IJPT
1735-2657/15/142-35-40
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 14: 35-40, 2015

ORIGINAL ARTICLE

Effects of *Pistacia Vera* Hydro-Alcoholic Extract on Carbon Tetrachloride-Induced Hepatotoxicity in Male Rats

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Received October 27, 2014; Revised February 4, 2015; Accepted February 17, 2015

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

Oxidizing agents play a major role in the pathogenesis of liver diseases. Pistacia vera (P. vera), contains many antioxidant substances including coenzyme 10, vitamin E and beta-carotene. The current study was designed to evaluate the probable effects of P. vera on carbon tetrachloride (CCl4)-induced hepatotoxicity in male rats. Frothy male Wistar rats were randomly divided into five groups including normal, hepatotoxic (CCl4) and P. vera 10, 50, and 100 mg/kg + CCl4. P. vera extract (p.o., daily for 4 weeks) and CCl4 (1 ml/kg two times during the first week of study, 50% v/v in olive oil, i.p.) were administered to the animals. Serum analysis was performed to assay the levels of aspartate aminotransferase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP), total protein (TP), high density lipoprotein (HDL) and low density lipoprotein (LDL). Hepatic necrosis and inflammation were evaluated by histopathological examination (hematoxylin and eosin staining) of liver section. CCl4-induced hepatotoxicity was evidenced by considerable increase in serum levels of ALT, AST and LDL ($p< 0.05$). Also, administration of CCl4 induced congestion in central vein and lymphocyte infiltration. Gavage of hydro-alcoholic extract of P. vera (10, 50, 100 mg/kg) significantly decreased mentioned indices ($p<0.05$). Histological analysis demonstrated that gavage of different doses of extract significantly decreased inflammation and tissue necrosis ($p< 0.05$). Our findings indicate hepatoprotective effects of the hydro-alcoholic extract of P. vera on CCl4-induced hepatotoxicity in rats.

Keywords: *Hepatotoxicity, Pistacia vera, Rat, Carbon tetrachloride*

Liver is the largest organ in the body that performs various functions including metabolism of nutrients and detoxification of toxic and chemical agents [1]. Despite the tremendous advancements in the treatment of liver diseases, hepatic disease remains as an unsolved health problem that has high mortality rates [1-2]. Drug-induced liver injury is known as a major cause of acute liver failure [3-4]. In general, hepatotoxicity of a drug is in the form of direct injury caused by the drug itself or its intermediate metabolites [4]. Drug -induced liver toxicity is a major challenge for pharmaceutical industry that is often noted as the commonest single phenomenon of the drugs.

Carbon tetrachloride (CCl_4) is a potent liver toxin that causes serious central lobular necrosis and is used to induce experimental liver injury in laboratory animals [5]. CCl_4-induced hepatotoxicity is characterized by steatosis, necrosis and increased levels of liver enzymes [6]. Previous studies have evidenced the conversion of CCl_4 to a free radical named three chloromethyl (CCl_3) by cytochrome P_{450}. subsequently, CCl_3 stimulates kupffer cells to further produce and release other free radicals and consequently result in oxidative stress and liver injury [7-9].

Pistacia vera (*P. vera*) belongs to the family of Anacardiaceae and is a plant native to Iran. *P. vera* has

attracted researchers interest because of various nutritional and medicinal properties that are found in its leaves, seeds and resins. including antioxidant, antimicrobial and anti-inflammatory activities that are likely attributable to its rich flavonoid contents [10,11]. In addition, *P. vera* is a rich source of phenolic compounds that are well known for their antioxidant properties [10,12-13]. Moreover, several studies have already confirmed promising antioxidant activities of herbal extracts (like red lentil or crocus sativus stigmata) that prevent and/or cure hepatotoxicity induced by different toxins [14, 15].Here in this study, the hepatoprotective effects of hydro-alcoholic extract of *P. vera* seeds on CCl_4-induced hepatotoxicity is thoroughly investigated in male Wistar rats.

MATERIALS AND METHODS

Animals

Forty male Wistar rats (200-250 g) were purchased from Rafsanjan University of Medical Sciences. The animals were housed under 12 h dark/light cycles, the temperature was set at 25°C and all animals were fed *ad libitum* until the end of study. Animal treatments and interventions were approved by Animal Ethics Committee of Rafsanjan University of Medical Sciences.

Preparation of Pistachio extract

Dried *P. vera* fruits (pistachio) from *Akbari* species with genetic code of *M30* were purchased from an herbal pharmacy in Rafsanjan, Iran. The plant originality authenticated by Hamid Alipour; an expert teacher in Pistachio Research Institute of Iran. Pistachio were ground into fine powder. The amount of 300 g of the pistachio powder was mixed with 900 ml ethanol (80%), as solvent. The mix was macerated and incubated for 12 h at 50°C. The extraction process was repeated three additional times using slag from the previous stage. The vehicle of the extract was evaporated using rotary evaporator (Rotavap, England). The extract was frozen and stored at -20°C and then dissolved in dimethyl sulfoxide 2.5% (DMSO, Sigma-Aldrich, Germany), and further diluted with DW at 1:9 ratio, and administered to the proposed animals through gavage.

Induction of hepatotoxicity by CCl_4

CCl_4 was purchased from Merck Company (Germany). The stock solution was diluted at 1:1 ratio (50%) using olive oil, and administered intraperitoneally at dose of 1 ml/kg, 2 times per week during the first week of study (Saturday and Wednesday).

Animal grouping and treatments

After 1 week resting, the male Wistar rats were randomly divided into 5 groups of 8 animals. Group 1(control) received no interventions. Group 2 (hepatotoxic) received CCl_4 as explained above. Groups 3–5 (the extract groups) received CCl_4 plus three doses of 10, 50 and 100 mg/kg/day extract respectively (p.o., daily for 4 weeks).

Sampling

Histopathologic examination and measurement of biochemical factors in blood samples were done to evaluate liver function. Ketamine (90 mg/kg) and xylazine (10 mg/kg) were used as sedative agents. Blood samples were taken from retro-orbital vein 48 h after the last treatment and centrifuged at 3000 rpm for 15 min to separate serum. Aspartate aminotransferase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP), total protein (TP) were assayed using standard procedures. Serum high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were determined using biochemical methods. For histopathological evaluation, the animals were kept under anesthesia and their livers were aseptically removed.

Histopathology

The livers were immersed in 10% neutral buffered formalin solution for 1 week for tissue fixation. Two millimeters thick sagittal sections was prepared from each liver and further processed using a tissue processor (Sukara Fine Technical, Japan). Subsequently, 4 to 5 µm thick sections were cut using paraffin microtome (SLEE Medical Mainz, German) and stained with Hematoxylin and Eosin (H&E). The liver sections were observed by experienced pathologists using light microscope (Olympus CH30, Japan) and then graded for the intensity of inflammation and necrosis. All slides were coded and introduced to the pathologist in a blind manner. The intensity of inflammation and necrosis in portal and lobular spaces were scored from 0 to 3. For inflammation, score of 0 indicatesno lesions and score of 3 indicates the most severe inflammatory cells infiltration. The same criteria was applied (0: No necrosis, 1: mild necrosis, 2: moderate necrosis and 3: severe necrosis) for scoring the intensity of necrosis [16].

Statistical Analysis

SPSS version 18 statistical software was used for statistical comparisons. Results were reported as mean±SEM. Differences between the groups were determined using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. LSD post-hoc test was performed for analysing the differences in serum level of LDL among groups. P value less than 0.05 was considered statistically significant.

Published online: July 12, 2015

Table 1. Histopathology findings and blood factors in rats

Measured index	**Study groups**				
	Control	***CCl_4- induced Hepatotoxicity***	***CCl_4+Pistachio 10mg/kg***	***CCl_4+Pistachio 50mg/kg***	***CCl_4+Pistachio 100mg/kg***
Inflammation	0.14±0.14	2±0 [a]	0.71±0.28 [b]	0.33±0.16 [b]	1±0.23 [b]
Necrosis	0±0	0.75±0.25 [a]	0.28±0.18 [b]	0±0 [b]	0.5±0 [b]
AST (U/l)	156.6±4.3	257.6±44.6 [a]	169.4±11.7 [b]	123.2±10.1 [b]	173.6±9.8 [b]
ALT (U/l)	89.6±5	142.6±33.5 [a]	87.4±2 [b]	81.5±2.7 [b]	95.0±3.9 [b]
ALP (U/l)	453.3±44.5	345.2±34.5	437.4±50.2	419.4±31.6	447.1±28.4
HDL (mg/dl)	36±1.1	39±3.7	35.2±2	35.6±2.1	35.1±1.8
LDL (mg/dl)	39±1.7	48.6±5.8 [a]	36.4±1.3 [b]	30.3±2.2 [b]	38.6±1.1 [b]
TP (g/dl)	7.0±0.24	6.9±0.17	6.9±0.1	7.3±0.1	7.0±0.07

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TP: Total protein. [a] Significant differences ($p< 0.05$) versus control. [b] Significant differences ($p< 0.05$) versus CCl_4.

RESULTS

Blood markers of hepatotoxicity

Following intraperitoneal application of two doses of CCl_4; AST, ALT and LDL levels were significantly increased in comparison with control group (all $p<0.05$). However, the changes in ALP, HDL levels and TP were not significant (all $p> 0.05$). Also, serum ALT, AST and LDL levels were significantly lower in the treatment groups that received 10, 50 and 100 mg/kg hydro-alcoholic extracts of the pistachio (all $p< 0.05$), as shown in Table 1.

Histopathology of liver sections

H&E staining was used (Fig 1) to evaluate the effects of pistachio extract on the histological changes in the liver, Injection of CCl_4 induced severe pathological changes, including inflammation and necrosis (1B). Four weeks daily treatment of CCl_4-exposed rats with pistachio extract led to remarkable decrease in pathological changes of liver (Fig. 1C-E). A scoring system was implemented for quantification of pathological changes. Statistical comparison between the studied groups showed that the amount of inflammation and necrosis have significantly increased in the animals when exposed to CCl_4 in comparison to control mice ($p< 0.05$). Mean scores of inflammation and necrosis among pistachio extract-treated rats (groups 3-5) were significantly lower than those of the hepatotoxic group (all $p< 0.05$). The greatest decrease in the rate of hepatic injury was related to rats treated with 50 mg/kg dose of the extract, as shown in Table 1.

DISCUSSION

The liver is the main organ in the body that functions as a detoxifying and neutralizing any exogenous or endogenous toxic materials. The total capacity of the liver for scavenging of toxic materials is limited and a serious oxidative stress would occur when an excessive amount of free radicals is produced. The liver is the first target tissue for exogenous toxic materials passing through intestinal mucous membranes and therefore is prone to damage induced by the toxins. Drug-induced liver injury is the major cause of acute or chronic liver failure. In the plant-derived materials are the major source of antioxidants. In fact, ingestion of plant-based foods can help our body to better combat with continuously produced free radicals and further potentiate scavenging capacity of the liver. *P. vera* is a valuable plant that grows in Iran and its leaves, seeds and resins contain many anti-oxidative and anti-inflammatory materials. The obtained results show that oral administration of hydroalcoholic extract of *P. vera* on experimentally-induced hepatotoxicity improves liver functional factors, including serum ALT, AST and LDL levels, and histologic signs of the hepatotoxicity, including inflammation and necrosis. It has been observed that intraperitoneal administrations of CCl_4 in rats increases serum ALT, AST activities and LDL levels when comparing to normal animals. Banda et al., have also reported similar findings after hepatotoxicity in rats induced by CCl_4. They have also reported severe pathological changes in the liver after CCl_4 exposure that are consistent with our observations [17]. It is demonstrated that CCl4 is primarily metabolized by P450 cytochrome enzyme complex to produce a $CCl_3^{\bullet}$ free radical. The $CCl_3^{\bullet}$ radicals strongly react with lipid membranes of the hepatic cells and subsequently causes tissue necrosis and release of hepatocyte specific enzymes into the circulation. Rahmani et al. have also observed a significant increased lipid peroxidation in CCl_4-exposed animals. Malondialdehyde was used as a marker of lipid peroxidation in the experiments [14]. We also found that treatment of hepatotoxicity in rats with the pistachio hydro-alcoholic extract during four

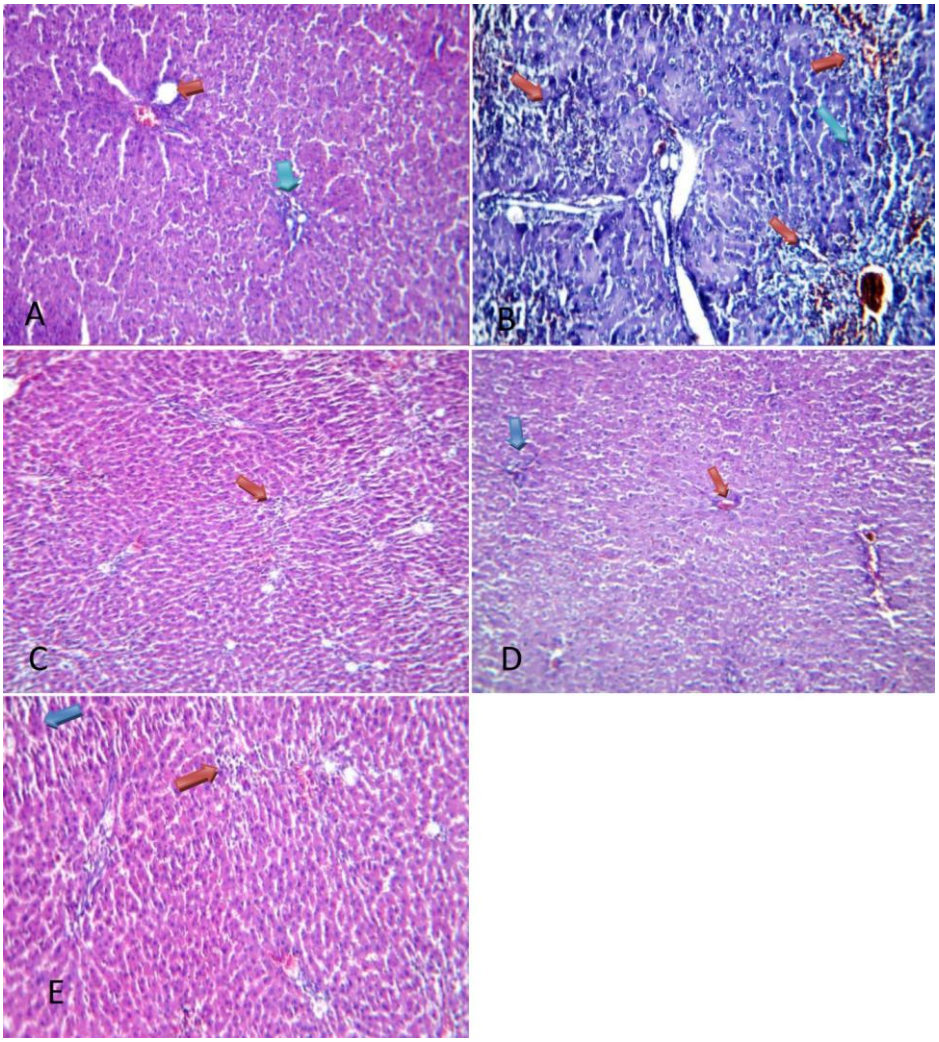

Fig 1. Effect of *P. vera* extract on CCl_4-induced hepatotoxicity in rats (100× magnification and H&E staining). **A**: H&E stain of liver section from normal group showing normal lobular structure, red arrow points to central vein and blue one to portal space; **B**: Liver section from CCl_4-exposed group (not treated with extract) showing severe inflammation, central necrosis and marked congestion, although, infiltration of inflammatory cells and regenerative hepatic cells are also evident, red arrows points to necrosis and blue one to regenerative hepatic cells; **C**: Liver section from CCl_4-exposed rats treated with 10 mg/kg dose of *P. vera* extract showing few inflammatory cells, red arrow points to inflammation; **D**: Liver section from CCl_4-exposed rats treated with 50 mg/kg dose of *P. vera* extract lacking evidences of hepatocellular necrosis or infiltration of inflammatory cells, red arrow points to central vein and blue one to portal space ; **E**: Liver section from CCl_4-exposed rats treated with 100 mg/kg dose of *P. vera* extract showing moderate inflammation and necrosis, red arrow points to inflammation and blue one to necrosis.

weeks reduce ALT, AST activities and LDL levels the serum.. The best outcomes were related to mice receiving 50 mg/kg dose of the extract.

Several other plant extracts have also been reported to have considerable therapeutic effects on liver injury induced by chemical agents. for example, administration of poly phenolic extracts from chicory (Cichorium intybus) resulted in wholly normalization of the serum AST and ALT levels in mice exposed to thioacetamide, a hepatotoxic organosulfur compound [18]. Rafiei et al. have also reported similar effects from barberry extract upon administration to CCl4 induced hepatotoxic animals [19]. These plants have notable amounts of antioxidant substances with hepatoprotective effects. The pistachio has also antioxidant properties that may be attributable to its flavonoid and polyphenolic contents [20-21]. Moreover, previous studies have reported that pistachio elicits significant antioxidant activity similar to the synthetic antioxidant [22-24]. Parvardeh and colleagues have studied the hepatoprotective effect of hydro-alcoholic extract of P. vera resin in rats. They administered the resin extract before exposure of rats to CCl_4 and the preventive treatment resulted in reduced serum ALT levels in mice, although AST levels remained high. However, in our study therapeutic administration of Pistachio extract for four weeks in the rats with hepatotoxicity resulted in significant reduction in both serum ALT and AST levels. Pathologic observations of liver sections were also consistent with the results of blood indices. Our data indicates that the hepatic inflammation and necrosis of hepatotoxic rats treated

Published online: July 12, 2015

with 10, 50 and 100 mg/kg doses of the extract were significantly different from that of the group with hepatotoxicity.

As previously noted and similar to the results achieved for other plants in the literature, our observations and findings can be attributed to the antioxidant ingredients of Pistachio that probably inhibit lipid peroxidation and consequently inhibition of oxidative stress. Therefore, the cell membranes remain intact and as a result cells are prevented to enter the necrosis step [25-27]. It is worth to mention that there were no significant changes in serum ALP, HDL levels and TP concentrations of all studied groups.

CONCLUSION

Hepatoprotective effects of the hydro-alcoholic extract of *P. vera* on CCl_4-induced hepatic damage in 40 male Wistar rats were observed in the present study. Probably, antioxidative properties of the extract helped hepatic cells to obviate CCl_4-induced necrosis and inflammation. The results obtained here and the reports from previous studies suggest that Pistachio extract may function as a good candidate for the treatment or prevention of liver failure. However, further investigations are required to unveil the molecular identification of the active ingredients and elucidation of the mechanisms involved in the effect.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Published online: July 12, 2015