ^{**}	0.67±0.07 ^{**}	40.75±5.29 ^{**}

Significant value: [†] P<0.01 Vs normal control, ^{*}P<0.05; ^{**}P<0.01 Vs toxic control, One way ANOVA followed by Dunnett's Test, SGOT (serum glutamate oxaloacetate transaminase), SGPT (serum glutamate pyruvate transaminase), ALP (alkaline phosphatase), IU/l (international units per liter), g/dL (grams per deciliter), KA unit (king armstrong unit).

Table 2: Effects of aqueous extract of *Punica granatum* on biochemical parameters in hepatic tissue. Data were presented as mean±SD, No. of animals (n) =6

Groups	Parameters			
	TBARS (nanomole of MDA/mg protein)	GSH (µmole GSH/mg protein)	SOD (nanomoles of H2O2 consumed/min/mg protein)	Catalase (U/mg protein)
Control	0.083±0.006	0.86±0.23	6.26±1.07	8.54±0.67
Toxic Control (GalN)	0.212±0.02 ^u	0.292±0.10 ^u	1.80±0.80 ^u	2.05±0.45 ^u
<i>P. granatum</i> (500 mg/kg) (per se)	0.084±0.004	0.82±0.33	6.21±0.81	8.49±0.81
<i>P. granatum</i> (250 mg/kg)+(GalN)	0.172±0.02*	0.457±0.25*	2.93±0.78	2.99±0.73*
<i>P. granatum</i> (500 mg/kg)+(GalN)	0.094±0.02**	0.719±0.30**	5.59±1.15**	6.96±0.89**
Positive Control (Silymarin)	0.087±0.01**	0.788±0.18**	5.99±0.97**	8.11±1.77**

Significant value: ^u P<0.01 Vs normal control, *P<0.05; **P<0.01 Vs toxic control, One way ANOVA followed by Dunnett's Test, TBARS (thiobarbituric acid reactive substances), GSH (glutathione), SOD (superoxide dismutase) and CAT (catalase), MDA (malondialdehyde), U/mg (unit per miligrams).

DISCUSSION

Liver injuries induced by D-GalN are the best-characterized system of xenobiotic induced hepatotoxicity and commonly used models for the screening of antihepatotoxic and/or hepatoprotective activities of drugs. The changes associated with D-GalN induced liver damage are similar to that of acute viral hepatitis [40, 41]. Hence, D-GalN mediated hepatotoxicity was chosen as the experimental model. D-GalN acts directly or indirectly and alters antioxidant status that makes certain organs, more susceptible to oxidative stress. Several studies have shown that D-GalN causes alteration of liver marker enzymes [42]. The toxicity of D-GalN results from inhibition of RNA and protein synthesis in the liver. The metabolism of D-GalN may deplete several uracil nucleotides including UDP-glucose, UDP-galactose and UTP, which trapped in the formation of uridine-diphospho D-galactosamine. Accumulation of UDP-sugar nucleotide may contribute to the change in the rough endoplasmic reticulum and to the disturbance of protein metabolism [40, 41].

Phenolic compounds are known to be powerful chain breaking antioxidants and are important constituents of plants. Phenolic compounds may contribute directly to antioxidative action [43]. It has been recognised that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. Rutin and quercetin are flavonoids which is a very potent antioxidant, present in *P. granatum* fruit [44]. Antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms and thereby help in preventing the free radical induced diseases.

Since the antioxidant activity is important in providing protection against hepatic damage [45], in the present study, we have attempted to demonstrate the hepatoprotective activity of the extract of *P. granatum* through antioxidant activity. The *in vitro* assay provides an indication of effective results for aqueous extract of *P. granatum* fruit as compared to metanolic extract which further helpful in the selection of aqueous extract for *in vivo* study.

In the present research the elevated level of AST, ALT, ALP, and bilirubin levels induced by D-GalN administration was significantly reduced by aqueous extract of *P. granatum* fruit pre-treatment suggesting that its hepatoprotective activity might be due its effect against cellular leakage and loss of functional integrity of the cell membrane. GSH is an important endogenous antioxidant system that is found in particularly high concentration in liver and it is known to have key functions in protective processes. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. Excessive production of free radicals resulted in the oxidative stress, which leads to damage of macromolecules and can induce lipid peroxidation *in vivo*. Elevation of GSH level by *P. granatum* shows its protective effects against oxidative stresses induced GSH depletion [46].

In our study, D-GalN treatment produced the elevation in the levels of TBARS and depletion in GSH, SOD and CAT. Pre-treatment of the rats with aqueous extract of *P. granatum* fruit significantly reduced the elevated levels of TBARS and increased the concentration of GSH, SOD

and CAT. The 500 mg/kg dose of *P. granatum* showed more pronounced effects as compared to other doses (250 mg/kg), as shown by percent protection indicating improved metabolic activity and cellular stability. In summary, this investigation showed that the *P. granatum* fruit at a higher dose (500 mg/kg) possesses considerable protective effect against D-GalN induced liver injury. This protective effect of aqueous extract might be due to its antioxidant property.

CONCLUSION

From the results of the current investigation, it can be concluded that aqueous extract of *P. granatum* fruit has the ability to change the levels of enzymatic antioxidant parameters and significantly increase the endogenous antioxidant defense mechanisms in D-GalN induced hepatotoxicity. Our finding also showed that aqueous extract of *P. granatum* fruit treatment significantly normalizes the changes in serum markers of D-GalN treated rats. From the results obtained, we suggested that aqueous extract of *P. granatum* fruit may be developed as an effective hepatoprotective agent.

AUTHORS CONTRIBUTION

Most of experimental part of the work and writing of the manuscript was done by the first author Mohd Amir. *In vitro* study by MTT assay was done by Mohd Nasar Mallick. Before submission reading and correction of the manuscript was done by Niyaz Ahmad and Abuzer Ali. Design of the research work was done by corresponding author Dr. Mohd Mujeeb (Main Supervisor). Dr. Sayeed Ahmad and Dr. Mohd Akhtar was Co-supervisor.

CONFLICT OF INTERESTS

Declared none

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