Research Article

Hepatoprotective effect of aqueous extracts of root and peel of *Punica granatum* in wistar rats

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Abstract

Objective: To evaluate the hepatoprotective effect of aqueous extracts of root (RAE) and peel (PAE) of *Punica granatum*. **Materials and Methods:** This study was conducted on adult albino Wistar rats of either sex weighing 150-200 g. Animals were divided into five groups (n=5). Liver injury was produced by carbon tetrachloride (CCl_4) 1 ml/kg dissolved in olive oil (1:1) given intraperitoneally on day 1 and day 4 of the study duration of 14 days. Silymarin (50 mg/kg/d) orally was used as standard drug. Test groups received aqueous extract of *P. granatum* root (RAE) at doses of 200 and 400 mg/kg/day and aqueous extract of *P. granatum* (PAE) peel at doses of 200 and 400 mg/kg/day orally along with CCl_4. On the 15th day, the hepatoprotective effect of RAE and PAE was evaluated by assessment of physical parameters, histopathological examination and biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total serum bilirubin in blood sample. **Results:** The administration of RAE of *P. granatum* at doses of 200 and 400 mg/kg/day orally, exhibited a highly significant decrease in the rise of mean serum AST, ALT, ALP, and total bilirubin as compared to CCl_4 treated group ($p<0.001$). PAE 200 mg/kg/day showed highly significant reduction in serum AST, ALT and serum total bilirubin ($p<0.001$). PAE 200mg/kg/day showed significant reduction in ALP ($p<0.01$). PAE 400mg/kg/day showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin when compared to CCl_4 treated group ($p<0.001$). Histopathological examination of the liver also suggested hepatoprotective effect of RAE and PAE of *P. granatum* by restoration of hepatic architecture toward normal. Maximum decrease in the extent of centrilobular necrosis was observed in RAE 400 mg/kg/day treated rats when compared to CCl_4 treated group. **Conclusion:** This study demonstrated hepatoprotective activity of RAE and PAE of *P. granatum* against CCl_4 induced liver injury in rats.

Keywords: *Punica granatum*, Silymarin, hepatoprotective activity, Carbon tetrachloride

Introduction

Liver is a vital organ of body and maintains body's metabolic homeostasis (Cullen, 2005). It is an important site for the metabolism of carbohydrates, proteins and lipids. It synthesizes many regulatory enzymes, hormones and stores many nutrients necessary for the daily housekeeping function of the body (Ramadori et al., 2008). Liver acts as a primary organ for detoxification of endogenous as well as exogenous compounds and it is a multitasking organ working round the clock for maintaining the homeostasis of body (Yadav et al., 2008). Because of the alarmingly increasing number of patients of chronic liver disease worldwide, the study of liver ailments and development of drugs for various liver diseases has become one of the priority areas of research (Corless et al., 1983).

Liver diseases which include infectious, metabolic, autoimmune and drug toxicities are a major cause of concern. India is known to have a large burden of viral hepatitis with limited available national surveillance data (Kumar et al., 2015). Viral hepatitis affects about 400 million people globally and every year 6-10 million people are newly infected (WHO, 2016). The Integrated Disease Surveillance Programme of India's National Center for Disease Control (NCDC) reported 290,000 cases of acute viral hepatitis in India in 2013 (Kosanam et al., 2015). About 1.4 million people die each year from hepatitis, globally (WHO, 2016).

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Liver cirrhosis is a significant cause of global disease burden and mortality (Mokdad et al., 2014).

Drugs and chemicals induce liver injuries which are recognized as significant cause of acute, acute-on-chronic, and less commonly, chronic liver disease (Sonderup, 2011). Several environmental toxins and carcinogens may be converted into reactive intermediates during metabolism by the liver resulting in liver damage. There is increasing evidence that free radicals and reactive oxygen species play a crucial role in various steps that initiate and regulate the progression of liver diseases (Khan et al., 2012). Treatment options for common liver diseases such as drug induced hepatitis, fatty liver and chronic hepatitis are very few. The effectiveness of interferons, penicillamine and corticosteroids given in different liver diseases is inconsistent and these treatments have considerable incidence of side effects (Luper, 1998).

Drugs that have been designed so far to decrease the intensity of liver injury may act by free radical scavenging property (Khan et al., 2018). However, still there is no cure for majority of liver diseases (Roy et al., 2010). In traditional system of medicine, plants are claimed to be effective and used successfully to alleviate liver disorders but evidence for efficacy is sparse (Chaudhary et al., 2010).

Still there is a need to explore the hepatoprotective potential of number of plants. Different models for inducing hepatotoxicity have been used to simulate disease pathology in experimental animals. Carbon tetrachloride (CCl_4) induced liver toxicity is one of the widely used and consistent models for inducing liver injury. It produces acute liver injury which is histopathologically similar to acute hepatitis, hence this model is chosen and liver damage is assessed with the help of physical parameters, liver function tests and histopathological examination.

Punica granatum (Pomegranate) is a medium-sized, deciduous tree, found throughout India, commonly known as Anar. Various parts of *Punica granatum* are used in conditions like diarrhoea, dysentery and worm infestations in indigenous systems of medicine (Khare, 2004; Ayurvedic Pharmacopoeia of India, 2003). Many pharmacological properties of different parts of *Punica granatum* plant viz. anti-inflammatory, analgesic, antiarthritic, antiulcer, anthelminthic, nephroprotective, hepatoprotective, antidiabetic and antioxidant properties have been reported (Bhowmik, 2013; Das et al., 2012). *Punica granatum* was selected on the basis of its folklore claim and experimented for potential hepatoprotection in albino Wistar rats.

Materials and methods

This study was carried out from January 2015 to October 2016 in Department of Pharmacology and Department of Anatomy, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh.

Plant Material

Punica granatum root and peel were obtained from an orchard at Aligarh, Uttar Pradesh, India. They were identified and authenticated by Mr. M. Badruzzaman Siddiqui (Associate Professor) of Department of Botany, Aligarh Muslim University, Aligarh. A voucher specimen was deposited in the herbarium for future reference with number (42931). Parts of the plant (peel and root) were collected, thoroughly washed, chopped, shade dried and pulverized in electric grinder. The powder so obtained was extracted in distilled water.

Preparation of aqueous extracts

The roots and peels were washed and dried under shade for 7 days. They were pulverized into powder using electric blender. 100 gm of finely powdered peel and root were extracted in 300 ml distilled water for 72 hours with the help of Soxhlet apparatus. Thereafter, the mixture was filtered using Whatman No.1 filter paper. The filtrate was concentrated using water bath at a temperature of 50°C; then evaporated to dryness to give a dark brown solid paste. The extracts obtained were collected in Petri dishes and air dried for a week. The dried mass thus obtained was weighed, its yield calculated, sealed with aluminium foils and then stored in refrigerator for further experimental work.

Experimental animals

Adult albino wistar rats of either sex weighing 100-200 grams were obtained from Central Animal House, JNMC, Aligarh Muslim University. The animals were housed in polypropylene cages bedded with paper strips in Pharmacology section of Central Animal House. Animal room was well ventilated and maintained under standard conditions (Temperature 27±3°C and 12 hours light/dark cycle) throughout the experimental period. All animals were fed with standard pellet diet (Ashirvad Industries, Chandigarh) and water ad libitum. They were acclimatized to the laboratory condition for one week prior to the experiments.

Ethical approval for study

The study protocol was approved by Institutional Animal Ethics Committee (IAEC) on 04.03.2015 (Reg. no. 401/RO/C/2001/CPCSEA). All animal experiments were carried out as per the rules and regulations of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) under the "Guidelines for Care and Use of Animals in Scientific Research".

Chemicals and Instruments

Carbon tetrachloride (CCl_4), Silymarin (Silybon

suspension), Formalin (10%), Xylene, Haematoxylin, Paraffin wax, Hydrochloric acid, Soxhlet extraction apparatus, Microtome Machine, Block holder, Slide Warmer, Electronic balance, Microscope

Hepatotoxicity induction

Hepatic damage was induced by method of Dongare et al. (2013). A 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) was given on Day1 and Day 4 at 9:00 am. Standard drug used was silymarin, given in a dose of 50 mg/kg/day by oral route for a period of 14 days (Syed et al., 2014). The test drugs dissolved in distilled water were given by oral route at 1:00 pm for a period of 14 days simultaneously with CCl_4 treatment. The animals were sacrificed on 15th day.

Animal grouping

Animals were divided into 7 groups, consisting of normal control group, negative control group, positive control group and 4 test groups. Each group consisted of five animals of either sex (n=5). Drugs were administered by oral route for 14 days using feeding cannula. In normal control group, only distilled water was given. In negative control group, only CCl_4 was given. Silymarin and CCl_4 were given in positive control group. Test groups were administered with test drugs and CCl_4. On 15th day, rats were sacrificed and blood was collected.

Table 1. Treatment Schedule

Groups (n=5)	Treatment given	Dosage
Normal Control	Distilled water	1ml/100g/day p.o.×14 Days
Negative control	CCl_4	1ml/kg i.p. on day 1 and day 4
Positive control	Silymarin	50 mg/kg/day p.o.×14 Days
RAE (200)	CCl_4	1ml/kg i.p. on day 1 and day 4
	RAE	200mg/kg/day p.o.×14 Days
RAE (400)	CCl_4	1ml/kg i.p. on day 1 and day 4
	RAE	400 mg/kg/day p.o.×14 Days
PAE (200)	CCl_4	1ml/kg i.p. on day 1 and day 4
	PAE	200 mg/kg/day p.o.×14 Days
PAE (400)	CCl_4	1ml/kg i.p. on day 1 and day 4
	PAE	400 mg/kg/day p.o.×14 Days

RAE: Root aqueous extract; PAE: Peel aqueous extract

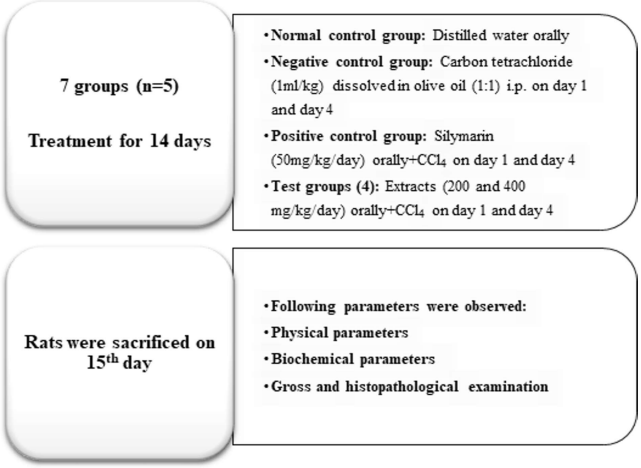

Figure 1. Groups and treatment plan

Blood sample and liver tissue collection

Rats were anaesthetized using Ketamine Xylazine solution. 10ml ketamine HCl (100mg/ml) and 1ml Xylazine (10mg/ml) were mixed in a sterile vial and administered to the rats at a dose of 0.1ml/100g intraperitoneally (Wellington et al., 2013). A line was drawn on the ventral surface of rat's body in midline from a line joining the iliac crest (caudal end) to 2 cm above the sternum (cranial end). Incision was given on the skin at caudal end with the help of a scissors and slowly extended up to the drawn mark at cranial end. Skin flap was rolled out and pinned. Abdominal muscle was incised from caudal to cranial end and ribs were cut on the right side of sternum. Rib cage was then retracted to expose the heart. The diaphragm was incised to give better field for cardiac puncture.

A 5 ml syringe was inserted into the left ventricle and blood was withdrawn with as little pressure as possible (to avoid haemolysis). The gold top labelled vials were kept at 4°C till the final analysis was done (Morton et al., 1993). Blood was centrifuged at 5000 rpm for 10 minutes and plasma was extracted.

After opening the abdomen, liver was visualized in the right upper quadrant. Liver was dissected out after removing its attachments with the help of scissors. Liver was kept in 10% formalin for histopathological examination.

Hepatoprotective effect

Weight of liver of rat in grams

Liver was dissected out and dried gently by using blotting paper. Electronic balance (least count 0.001g) was used to measure wet weight of liver.

Volume of liver of rat in millilitres

Liver was dissected out and hanged with the help of a hook and thread. Liver was then dipped into the measuring cylinder half filled with normal saline. Volume of liver was calculated by measuring the displacement of normal saline in the measuring cylinder.

Biochemical tests

Estimation of Bilirubin was done according to Malloy and Evelyn method (1937). Estimation of Serum Alkaline phosphatase (ALP) was done according to the method of Marsh et al., 1959. Estimation of Serum Aspartate transaminase (AST) was done according to Reitman and Frankel method (1957). Estimation of Serum Alanine transaminase (ALT) was done according to Reitman and Frankel method (1957).

Percentage of hepatoprotection (Khan et al., 2017)

The percentage of hepatic protection shown by ethanolic

and aqueous extracts of root and peel of *Punica granatum* was calculated by using the following formula:

$$H = \left[1 - \frac{(T - C)}{(N - C)}\right] \times 100$$

Where, H = Percentage of Hepatoprotection

T = Mean value of Test Group

N= Mean value of Negative Control group

C = Mean value of Normal control group

Gross and histopathological examination

Dissected liver was grossly examined for any obvious pathology, then preserved in 10% Formalin and processed in the Post Graduate Histology laboratory, Department of Anatomy, J.N.M.C., A.M.U., Aligarh. The sectioned tissue was placed in a plastic tissue cassette, fixed and processed for making blocks.

Statistical analysis

The results were presented as Mean ± Standard Error of Mean (SEM). The groups were compared by One-way analysis of variance (ANOVA) followed by Tukey HSD test to analyse statistical significance. P value of less than 0.05 was considered to be significant.

Results

The aqueous extracts of root and peel of *Punica granatum* were prepared by soxhlet extraction using distilled water. The yield of aqueous extract of root was 5.99% and the yield of aqueous extract of peel was 35.77%.

Physical parameters were evaluated by measuring weight of liver and volume of liver of rat. The weight of rats in all groups was recorded on Day 1 and Day 15 of study. However, no observable change in weight of rats was recorded. There was significant increase in weight of liver as well as volume of liver in negative control group when compared with normal control group ($p<0.001$). Positive control group showed a decrease in weight of liver as well as volume of liver when compared with negative control group ($p<0.05$). RAE 200mg/kg/day showed a decrease in weight of liver and volume of liver as compared to negative control group but the decrease was not significant ($p=0.990$; $p=1.000$). RAE 400mg/kg/day also showed a decrease in weight of liver and volume of liver as compared to negative control group but the decrease was not significant ($p=0.990$; $p=1.000$). PAE 200mg/kg/day showed a decrease in weight of liver and volume of liver as compared to negative control group but the decrease was not significant ($p=1.000$; $p=1.000$). PAE 400mg/kg/day also showed a decrease in weight of liver and volume of liver as compared to negative control group but the decrease was not significant ($p=1.000$; $p=1.000$) (Table 2).

Blood samples were collected on 15th day and serum was analysed for biochemical parameters: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP) and Total serum bilirubin.

Table 2. Effect of aqueous extracts of root and peel of *Punica granatum* (RAE and PAE) on weight and volume of liver of rat

Groups (n = 5)	Weight of liver in g (Mean ± SEM)	Volume of liver in ml (Mean ± SEM)
Normal control	3.86 ± 0.16	3.86 ± 0.16
Negative control	5.40 ± 0.30***	5.30 ± 0.30***
Positive control	4.31 ± 0.08*	4.30 ± 0.05*
RAE (200)	5.10 ± 0.17	5.10 ± 0.12
RAE (400)	5.10 ± 0.11	5.12 ± 0.13
PAE (200)	5.19 ± 0.18	5.36 ± 0.20
PAE (400)	5.26 ± 0.15	5.26 ± 0.13

Negative control group was compared with Normal control group and all other groups were compared with Negative control group, *p< 0.05, **p<0.01 and ***p<0.001 were considered significant.

Table 3. Effect of aqueous extract of root and peel of *Punica granatum* (RAE and PAE) on biochemical parameters

Groups (n = 5)	AST (IU/L)	ALT (IU/L)	ALP (KAU/dl)	Total Bilirubin (mg/dl)
Normal control	35.20 ± 4.45	32.00 ± 6.78	51.00 ± 4.04	0.27 ± 0.01
Negative control	120.00 ± 7.54***	133.60 ± 8.23***	89.40 ± 5.91***	0.42 ± 0.04***
Positive control	46.80 ± 4.75***	53.80 ± 3.53***	55.40 ± 3.12***	0.28 ± 0.01***
RAE (200)	48.20 ± 4.32***	58.60 ± 1.54***	56.80 ± 3.38***	0.30 ± 0.00***
RAE (400)	47.80 ± 4.57***	51.00 ± 7.09***	52.70 ± 2.08***	0.27 ± 0.02***
PAE (200)	52.60 ± 2.84***	56.60 ± 2.38***	63.40 ± 2.84**	0.29 ± 0.01***
PAE (400)	49.40 ± 2.87***	53.80 ± 2.67***	61.00 ± 1.45***	0.28 ± 0.01***

Negative control group was compared with Normal control group and all other groups were compared with Negative control group, * p< 0.05, **p<0.01 and ***p<0.001 were considered significant.

Normal control group which was given only distilled water served as baseline for biochemical parameters as shown in Table 2. There was highly significant rise in AST, ALT, ALP and total serum bilirubin ($p<0.001$) in negative control group when compared with normal control group. Positive control group showed highly significant decrease in AST, ALT, ALP and total serum bilirubin ($p< 0.001$) when compared with negative control group (Table 3). Aqueous extract of root of *Punica granatum,* RAE 200mg/kg/day showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin ($p<0.001$). RAE 400mg/kg/day also showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin when compared to CCl_4 treated group ($p<0.001$) (Table 3). Aqueous extract of peel of *Punica granatum,* PAE 200mg/kg/day showed highly significant reduction in serum AST, ALT and serum total bilirubin ($p<0.001$). PAE 200mg/kg/day showed significant reduction in ALP ($p<0.01$). PAE 400mg/kg/day showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin when compared to CCl_4 treated group ($p<0.001$) (Table 3).

Carbon tetrachloride (CCl_4) induced hepatic damage raised the level of enzymes as serum AST, ALT, ALP and Total serum bilirubin and percentage of hepatoprotection against CCl_4 induced injury was calculated (Syed et al., 2014). Silymarin provided highest percentage of hepatoprotection by reducing AST, ALT, ALP and total bilirubin against CCl_4 induced liver injury. PAE 200mg/kg/day and 400mg/kg/day have shown hepatoprotection against CCl_4 induced liver injury (Table 4). Aqueous extract of root of *Punica granatum,* RAE 200mg/kg/day showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin ($p<0.001$). RAE (400mg/kg/day) also showed highly significant reduction in serum AST, ALT, ALP and total serum bilirubin when compared to CCl_4 treated group ($p<0.001$). RAE provided high percentage of hepatoprotection in serum AST, ALT, ALP and total serum bilirubin against CCl_4 induced liver injury. The hepatoprotection provided by RAE (400mg/kg/day) was comparable to silymarin (Table 4).

Table 4. Percentage of hepatoprotection showed by aqueous extract of root and peel of *Punica granatum* (RAE and PAE)

S. No.	Groups (n = 5)	Percentage of hepatoprotection (%)			
		AST	ALT	ALP	Total Bilirubin
1	Positive control	86.32	79.80	88.54	93.33
2	RAE (200)	84.67	73.82	84.90	80.00
3	RAE (400)	85.14	81.30	95.57	100.00
4	PAE (200)	79.48	75.79	67.70	86.67
5	PAE (400)	83.25	78.54	73.96	93.33

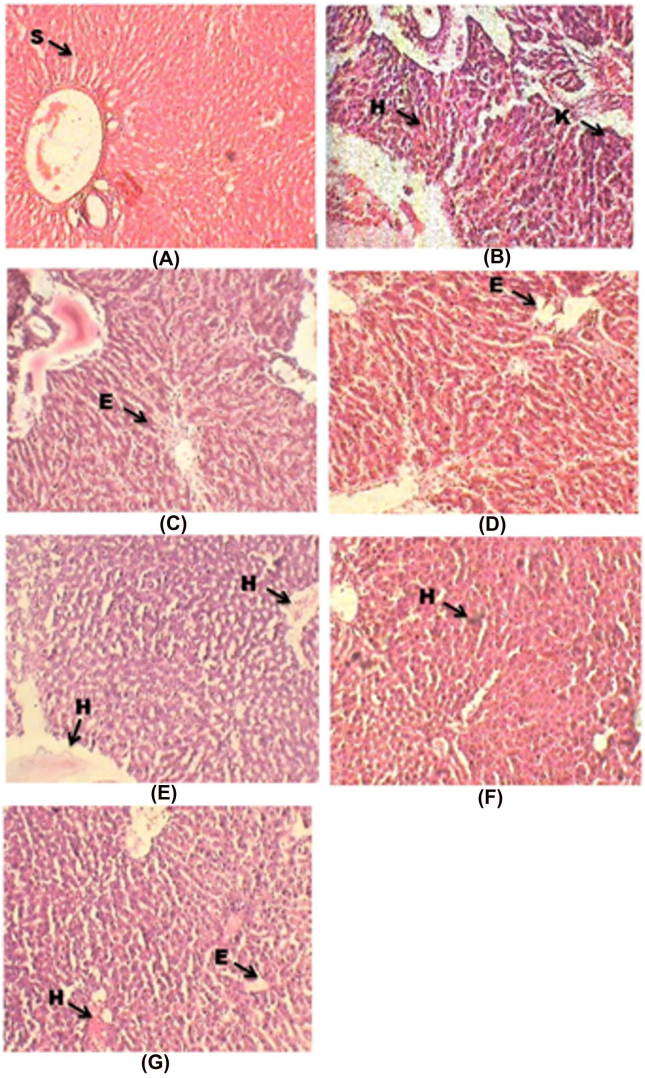

Figure 2. Photomicrograph of histopathology of liver. All sections were stain with H&E, and seen at 10X.

(A) Normal control: Liver section showing central vein surrounded by cords of normal hepatocytes separated by sinusoids (S)

(B) Negative control: Liver section showing hepatocytes having edema, degeneration and necrosis. Sinusoidal hemorrhages (H) are present. RBCs are present in central vein. Few kupffer cells (K) are seen

(C) Positive control: Liver section showing improvement in lobular architecture. Sinusoidal edema (E) and congestion are nearly absent

(D) RAE 200: Liver section showing some edema (E) and congestion in periportal region. Hepatocyte disruption is less. Few kupffer cells are visible

(E) RAE 400: Liver section showing some dilatation of central vein. Lobular architecture and hepatocytes (H) show less disruption

(F) PAE 200: Liver section showing mild disruption of architecture of lobules. Central vein shows dilatation, edema and hemorrhages (H)

(G) PAE 400: Liver section showing lobular architecture which is not well maintained. Sinusoidal and periportal edema (E) is decreased. Few hemorrhages (H) are seen.

Liver of normal control group was brown in colour. Negative control group liver was reddish brown in colour and slightly enlarged. No obvious changes were observed in liver of test groups on external appearance when compared to negative control.

Liver tissue from all groups were analysed histologically. Histological examination of liver from normal control group showed normal architecture of liver. Findings of negative control group were compared with normal control. Histopathology of test groups was compared with negative control group.

Discussion

Liver plays a key role in the maintenance of normal physiology and body's metabolic homeostasis (Sande, 2015). Liver helps in detoxifying chemical compounds before they enter the blood circulation (Maronpot et al., 2010). Human beings are susceptible to injury by these compounds upon exposure to chemical substances present in the occupational environment, consumption of contaminated food or synthetic drugs which are foreign to body organs. All these compounds may produce a variety of toxic manifestations (Sharma et al., 2012). Liver is responsible for detoxification of these substances and therefore xenobiotic induced liver injury can occur (Corless et al., 1983).

Microsomal drug metabolizing enzyme system of liver plays a major role in the detoxification process and performs the function of biotransformation of xenobiotics as well as metabolism of important endogenous substrates (Cederbaum, 2015). Most drugs do not cause damage to liver directly but can lead to the production of reactive metabolites by a process known as bioactivation, thus causing secondary injury to liver (Geubel et al., 2000). Accumulation of chemically reactive metabolites, which if not detoxified, can lead to covalent modification of biological macromolecules resulting in toxicity (Sturgill et al., 1997). Liver is prone to drug induced injury because of its central role in xenobiotic metabolism (Russmann, 2009).

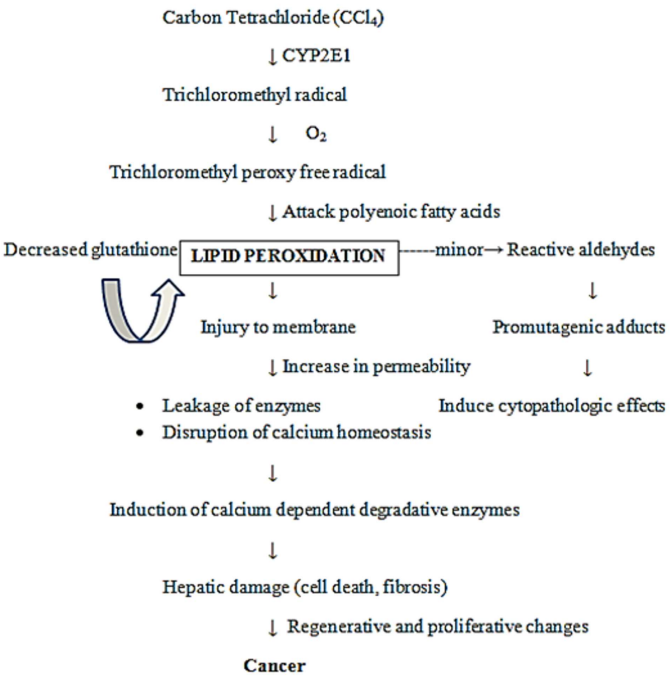

Figure 3. Proposed mode of action for carbon tetrachloride (Manibusan et al., 2007)

Liver diseases are mainly caused by excess consumption of alcohol, infections, autoimmune disorders, toxic chemicals as aflatoxin, carbon tetrachloride, chlorinated hydrocarbons and therapeutic agents. There is no effective treatment for liver ailments other than removal from exposure to the causative agent (Wolf, 1999). The options available for treatment of liver diseases are limited (Singh, 2011). There is a need of effective therapeutic agents with low incidence of side effects. Plant derived products have been used traditionally for the prevention and treatment of liver diseases worldwide. Clinical researches have confirmed the efficacy of several plants in the treatment of liver disease (Luper, 1998). Therefore, many researchers have tried to explore the hepatoprotective effects of a variety of plants (Pandey et al., 2014). Basic scientific research has uncovered the mechanisms by which some plants produce their therapeutic effects. The hepatoprotective agents protect the liver against oxidative stress and damage, facilitate regeneration by proliferation of hepatic parenchymal cells and arrest growth of fibrous tissue (Fraschini et al., 2002).

Carbon tetrachloride (CCl_4) is a well-known toxicant and exposure to this chemical is known to induce oxidative stress by the formation of free radicals (Figure 3). Carbon tetrachloride induced hepatotoxicity results from its bioactivation which leads to formation of reactive intermediates (CCl_3*, CCl_3OO*). These reactive intermediates covalently bind to macromolecules, produce lipid peroxidation resulting in membrane injury and leak of cytosomal enzymes (Manibusan et al., 2007).

Disruption of cell membrane integrity of hepatocytes is accompanied by release of liver enzymes into circulation, which forms the basis for analysis of serum enzymes (Amacher, 1998). Aminotransferases are normally present in hepatocytes and are found in low concentrations in serum. These enzymes are released into blood in large amounts when there is damage to liver cell membrane leading to increased membrane permeability. Altered levels of aminotransferases are sensitive indicators of liver cell injury (Lee, 2003). In liver, alkaline phosphatase (ALP) enzyme is localized to microvilli of bile canaliculus. ALP level is usually elevated in intrahepatic cholestasis as cholestasis leads to injury of canalicular membrane and

transporters. Bilirubin conjugation and excretion takes place in liver. Therefore, metabolic and excretory functions of liver can be assessed by estimating total serum bilirubin levels. Elevated total serum bilirubin in patients with xenobiotic induced liver disease indicates severe liver injury (Pratt et al., 2005). The efficacy of any hepatoprotective drug is dependent on its capability of protecting against harmful effects that are produced by the offending agents (Gole et al., 2002).

In the present study, aqueous extracts of peel and root of *Punica granatum* were given in different doses along with CCl_4 administration.

Weight of liver and volume of liver of rat were increased in negative control group as compared to normal control group, indicating congestion and engorgement of liver produced by CCl_4 and these changes were highly significant ($p<0.001$) (Table 2). RAE and PAE in doses of 200mg/kg/day and 400mg/kg/day showed decrease in weight and volume of liver but the reduction was not significant when compared to negative control group (Table 2).

Estimating the activities of serum marker enzymes like Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) is a useful quantitative marker of the extent and type of hepatocellular damage. Elevated levels of hepatic Alkaline phosphatase in blood is a feature of obstructive jaundice. The tendency of these enzymes to decrease in plant extract administered group gives an indication of hepatoprotective activity of the plant extract. Simultaneous fall in the level of raised total serum bilirubin suggests the improvement of the biliary function after CCl_4 induced liver injury (Rajendran et al., 2009).

In this study, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP) and total serum bilirubin were found to be significantly elevated ($p<0.001$) after CCl_4 administration (Table 3) indicating acute hepatocellular damage and biliary obstruction. These findings were similar to studies done by Ramakrishna et al., 2011 and Surendran et al., 2011.

Levels of serum AST, ALT, ALP and total serum bilirubin were found to be significantly decreased in silymarin (50mg/kg/day) treated group ($p<0.001$) (Table 3). Silymarin provided hepatoprotection by decreasing levels of AST by 86.32%, ALT by 79.80%, ALP by 88.54% and total bilirubin by 93.33% (Table 4). Silymarin has been proven to show hepatoprotective properties in different models of hepatotoxicity (Sharma et al., 2012; Roy et al., 2010). The hepatoprotective effect offered by silymarin is probably due to membrane stabilizing action, free radical scavenging property, inhibition of lipid peroxidation, modulation of hepatocyte Ca^{2+}, enhanced detoxification, protection against glutathione depletion, promotion of ribosomal RNA synthesis and stimulation of liver regeneration (Salam et al., 2007).

Aqueous extract of root of *Punica granatum* (RAE) 200mg/kg/day and 400mg/kg/day significantly decreased the raised level of liver enzymes and total bilirubin ($p<0.001$) (Table 3). Percentage hepatoprotection offered by RAE 200mg/kg/day resulted in decrease of AST by 84.67%, ALT by 73.82%, ALP by 84.90% and total bilirubin by 80.00% (Table 4). Percentage hepatoprotection offered by RAE 400mg/kg/day resulted in decrease of AST by 85.14%, ALT by 81.30%, ALP by 95.57% and total bilirubin by 100% (Table 4).

Aqueous extract of root of *Punica granatum* showed highly significant hepatoprotection by decreasing the level of raised liver enzymes and bilirubin. Aqueous extract of root of *Punica granatum* has probably been evaluated for the first time for its hepatoprotective activity in the present study.

Aqueous extract of peel of *Punica granatum* (PAE) 200mg/kg/day and 400 mg/kg/day produced highly significant reduction in serum AST, ALT, ALP and total bilirubin ($p<0.001$) (Table 3). PAE 200mg/kg/day provided hepatoprotection by decreasing the level of AST by 79.48%, ALT by 75.79%, ALP by 67.70% and total bilirubin by 86.67% (Table 4). PAE 400mg/kg/day provided hepatoprotection by decreasing the level of AST by 83.25%, ALT by 78.54%, ALP by 73.96% and total bilirubin by 93.33% (Table 4). Khalil, 2004 has also reported hepatoprotection with aqueous extract of peel of *Punica granatum* in acetaminophen treated rats.

Aqueous extract of peel offered protection against CCl_4 induced liver injury by decreasing the elevated levels of serum AST, ALT, ALP and total bilirubin.

On gross examination, normal liver possessed brown colour but liver of negative control group (CCl_4 treated rats) was slightly enlarged and reddish brown in colour. These findings may be due to edema and congestion. No remarkable changes in colour and size of liver were observed in test groups when compared with negative control.

Comparative histopathological study of liver from different groups of rats corroborated the hepatoprotective efficacy of *Punica granatum*. Histological study of liver of normal control rats showed normal architecture of hepatic lobules, hepatocytes arranged in rows, normal components of portal triad and normal central vein and sinusoids (Figure 2A) whereas the liver of CCl_4 treated rats showed disruption of hepatic architecture, edema and congestion in lobular sinusoids, edematous, degenerated and necrosed hepatocytes, dilated central vein, presence of few kupffer cells and inflammatory cells (Figure 2B).

Burk et al., 1983. reported that CCl_4 is metabolized to CCl_3* in large quantities by cytochrome P-450 enzyme system in the poorly oxygenated centrilobular regions of liver. There is much greater metabolism of CCl_4 under low oxygen tension and CCl_3* is converted to CCl_3OO* which can bind covalently and can cause lipid peroxidation. As metabolic activation of CCl_4 takes place in centrilobular region, this region suffers more injury due to formation of hepatotoxic metabolites.

In present study, liver of rats treated with standard drug silymarin showed normal hepatocytes, reduced sinusoidal edema and congestion, normal hepatic architecture and absence of inflammatory cells (Figure 2C) which is comparable with liver of normal control group. Similar histopathological findings in liver of silymarin treated rats have also been reported previously (Freitag et al., 2015; Salam et al., 2007).

Aqueous extract of root of *Punica granatum,* 200mg/kg/day treated group showed decreased sinusoidal edema and less disruption of hepatocytes, decreased periportal edema and dilatation of components of portal triad (Figure 2D). RAE 400mg/kg/day treated groups showed normal architecture of components of portal triad with reduced periportal edema, nearly normal architecture of hepatocytes, less dilatation of central vein and decreased sinusoidal edema and congestion (Figure 2E).

PAE 200mg/kg/day treated group showed slight disruption of lobular architecture, decreased sinusoidal edema and less disruption of hepatocytes, periportal edema, dilatation of central vein and few hemorrhages (Figure 2F). PAE 400mg/kg/day treated groups showed less components of portal triad showing dilatation, reduced periportal edema, nearly normal architecture of hepatocytes, less dilatation of central vein and decreased sinusoidal edema and congestion (Figure 2G).

Administration of *Punica granatum* with CCl_4 caused marked improvement in histology of liver section of rats treated with *Punica granatum* as compared to that seen in rats administered only CCl_4, indicating the possibility of inducing accelerated regeneration of liver by *Punica granatum*, reduction in centrilobular necrosis and fatty infiltration and thereby improvement of hepatic architecture.

Aqueous extract of root and peel of *Punica granatum* in doses of 200mg/kg/day and 400mg/kg/day showed comparable hepatoprotective activity. Therefore, this study suggests that aqueous extract of root and peel of *Punica granatum* possess the potential of protecting liver from peroxidative damage in carbon tetrachloride treated rats.

RAE 200mg/kg/day and PAE 200mg/kg/day showed comparable hepatoprotective activity as shown by highly significant decrease in biochemical parameters i.e., serum AST, ALT, ALP and total serum bilirubin when compared with CCl_4 treated group (Table 3). RAE 400mg/kg/day and PAE 400mg/kg/day also showed comparable hepatoprotective activity as evident by highly significant decrease in biochemical parameters i.e., serum AST, ALT, ALP and total serum bilirubin when compared with CCl_4 treated group (Table 3).

Qnais et al. (2007) reported that phytochemical analysis of the aqueous extract of *Punica granatum* peels revealed the presence of flavonoids and alkaloids. Several compounds have been isolated from aqueous extracts of *Punica granatum* such as anthocyanins, tannins, saponins, terpenoids, polypeptides, lectins, punicalagin, ellagic acid, hydroquinone pyridinium, delphinidin, cyanidin and pelargonidin. Punicalagin and tannins have antiproliferative and antioxidant activities (Noda et al., 2002).

Ellagitannins including punicalin, punicalagin and numerous piperidine alkaloids were also isolated from roots of *Punica granatum* (Rosenblat et al., 2006; Neuhofer et al., 1993).

Polyphenols are bioactive compounds which are the largest group among natural antioxidants (Tiwari et al., 2011). Many polyphenolic compounds have been identified that include mainly flavonoids, phenolic acids, lignans, stilbenes, coumarins, tannins, xanthones and chromones (Pandey et al., 2009). Flavonoids are divided into six major subclasses: flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones. Plant polyphenols are non-nutritive, hydrophilic components found in small amounts in plant derived products (Singh et al., 2000). Polyphenols have beneficial pleiotropic effects on health, protecting against development and progression of diabetes, ageing, neurodegenerative diseases, where the role of oxidative stress is implicated. As antioxidants, polyphenols may provide protection against oxidative damage and therefore decrease the risk of development of diseases associated with oxidative stress (Uzma et al., 2011; Pandey et al., 2009).

Root and peel of *Punica granatum* have been reported to have phenolic punicalagins, gallic acid, catechin, quercetin, rutin, flavonols, flavones, flavonones and anthocyanidins on phytochemical analysis (Osman et al., 2011). Previous studies have also reported that peel of *Punica granatum* possesses free radical scavenging property (Ali et al., 2014). Presence of antioxidants in root and peel of *Punica granatum* may be responsible for protection against CCl_4 induced liver injury.

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Conflicts of interest: None

Ethical consent

The study protocol was approved by Institutional Animal Ethics Committee (IAEC) on 04.03.2015 (Reg. no. 401/RO/C/2001/CPCSEA). All animal experiments were carried out as per the rules and regulations of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) under the "Guidelines for Care and Use of Animals in Scientific Research".

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