Effect of Flax Seed Oil on Acute Carbon Tetrachloride-Induced Hepatic Injury and Determination of Hepatic Apoptosis in Rats

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ABSTRACT

Aims: The present study was designed to evaluate the hepatoprotective activity of flaxseed oil (FSO) on liver lesions induced by carbon tetrachloride (CCl_4) in rats by measurement of caspase 3, 8 and 9 activities in cellular apoptosis, ALT activities, triglyceride, total protein, total cholesterol and liver MDA levels.

Place and Duration of Study: Faculty of Veterinary Medicine, Department of Pathology, Erciyes University, Kayseri, between June 2017 and July 2018

Methodology: In this study 32 male Wistar albino rats were divided into four groups ~~including~~ of 8 animals in each. The first group was identified as the control and received 0.9% NaCl and the second group was given 4 ml/kg FSO by gavage for 4 weeks. The third group received an intraperitoneal dose of 1.0 ml/kg CCl_4 twice in the first week. The fourth group received an intraperitoneal dose of 1.0 ml/kg CCl_4 twice in the first week and simultaneously 4 ml/kg FSO by gavage for 4 weeks.

Results: Histopathological examination of CCl_4 group showed intense macro and micro vesicular steatosis in hepatocytes, necrosis, lymphocytes rich mononuclear cell infiltration in portal area and parenchyma. The flaxseed oil application did not ameliorate the histological changes induced by CCl_4, however reduced the activity of caspase 3, 8 and 9 by a limited number. CCl_4 administration produced significantly elevated levels of serum ALT activity, total cholesterol, triglyceride and liver MDA levels, and these increases were not normalized with FSO treatment. In addition, decreased serum total protein levels in CCl_4 treated group were ameliorated by FSO application.

Conclusion: The results indicate that the antioxidant properties of FSO do not have an ameliorative effect in either the histopathological lesions or biochemical parameters against CCl_4-induced hepatotoxicity in rats. In addition, it was concluded that duration-dependent further research results are needed to determine the effects of flaxseed oil in high doses that can give the best results without side effects.

Comment [WU1]: State the route of administration

Comment [WU2]: Change to Per os to sound more scientific

Keywords: Histopathology, immunhistochemistry, carbon tetrachloride, flaxseed oil, rat.

1. INTRODUCTION

Liver disease is considered a major health problem in the world, as the liver is an important organ that when exposed to toxic substances and other various factors can be damaged [1, 2]. Carbon tetrachloride (CCl_4) ~~is~~ has been used to induce acute and chronic hepatotoxicity and manifests its effects at biochemical and cellular organelle level [3, 4]. Free radical derivatives result from the formation of oxidative stress and produce lipid peroxidation by

acting on unsaturated fatty acids in the cell membrane [3, 4, 5, 6]. Blocking or delaying the reaction of the oxidation chain is one of the strategies used to prevent oxidative stress-induced hepatotoxicity. Therefore, intake of oxygen radical scavengers such as antioxidants may be a good defense mechanism for hepatoprotection.

Apoptosis is triggered by a successive activation of caspases dividing the “death substrates" required in nonapoptotic cells for processes such as cell cycle control, DNA repair, cell signaling and structural integrity. Caspases represent a group of cysteine proteases that are activated by proteolytic division when a cell is found to have inactive proenzymes and decides to commit a solitary apoptotic suicide [7, 8, 9]. The intrinsic caspase-9 and extrinsic caspase-8 apoptotic pathways both contribute to the activation of caspase-3 that leads to apoptosis [8, 10]. There is a histopathological increase in caspase 3 activation in CCl_4-induced liver toxicity [7, 11, 12].

Phenolic substances, including flavonoids, cinnamic acid derivatives, coumarins, tocopherols and phenolic acids, are the most important groups of natural antioxidants [13, 14]. Some plants such as rosemary, sage, oregano, flaxseed oil, garlic, olive leaf, pomegranate seed and tea extracts are used as natural antioxidant sources to prevent lipid peroxidation due to the phenolic compounds in their contents [15, 16].

This study aimed to determine the effects of FSO, which is known to have various biological activities, on CCl_4 induced hepatic damage by assaying serum ALT activity, triglyceride, total protein, cholesterol and liver MDA levels as well as the Immunohistochemical analyses of apoptosis by caspase 3, caspase 8 and caspase 9 activities of liver tissues in rats.

2. MATERIAL AND METHODS

2.1. Materials

Flaxseed oil (FSO) used in the study is commercially available from BUKAS (Industry and Trade. Inc. Izmir/Turkey) and its components are shown in Table 1.

Table 1. Fatty acid composition of the flax seed oil used in the experiment.

Saturated Fatty Acid	**Percentage**
Palmitic Acid	5.11
Palmitoleic Acid	0.07
Margaric Acid	0.07
Stearic Acid	3.19
Unsaturated Fatty Acid	**Percentage**
Oleic Acid (Omega 9)	16.33
Linoleic Acid (Omega 6)	16.04
Linolenic Acid (Omega 3)	58.86
Arachidic Acid	0.11
Eicosenoic Acid	0.10
Behenic Acid	0.05
Total	**100**

2.2. Animals

Experiments were performed using 32 adult male Wistar albino rats weighing 200–250 g weighing. The experiments were carried out in accordance with the Guidelines for Animal Experimentation approved by the Erciyes University, Experimental Animal Ethics Committee (permit no: 16/008), and the experimental procedures were performed in Erciyes University Experimental Research and Application Center in Kayseri, Turkey. The animals were kept in a special room at a constant temperature of 22°C ± 2°C and controlled humidity (50% ±5%) with 12-h light/dark cycles and had free access to diet and tap water.

2.3. Experimental protocol

The rats were divided into 4 groups, each containing 8 animals. The first group (control group) were administrated with 0.9% NaCl (1 mL/kg); second group was given 4 mL/kg FSO through gavage for 4 weeks each day. The third group was injected with CCl_4 (1 mL/kg, 1:1 mixture with corn oil) (Merck, France, 1.02222) twice in the 1st week. The fourth group, were administered with CCl_4 (1 mL/kg, 1:1 mixture with corn oil) twice ~~twice~~ in the 1st week and simultaneously 4 mL/kg FSO through gavage for 4 weeks.

Comment [WU3]: State route of administration

2.4. Collection and processing of samples

The rats were anesthetized with intramuscular 80 mg/kg ketamine (alfamine, 100 mg/mL, Ata-Fen, Turkey) and 12 mg/kg xylazine (alfazyne, 20 mg/mL, Ata-Fen, Turkey) injection [17] 24 hrs after the last CCl_4 application. After the chest cavities were opened, intracardiac blood samples were taken and placed in anticoagulant and coagulant tubes and necropsies were performed. Blood samples were centrifuged at 3000 rpm for 10 min and then the serum and plasma were separated and stored at -20°C until analyses were done. All tissue samples were placed in a 10% buffered neutral formalin solution for light microscopic examination [18]. A portion of the liver tissue was stored at -80°C until the day of study to determine MDA. Serum ALT activity, triglyceride, total protein, albumin and cholesterol levels were determined by using commercial kits (Roche Cobas Kit-Switzerland) with auto-analyzer (Roche Cobas 8000) in the Gulser- Dr. Mustafa Gundogdu Central Laboratory at Erciyes University. Liver tissue MDA (Cayman, USA, cat no. 10009055) levels were determined with ELISA (CayQuant Bio-Tek, ELx50, USA) by using commercial kits.

Following fixation in neutral formalin solution (10%), liver tissue specimens were rinsed overnight, under tap water. Then, all tissue samples were dehydrated in graded alcohol and cleared in xylene, embedded in paraffin wax, and sectioned (thickness, 5 µm), for histopathological evaluation. After staining with hematoxylin and eosin [18] sections were examined with a light microscope. To demonstrate caspase activity in tissues, the Avidin Biotin Peroxidase Complex (ABC) technique was performed according to the standard procedure provided in the commercial kit (Zymed, Histostain Plus Kit, California, USA). Anti-caspase-3 (active) (Novus NB100-56113) (dilution ratio 1/2000), anti-caspase-8 (Abcam ab25901) (dilution ratio 1/100) and anti-caspase-9 (Abcam ab25758) (dilution ratio 1/100) were used as primary antibodies. As a negative control PBS was applied to liver tissues and as a positive control; primary antibodies were applied to the control tissues recommended by the primary antibody manufacturers. For lipid staining, liver tissues fixed normally with 10% buffered neutral formalin for 24 hours and then fixed in 0.1% Osmium Tetroxide (OsO_4) for 8 hours. After standing 8 hours in OsO_4, the tissues proceeded with the processing, embedding and sectioning and then stained with HxE [18].

All sections were semi quantitatively evaluated for hepatocyte steatosis, inflammation, necrosis and fibrosis using ten different places in each section for the aforementioned parameters by two pathologists and the mean percentile values within the groups were calculated. The values obtained in each group were evaluated statistically and the importance between the groups were recorded. The significance of the difference between

the experimental and control groups for liver tissue damage score were done by the Kruskal-Wallis test. Statistical analyses were carried out using SPSS 20.

3. RESULTS AND DISCUSSION

In both the control (group 1) and FSO (group 2) groups, no clinical signs were observed, whereas in the CCl_4 and CCl_4+FSO groups, the most remarkable signs were exhaustion, dysorexia, weakness and hypersalivation.

The histopathological examination of the rats revealed normal liver tissue samples in groups 1 (Figure 1A) and 2 (Figure 1B). The histopathological examination of liver tissues in the carbon tetrachloride group (group 3), revealed dense macro and micro-vascular fat vacuoles in the hepatocytes (Figure 1C). In these areas, the remark cords were damaged. Especially close to the portal area, lymphocyte-rich mononuclear cell infiltrations and Kupffer cells were increased in number and focal hemorrhage areas (Figure 1D) were seen. Large necrotic areas of the liver parenchyma were noted and necrosis could not be clearly classified. The area was transformed into a pink homogeneous mass with necrotic changes, and microvascular fat vacuoles were evident in the hepatocytes of these areas. The histopathological examination of the liver of rats in the FSO+CCl_4 group (group 4) had an appearance of lesions similar to group 3 (Figure 1E, 1F).

Comment [WU4]: What do you mean by this?

There was no positive staining in the hepatocytes for osmium tetroxide in Group 1 and 2. In both Group 3 (Figure 1G) and Group 4 (Figure 1H), it was noted that macro- and microvesicular lipid vacuoles were black in the hepatocyte cytoplasm after staining with OsO_4.

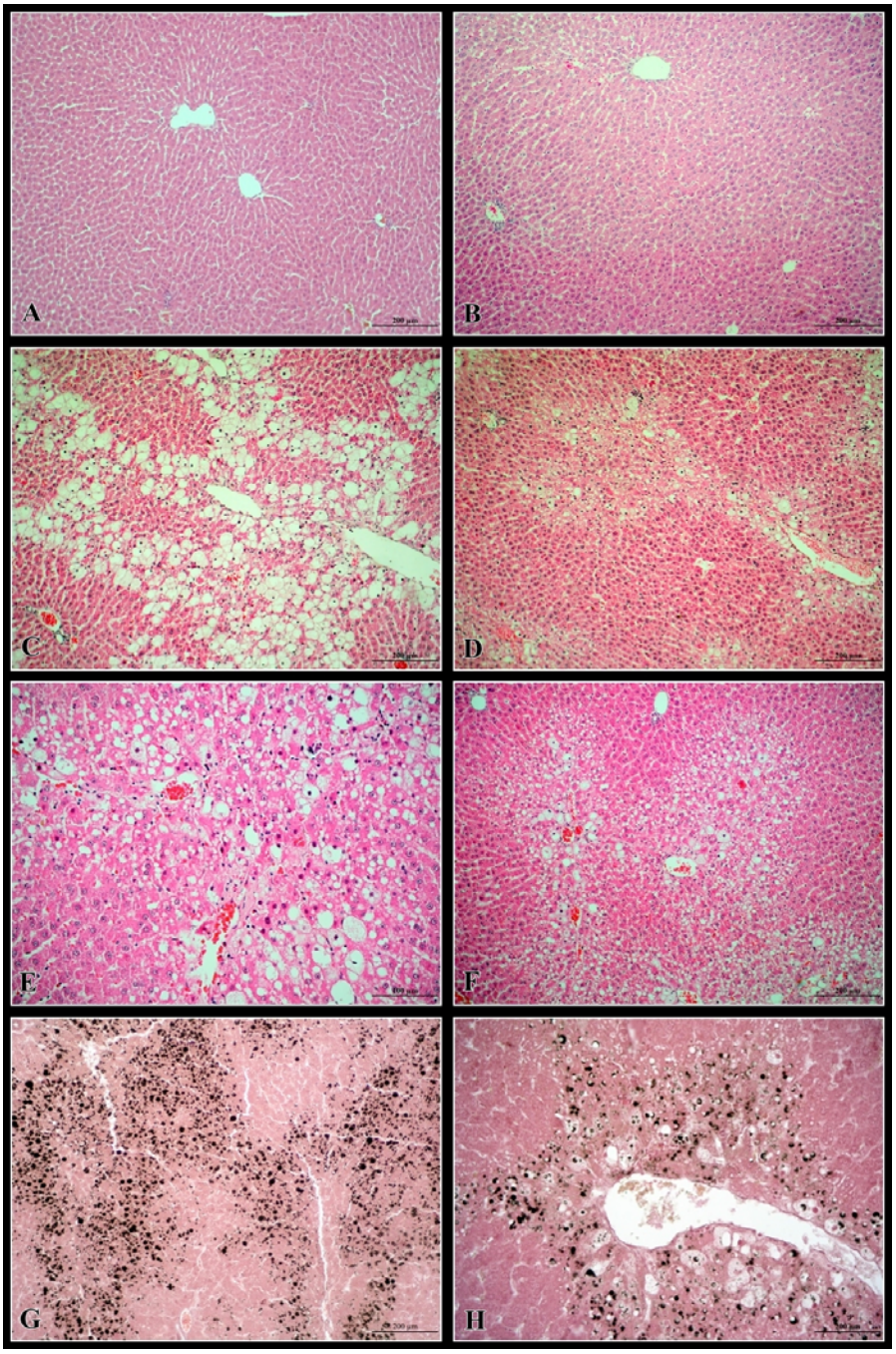
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Fig. 1. Histological analysis of the livers in carbon tetrachloride-induced acute hepatotoxicity; Normal appearance of the livers of the group 1 (A) and group 2 (B) groups. The appearance of micro-macro vesicular fat vacuoles in all parenchyma and increased numbers of infiltrating mononuclear cells, consisting predominantly of lymphocytes in group 3 (C, D) and group 4 (E, F), Liver, HxE. The appearance of black colored macro-micro vesicular fat vacuoles in hepatocyte cytoplasm in group 3 (G) and group 4 (H), Liver, (OsO_4-fixed) HxE.

The staining of caspase 8 in tissue sections of liver was negative in groups 1 and 2. However, in few a hepatocytes exposed to normal apoptosis, caspase 3 and caspase 9 were found to be positive (Figures 2). In the examined liver sections of group 3, caspase 3, caspase 8 and caspase 9 cytoplasmic immunopositive cells were detected particularly in the periphery of hepatocytes with lipid vacuoles (Figure 3A, 3B, 3C). In an immunohistochemical examination of group 4, the severity of positivity in caspase 3, caspase 8 and caspase 9 was similar to the CCl_4 group in hepatocytes in the periphery of the sentriacinar veins (Figure 3D, 3E, 3F).

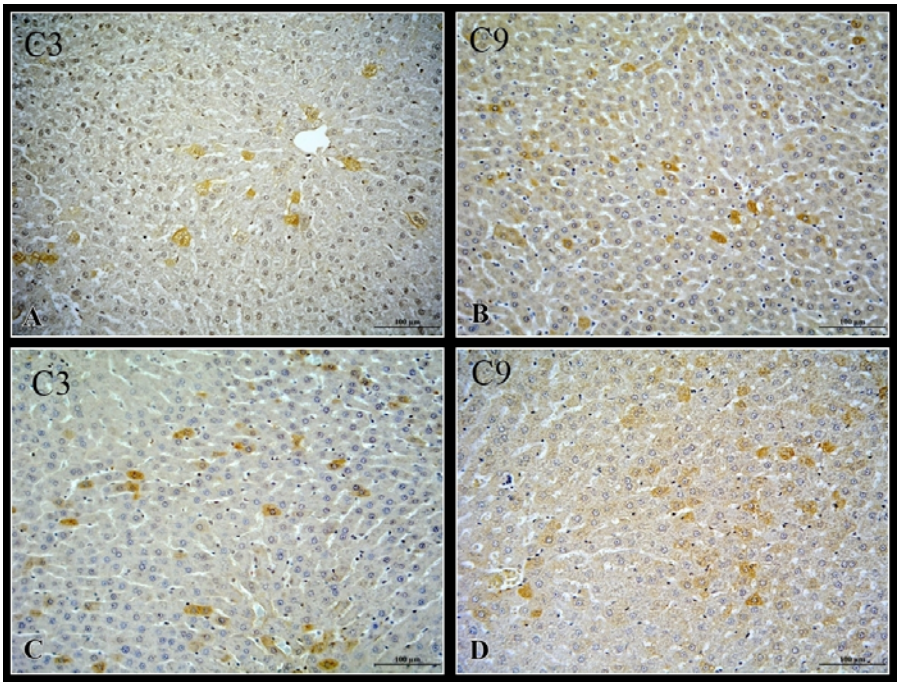

Fig. 2. Hepatic active caspase 3 (C3) and 9 (C9) expression. Hepatic caspase 3 and caspase 9 immunstaining of group 1 (A, B) and group 2 (C, D). ABC-P, Magnificaiton x100.

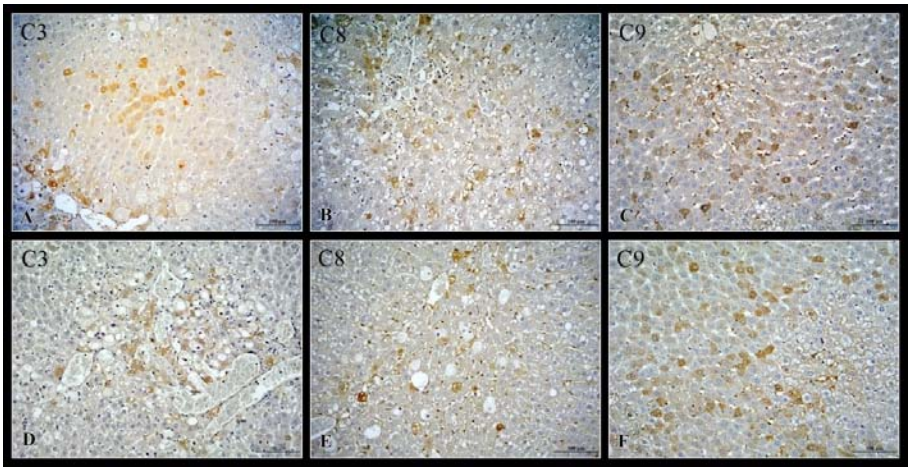

Fig. 3. Hepatic active caspase 3 (C3), caspase 8 (C8) and caspase 9 (C9) expression. Caspase 3, caspase 8 and caspase 9 immunoreactivity in the livers of CCl_4-intoxicated rats in group 3 (A, B, C) and group 4 (D, E, F) showed brown stained cytoplasm. ABC-P, Magnificaiton x100.

In both group 1 and 2, liver damage scores were found to be zero. The difference between groups 3 and 4 in terms of fibrosis, inflammation, steatosis and necrosis scoring was statistically insignificant (*P* < .001), (Table 2).

Table 2. Scoring system for hepatic damage in CCl_4 treated groups (n=8; *P* < .001).

	Control (N=8) Median (%25-%75)	CCl_4 (N=8) Median (%25-%75)	FSO (N=8) Median (%25-%75)	FSO+CCl_4 (N=8) Median (%25-%75)	P
Inflammation	0[a] (0-0)	2,0[b] (1,0-3,0)	0[a] (0-0)	2,0[b] (1,75-3,0)	***P* < .001**
Steatosis	0[a] (0-0)	3,5[b] (3,0-4,0)	0[a] (0-0)	3,0[b] (3,0-4,0)	***P* < .001**
Necrosis	0[a] (0-0)	3,0[b] (2,75-3,00)	0[a] (0-0)	2,0[b] (1,75-3,0)	***P* < .001**
Fibrosis	0[a] (0-0)	2,0[b] (2,0-2,25)	0[a] (0-0)	1,0[b] (1,0-2,0)	***P* < .001**

[a-b]: the difference between groups in the same line with different letters is statistically significant

At the end of the experiment, no statistically difference in biochemical parameters (serum ALT activity, triglyceride, total protein, cholesterol and MDA levels) were determined between Group 1 and 2 (Table 3). The present study showed a significant elevation in serum ALT activity, total cholesterol, triglyceride and MDA levels (*P* <.01) with a significant decrease in serum total protein levels (*P* >.05) after CCl_4 administration compared to the control group (Table 3). Serum ALT activities, total cholesterol, triglyceride and MDA levels

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were not affected by FSO administration. There was a significant increase in total protein levels in Group 4 when compared to the CCl_4 group.

Table 3. Effects of FSO on serum ALT activities, total protein, total cholesterol, triglycerides and MDA levels of rats in control and CCl_4 treated groups.

	CONTROL (N=8)	CCl_4 (N=8)	FSO (N=8)	FSO+CCl_4 (N=8)	P
ALT(U/L)	68,0[a] (65,0;81,5)	174,0[b] (72,0;810,0)	67,5[a] (62,0;71,25)	103,0[b] (69,5;190,5)	***P* < .01**
Total Protein(g/dL)	6,4[b] (6,1;6,5)	5,7[a] (5,6;5,9)	6,3[b] (6,2;6,6)	6,2[b] (6,0;6,5)	***P* > .05**
Total cholesterol (mg/dL)	66,0[a] 58,5;71,0	73,0[b] 72,5; 77,2	62,5[a] 59,7;67,2	70,0[b] 68,0;76,0	***P* < .01**
Triglycerides (mg/dL)	95,5[a] (72,7; 107,5)	220,0[b] (107,5; 239,0)	98,0[a] (79,5,0; 112,5)	167,5[b] (109,0;175,5)	***P* < .01**
MDA (µmoL/mg protein)	21,6[a] (20,1-23,4)	35,4[b] (24,3-38,3)	22,2[a] (19,5-24,3)	25,9[b] (25,7-33,2)	***P* < .01**

(n:8, FSO: flax seed oil, [a-b]: the difference between groups in the same line with different letters is statistically significant)

Comment [WU5]: Review the data in this table and replace , with . were appropriate.

Carbon tetrachloride activated in the hepatocytes to highly reactive trichloromethyl radical by the activation of cytochrome P450 enzyme, which initiated lipid peroxidation and caused hepatotoxicity. In the present study, large necrotic areas which could not be classified in the centrilobular and parenchyma areas, lymphocyte-rich mononuclear cell infiltrations, and sharply defined cytoplasmic lipid vacuoles in hepatocytes in all the parenchyma especially in centrilobular region were similar with other researcher's findings [19, 20, 21, 22] of different doses of CCl_4.

Experimental animal model studies that use extracts and oils of plants with an antioxidant content prevents lipid peroxidation, have become recently popular for the determination of the protective effects of toxic chemicals against liver damage [23, 24, 25] because they are cheap and easily accessible and have ~~and~~ low side effects. Tocopherols (all three forms: α, β, and γ) and flavonoids (flavone C- and O-glycosides) are found in flaxseed which is responsible for the nullification of lipid peroxidation [26, 27, 28, 29].

No studies have been conducted to evaluate the effects of FSO on histopathological lesions of liver in CCl_4-induced liver toxicity. Researchers using flaxseed extract [30, 31, 32], against CCl_4-induced the liver toxicity reported that flaxseed extract had ameliorative effects on liver necrosis, fat vacuoles and inflammatory cell infiltration. There are some studies using FSO to improve liver damage created by different toxic substances [33, 34, 35, 36, 37, 38]. In these studies, it was reported that FSO administration increased the numbers of Kupffer cells and decreased cytoplasmic lipid vacuole formation, degeneration and necrosis in hepatocytes as well as inflammatory cell infiltrations. In group 3 and group 4, the liver histology appearance was the same and this is proof that FSO did not have a beneficial effect on hepatotoxicity and this result suggests that there is a need for new studies to be done with FSO.

The studies conducted during the last decade are strongly suggestive that hepatocyte apoptosis is thought to be the first cellular response to toxic damage and the basis of cell

death in liver diseases [39, 40]. Carbon tetrachloride triggers caspase-3 dependent apoptosis [41] by damaging the plasma membrane and phospholipid bilayer in mitochondria [42]. Caspase-3 is required for initiator caspases such as caspase-8 and -9 in the membrane or mitochondrial pathways in response to different stimuli [43, 44]. In the present study, the increase in caspase 3, 8 and 9 activities in the CCl_4 administered groups were found similar to the findings of earlier studies [45, 46, 47, 48, 49, 50, 51]. The application of FSO partially reduced the activities of caspase 3, 8 and 9, and thus hepatocyte apoptosis. CCl_4 induced free radical formation, by decreasing endogenous antioxidant enzymes, induced hepatocyte apoptosis by caspase 3, 8 and 9, suggesting that both intrinsic and extrinsic pathways are used in CCl_4 toxicity.

Fadlalla et al. [52], reported that serum ALT activity, total cholesterol and liver MDA levels were increased in acute CCl_4 treated groups, which were decreased significantly in rats treated with FSO. In addition, several studies have shown that flaxseed oil or extract reduces increased ALT activity in liver damage caused by various toxicants in rats (such as ethanol, acetaminophen, lead, lead acetate, Thiacloprid). In the present study, serum ALT activity was not significantly decreased by FSO administration. Chavan et al. [33] stated that with paracetamol treatment decreased serum total protein levels and increased serum cholesterol and triglycerides level were normalized with FSO application. Naqshbandi et al. [53] reported that increased cholesterol levels decreased with FSO in the toxicity of cisplatin. Several studies have shown that increased levels of MDA due to lipid peroxidation have been reduced with the administration of flaxseed extract [30] and flaxseed oil [33, 34, 36, 38]. In the present study, total cholesterol, triglyceride, serum protein and liver MDA levels were not affected by FSO applications.

4. CONCLUSION

From the present study results, it could be concluded that FSO application did not cause any change in either the histopathological or the biochemical parameters against CCl_4-induced hepatotoxicity, which indicates that the damage in liver tissue did not improve. Nevertheless, other dose- and duration-dependent investigations need to be performed in order to understand the effects of flaxseed oil on tissues.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors GE and AA designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors GE and DYG managed the analyses of the study. Authors AA and DYG managed the literature searches. All authors read and approved the final manuscript.

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