Original Article

The effects of pumpkin seed oil on carbon tetrachloride induced chronic hepatic damage in rats

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Abstract

The present study determined the possible protective effect of pumpkin seed oil (PSO) on histopathological changes in liver tissue and serum alanine aminotransferase (ALT) activity, glucose, Non-Esterified Fatty Acids (NEFA) and triglyceride (TG) levels on chronic liver injury induced by carbon tetrachloride (CCl_4) in rats. A total of 80 Wistar rats were divided into eight groups of ten rats each: the first group served as the control, group II was given intraperitoneal 0.2 mL/kg CCl_4, twice a week for 8 weeks. Groups III-V were treated daily with PSO for 8 weeks (1, 2 and 3 mL/kg respectively). Groups (VI-VIII) were administered with intraperitoneal CCl_4 (0.2 mL/kg) twice a week and simultaneously PSO for 8 weeks (1, 2 and 3 mL/kg, respectively). Groups VII and VIII showed a partial decrease of steatosis in the hepatocytes while the findings in the Group VI were similar to Group II. Serum glucose, TG and NEFA levels, which were increased as a result of liver toxicity, decreased significantly with the treatment of 1, 2 and 3 mL/kg PSO. In addition, serum ALT activity also decreased in CCl_4 groups given 2 and 3 mL / kg PSO.As a result, using of PSO at a dose of 3 mL/kg is more effective than 1 and 2 mL/kg on the some biochemical and histopathological changes in CCl_4 induced liver damage. Further investigations on dosage and duration that produce the best result without side effects, need to be performed.

Keywords: Biochemical parameters, hepatotoxicity, histopathology, pumpkin seed oil, rat

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Received March 4, 2020.
Accepted July 20, 2020.

Introduction

The liver, in which the adverse and toxic effects of xenobiotics are most evident, plays a central role in their metabolism and excretion. Hepatic dysfunction or liver damage caused by excessive drug or xenobiotic exposure is known as hepatotoxicity and these chemicals are called hepatotoxins (Evans, 2009; Singh and Gupta, 2011; Khan *et al.,* 2012), one among them is carbon tetrachloride (CCl_4) (Kodavanti *et al.,* 1989; Bouhrim *et al.,* 2018).

Carbon tetrachloride is a well-known experimental model compound for producing chemical hepatic injury in laboratory animals (Evans, 2009; Bouhrim *et al.,* 2018; Breikaa *et al.,* 2013; Atasever and Yaman Gram, 2014). This hepatotoxin is transformed to trichloromethyl and proxy chloromethyl free radicals by hepatic microsomal cytochrome P450 and these free radicals react with many biologically important organic substances such as fatty acids, proteins, lipids, nucleic acids and amino acids (Khan *et al.,* 2012; Mahmoudzade *et al.,* 2017; Al-Assaf, 2013; Osman *et al.,* 2011).

Many natural compounds have gained much attention for the physiological functions involved in the detoxification process. In various studies (Andjelkovic *et al.,* 2010;; Fruhwirth and Hermetter, 2007; Murkovic *et al.,* 2004;), pumpkin seed oil which is one of these compounds and contains unsaturated fatty acid, antioxidant, proteins, mineral and phytosterols has been shown to have estrogenic, antiviral, antibacterial, antifungal, antihelminthic, anticarcinogenic and antioxidant effects as well as liver protective effects.

Some researchers (Mohammed et al., 2009; Aal, 2014; Seif, 2004; Ahmed *et al.,* 2015) have stated that PSO has an ameliorative effect on histopathological changes in liver toxicity. On the other hand, limited literature (Al-Assaf, 2013; Ohda *et al.,* 2006; Althnaian *et al.,* 2013; Abdel-Moneim *et al.,* 2015) has been found on the effect of the treatment of pumpkin seed oil on changes in biochemical parameters in liver toxicity. Therefore, this study focuses on an evaluation of the effectiveness of pumpkin seed oil on some biochemical (serum alanine aminotransferase activity and glucose, triglyceride, non-esterified fatty acid levels) and histopathological parameters on carbon tetrachloride-induced hepatotoxicity in rats.

Materials and Methods

Animal Model: Eighty male Wistar albino rats of body weight ranging from 250 to 300 g obtained from Erciyes University Faculty of Medicine, Hakan Cetinsaya Experimental and Clinical Research Center were used for the study. The animals were housed five per cage in a room under the appropriate conditions [controlled temperature (21 ± 2°C), humidity (50 ± 5%), air exchange (12 rpm) with 12-h of light/dark cycle] and were provided rodent chow and water *ad libitum*. They were maintained in accordance with the Guidelines for Animal Experimentation approved by Erciyes University, Experimental Animal Ethics Committee (permit no: 16/052 dated 09.03.2016).

Dose selection: Carbon tetrachloride at a dose of 0.2 mL/kg, 1:1 mixture with corn oil, twice a week for 8 weeks, which is well documented to induce chronic hepatotoxicity in rats (Khan *et al.,* 2012) was used in the study. Eraslan *et al.,* (2013) was taken as a reference while determining the dose of PSO. The dosage range was chosen 1, 2 and 3 mL/kg/body weight since there was no exact dose determination in chronic studies before with PSO.

Experimental Model: A total of 80 Wistar rats were divided into eight groups of ten rats each: the first group was used as a control which was given only intraperitoneal 0.9% NaCl (0.2 mL / kg / bw); group II was given intraperitoneal CCl_4 at a dose of 0.2 mL/kg, 1:1 mixture with corn oil, twice a week for 8 weeks. Groups III-V were daily treated with PSO through gavage directly to the stomach for 8 weeks (1, 2 and 3 mL/kg respectively). Groups VI-VIII were administered with intraperitoneal CCl_4 (0.2 mL/kg) twice a week and simultaneously PSO by gavage for 8 weeks (1, 2 and 3 mL/kg oral respectively).

Collecting of tissue and blood samples: All treated animals were anesthetized by ketamine (intramuscular [IM], 50 mg/kg) and xylazine (IM., 10 mg/kg) injection and then sacrificed by cervical dislocation 24h after the last administration of CCl_4. Systemic necropsy was performed after opening the chest cavity and collecting blood samples intracardially.The blood samples were centrifuged at 1300 g for 10 mins and the sera were stored in the -20°C before they were analyzed. Following fixation in a neutral formalin solution (10%), liver tissue specimens were thoroughly rinsed overnight under tap water. Then, all tissue samples were dehydrated in graded alcohol, cleaned in xylene and embedded in paraffin wax and sectioned (thickness, 5 µm), for histopathological evaluation. After staining with hematoxylin and eosin sections were examined under a light microscope.

Liver damage scoring method: Following hematoxylin and eosin staining all sections were semiquantitatively evaluated for hepatocyte steatosis, inflammation, necrosis and fibrosis.All liver samples were evaluated using ten different places in each section for the aforementioned parameters by two pathologists and the mean percentile values within the group were calculated. Steatosis, inflammation, necrosis and fibrosis were graded as 1 (mild, <33% of liver cells), 2 (moderate, 33% to 66% of liver cells), and 3 (severe, >66% of liver cells) (Schwimmer *et al.,* 2005). The values obtained in each group were evaluated statistically and the statistical significance between the groups was recorded (Table 1).

Biochemical Analysis: Serum ALT activity was determined with spectrophotometer (Shimadzu UV Model 1208) using commercial kits (Biolabo 80127, France). Serum glucose, triglyceride (TG) and Non-Esterified Fatty Acid (NEFA) levels (Randox, GL364, TR213, FA115, UK, respectively) were also determined with autoanalyser (RX Monaco, United Kingdom) using commercial kits.

Statistical Analysis: When the liver inflammation, steatosis, necrosis and fibrosis score values were compared between the groups, Kruskal Wall's test was performed with the Bonferoni corrected Mann Whitney U test where the difference was significant. Statistical analyses were carried out using SPSS 20. For biochemical data, one-way analysis of variance (ANOVA) was used for the differences between groups. When the F values were significant, Duncan's Multiple Range Test was performed. All data was expressed as means ±SD. A value of $P< 0.05$ was considered significant.

Table 1 Scoring system for hepatic damage in CCl_4 treated groups.

	Control (%25-%75)	CCl_4 group (%25-%75)	1 mL/kg PSO (%25-%75)	2 mL/kg PSO (%25-%75)	3 mL/kg PSO (%25-%75)	CCl_4+ 1 mL/kg PSO group Median (%25-%75)	CCl_4+ 2 mL/kg PSO group Median (%25-%75)	CCl_4+ 3 mL/kg PSO group Median (%25-%75)	P value
Inflammation	0	2 (2-3)^a	0	0	0	2 (2-2)^a	2 (1-2)^{ab}	1 (1-2)^b	0.003
Steatosis	0	2 (1.75-2)^a	0	0	0	2 (1.75-3)^a	2.5 (2-3)^a	1 (1-2)^b	0.008
Necrosis	0	0 (0-1)^a	0	0	0	1 (1-1)^b	1 (1-1)^b	1 (1-1)^b	<0.001
Fibrosis	0	1 (0-1)	0	0	0	1 (1-1)	1 (1-1)	1 (0-1)	0.313

Results

Histopathology results: Our group reported earlier, as part of this study (Atasever *et al*., 2020), livers from animals administered only PSO (Groups III-IV) and the control group (Group I) showed a normal liver histological architecture (Fig. 1). Histopathological examination of Group II displayed intensive macro- and micro lipid vacuoles in the cytoplasms of hepatocytes (Fig. 2a). Necrotic changes in the hepatocytes around central veins resulted in pink homogenous mass formation. Lymphocyte-rich mononuclear cell infiltrations especially close to the portal area and Kupfer cell hyperplasia in all parenchyma were observed (Figure 2b).

In Group VI, necrosis in hepatocytes, fatty changes in cytoplasm and cell infiltrations were also similar to Group II (Fig. 2c, d). The severity of mononuclear cell infiltration and steatosis in Group VII (Figure 2 e, f) and Group VIII (Figure 2 g, h) were less than Group II. No deaths were observed during the study.

Biochemical parameters: There was no difference in serum ALT activity between the control and the 1-3 mL/kg PSO given groups. The highest serum ALT activity was determined only in CCl_4 treated group and CCl_4+1 mL/kg PSO treated groups (P<0.001). The ALT enzyme activity increased by CCl_4 administration and evaluated as an indicator of liver function was not affected by 1 mL/kg PSO application. However, the administration of 2 and 3 mL/kg PSO administration to CCl_4 treated groups significantly reduced the high ALT activity resulting from CCl_4 administration (P <0.001). Additionally, there was no statistically significant difference between CCl_4+3 mL/kg PSO treated group and the control group in terms of ALT activity (Table 2) (Atasever *et al*., 2020).

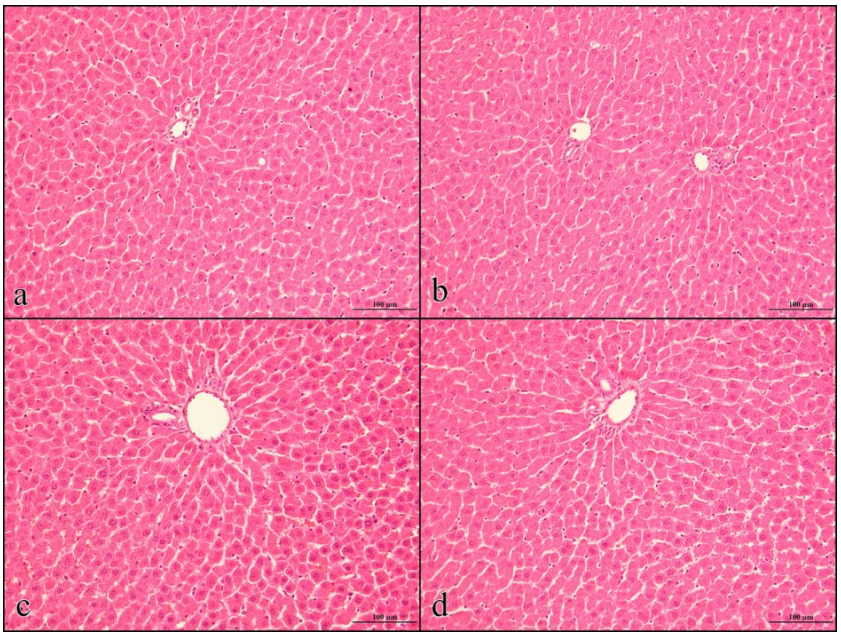

Figure 1 Normal appearance of the livers of the control (a) and PSO treated groups at doses of 1 (b) 2 (c) and 3 (d) mL/kg. HxE, Magnification x100.

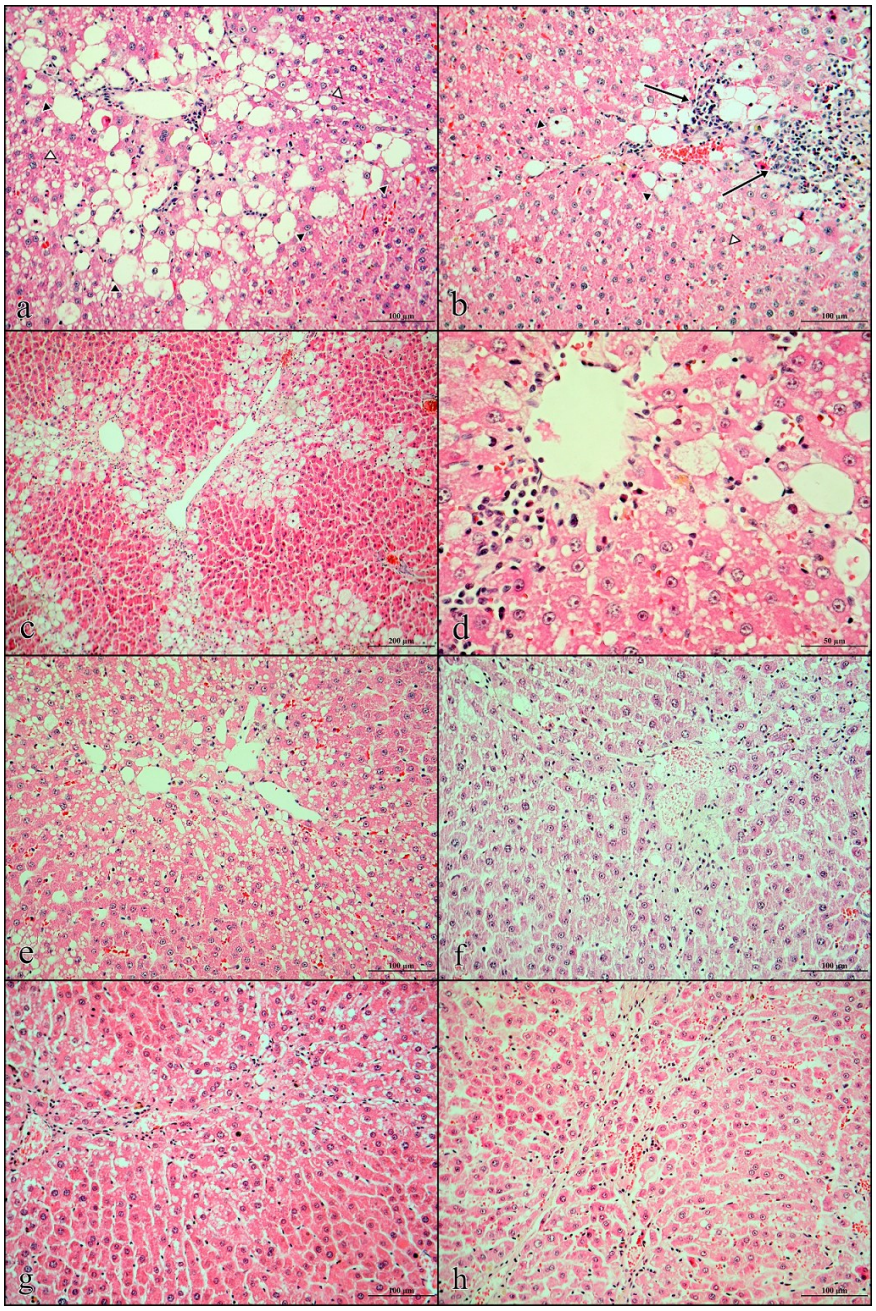

Figure 2 The appearance of micro (white arrowheads) and macro (black arrowheads) lipid vacuoles in all parenchyma and infiltrating mononuclear cells (arrows) in CCl_4 (a, b), and CCl_4+1 mL/kg PSO (c, d) treated groups and the severity of mononuclear cell infiltrations and steatosis were less in CCl_4+2 mL/kg PSO (e, f) and CCl_4+3 mL/kg PSO (g, h) treated groups. HxE, Magnification x100.

Table 2 Effects of PSO on serum ALT activities, Glucose, Triglyceride and NEFA levels of rats in control and CCl_4 treated groups.

	Reference ranges	Control	CCl_4 group	1 mL/kg PSO	2 mL/kg PSO	3 mL/kg PSO	CCl_4+1 mL/kg PSO	CCl_4+2 mL/kg PSO	CCl_4+3 mL/kg PSO	P value
ALT (U/L)	10-80 (Evans, 2009)	49.2 ± 2.4^{cd}	138.4 ± 17.7^{a}	39.6 ± 2.6^{d}	32.6 ± 3.0^{d}	39.8 ± 3.4^{d}	147.1 ± 6.1^{a}	93.1 ± 10.5^{b}	71.9 ± 9.8^{bc}	P <0.001
Glucose (mg/dL)	140-220, (Evans, 2009)	187.8 ± 2.6^{c}	239.6 ± 8.7^{a}	156.6 ± 3.4^{e}	156.8 ± 4.4^{e}	164.0 ± 4.6^{de}	211.6 ± 8.3^{b}	193.1 ± 4.7^{c}	177.0 ± 3.0^{cd}	P <0.001
Triglyceride (mg/dL)	49.7-197.7 (Evans, 2009)	111.0 ± 6.0^{b}	193.1 ± 4.7^{a}	126.3 ± 4.2^{b}	86 ± 7.9^{c}	86 ± 6.1^{c}	80.6 ± 8.5^{cd}	61.5 ± 4.7^{de}	54.3 ± 10.1^{e}	P <0.001
NEFA (mmol/L)	1.34±0.20 (Abdel-Moneim *et al.*, 2015)	0.97 ± 0.16^{b}	2.14 ± 0.15^{a}	1.24 ± 0.05^{b}	1.33 ± 0.3^{b}	1.40 ± 0.29^{b}	0.74 ± 0.16^{b}	0.88 ± 0.21^{b}	0.86 ± 0.31^{b}	P <0.01

(n:10, PSO: pumpkin seed oil, $^{a\text{-}e}$: the difference between groups in the same line with different letters is statistically significant)

When compared with the control group; it was found that all levels of PSO significantly decreased serum glucose levels (P <0.001) but did not affect NEFA levels. Also, serum TG levels were significantly decreased (P <0.001) in the 2 and 3 mL/kg PSO groups compared to the control and 1 mL/kg PSO groups.

In the same way, increased serum glucose, TG (P <0.001) and NEFA (P <0.01) levels due to liver damage in rats decreased significantly with 1-3 mL/kg PSO administration. There were also dose dependent decreases in serum glucose and TG levels. However, in terms of serum NEFA levels, no statistically significant difference was observed between CCl_4 groups treated with 1-3 mL/kg PSO.

Discussion

The liver plays a major role in the regulation of many blood constituents by metabolism, storage or excretory mechanisms. Some of its function indicators are enzymes, glucose and lipids. Many enzymes with increased activity in serum are considered to be indicative of hepatocellular damage as well as cellular degeneration or necrosis. However, alterations in serum or plasma glucose and lipid levels are also considered as indicators of liver damage (Evans, 2009; Singh and Gupta, 2011; Boone *et al*., 2005; Gad, 2007). Serum ALT, SDH and GDH activities are the best predictors of hepatocellular injury in rats. Of these enzymes, ALT is usually the most easily identified standard liver enzyme by most laboratories (Singh and Gupta, 2011; Gad, 2007). The increased serum enzyme level of ALT is an indicator of cellular damage and the functional integrity of liver cell membranes as supported by the pathological findings, characterised by steatosis related to hepatocellular damage consistent with the findings of some researchers (Plaa, 2000; Karagul *et al.,* 2000). Damage to hepatic cells causes a leakage of liver-specific enzymes into the blood (Al–Harbi and Al-Hasawi, 2014). Our group reported earlier, as part of this study (Atasever *et al*., 2020), ALT activity increased significantly in CCl_4-treated rats as indicated by several studies (Bouhrim *et al.,* 2018; Ahn *et al*., 2014; Li *et al.,* 2018, Yaman Gram *et al*., 2018).

In this study, pathological findings observed in the liver showing intense macro and micro lipid vacuoles in the cytoplasm of hepatocytes may be caused by alterations of glucose and lipid metabolism as reported by several researchers (Singh and Gupta, 2011; Bouhrim *et al.,* 2019). It was suggested that serum glucose levels increased with CCl_4 intoxication in rats (Khan *et al.,* 2012; Mahmoudzade *et al.,* 2017; Yaman Gram *et al.,* 2018, Martha *et al*., 2009). Similarly, in this study, serum glucose levels increased only in rats treated with CCl_4. The increase in serum glucose levels may result from a decreased secretion of insulin and amylase activities as a result of interference in the metabolic pathways of carbohydrates in CCl_4 induced hepatotoxicity (Khan *et al.,* 2012; Mahmoudzade *et al.,* 2017; Gram *et al.,* 2018). Elevated plasma concentrations of glucose and fatty acids may promote hepatic fatty acid and triglyceride uptake and synthesis and impair β-oxidation (Evans, 2009). Ohda *et al.,* (2006) reported that serum triglycerides and NEFA levels increased in naphthylisothiocyanate-induced hepatotoxicity in rats. In another study (Al-Assaf., 2013)) increases in serum TG and NEFA levels were found due to changes in lipid metabolism in CCl_4-induced hepatotoxicity in rats.

Some researchers (Althnaian *et al.,* 2013, Abdel-Moneim *et al.,* 2015) stated that CCl_4 intoxication caused a significant increase in the levels of triglyceride, cholesterol and NEFA levels in rats. In accordance with the findings of the above researchers (Al-Assaf, 2013; Ohda *et al.,* 2006; Althnaian *et al*., 2013; Abdel-Moneim *et al.,* 2015) in this study, the increases in serum triglyceride levels may result from the reduction of lipase activity, which can lead to decrease in triglyceride hydrolysis (Althnaian *et al.,* 2013). And also, serum NEFA levels increased in CCl_4 intoxication in the present study. Increases in NEFA levels are probably due to oxidative lipid degradation that can increase the synthesis of other major lipids and activate NADPH or NADH-dependent microsomal peroxidation (Abdel-Moneim *et al.,* 2015).

In this study, 1-3 mL/kg PSO treatment also ameliorated the metabolic function of the liver by restoration of the glucose, triglyceride and NEFA levels in serum to normal value in comparison with CCl_4 group. Pumpkin seed oil has been reported to increase the activity of the beta cells of the pancreas, leading to increased insulin secretion, which is effective in lowering blood glucose levels (Abd-elnoor, 2019). Triglyceride of rats received CCl4 and PSO was lower in group VI-VIII than those with either PSO or CCl_4 alone in this study. These results suggest that PSO may have a hypolipidemic effect, affecting energy metabolism in the presence of stress in rats. And also, administration of 2 and 3 mL/kg PSO to CCl_4 groups caused a significant decrease in serum ALT activities. However, administration of 2 and 3 mL/kg of PSO to CCl_4 groups resulted in a decrease in severity of mononuclear cell infiltration and steatosis in the liver parallel to the findings of researchers (Mohammed et al., 2009; Aal, 2014; Seif, 2004; Ahmed *et al.,* 2015) showing that liver damage was inhibited by the PSO and the number of fat vacuoles in the hepatocytes and the inflammatory cells in the portal area decreased due to the amount of pumpkin seed oil given maybe due to the liver-protective effects of polyphenol and the unsaturated fatty acid content in pumpkin seed oil.

On the whole, it can be concluded that PSO may have a beneficial effect on some biochemical and histopathological alterations induced by CCl_4. Use of PSO at a dose of 3 mL/kg is more effective than 1 and 2 mL/kg on the histopathological changes of CCl_4 induced liver damage. Further studies are needed to observe the effects of different doses of pumpkin seed oil on hepatotoxicity.

Acknowledgements

This study was supported by the Fund of Erciyes University Scientific Research Projects (Project no: TCD-2016-6791).

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