Original Article

Effect of fermented red radish (*Raphanus sativus L*) in carbon tetrachloride-induced liver injury in rats

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Abstract

In this study, the possible protective effect of red radish containing total phenolic content and antioxidant components on histopathological and immuno histochemical active caspase-3, TNF-α and Iba-1, serum ALT activity, triglyceride, total cholesterol, LDL-cholesterol, liver malondialdehyde (MDA) and nitric oxide (NO) levels were evaluated in CCl_4 induced liver injury in Wistar rats. The rats were randomly divided into six groups (n= 12 to each group). The first group was used as a control which was given only intraperitoneal 0.9% NaCl (0.2 mL / kg / bw); while fermented red radish (FRR) at a dose of 250 mg/kg or 500 mg/kg was given daily by intragastric administration to group II and group III, respectively for seven consecutive days. Carbon tetrachloride (CCl_4) diluted 1:1 ratio in corn oil and was given (1.5 mL/kg) to the rats in group IV by intraperitoneal injection. Group V and VI received FRR at a dose of 250 mg/kg or 500 mg/kg, respectively for seven consecutive days prior to CCl_4 injection. Pretreatment with FRR at 250 mg/kg and 500 mg/kg followed by CCl_4 exposure had no ameliorative effect on the histopathological changes in the liver. It is thought that FRR application has a partial antioxidative effect by decreasing the biochemical and lipid peroxidation levels in the control group but this is not effective in the treatment of damage to liver tissue. In conclusion, it has been concluded that new investigations should be done to determine the healing effects of red radish on fermented tissues using different doses to give the best results without any side effects.

Keywords: Histopathology, immunohistochemistry, liver, fermented red radish, carbon tetrachloride, rat

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Introduction

The liver is an important organ that is most exposed to toxic substances due to anatomical localization and its important functions and can be damaged by many factors (Kumar *et al.,* 2017). Carbon tetrachloride (CCl_4), a hepatotoxin, has been used to induce acute and chronic toxicity and manifests itself biochemically on cell organelles (Muriel and Mourelle, 1990). It has been accepted that the hepatotoxicity of CCl_4 results from oxidative stress that is followed by free radical formation. This free radical and reactive species effects the unsaturated fatty acids in the cell membrane which may cause cellular damage by initiating lipid peroxidation (Basu, 2003; Manibusan *et al.,* 2007). The oxidative stress associated with oxidative damage has been the focus of research in recent years. Since carbon tetrachloride inhibits protein synthesis, especially apolipoprotein synthesis, fatty liver occurs (Aruoma, 1994). There has been a growing interest in experimental studies in animal models in order to determine the antioxidant and hepatoprotective effects of plant origin drugs against liver damage caused by different chemicals (Kalantari *et al.,* 2009). Red radish and its products have been reported to have a strong hepatoprotective effect against CCl_4 toxicity by showing different protective effects such as free radical scavenging and antioxidative (Dash *et al.,* 2013; Kim *et al.,* 2013; Rafatullah *et al.,* 2008; Sadeek, 2011; Syed *et al.,* 2014). The health benefits of fermented foods are often linked to bioactive peptides synthesized by the microbial degradation of the proteins by bacteria involved in fermentation (Şanlier et al., 2019). Therefore, interest in the fermentation process and the use of the resulting fermented products have increased recently.

Lactic acid bacteria provide low-cost fermentation aimed at extending shelf life by preserving the sensory and nutritional properties of fresh vegetables and fruit. *L. plantarum* is one of the major lactic acid bacteria species used to ferment vegetables and fruit (Torres *et al.,* 2020). Fermentation increases antioxidant activity by ensuring the dissociation of vegetable fibers and a significant amount of bioactive substance release (Jing *et al.,* 2014, Kim *et al.,* 2017).

The main objective of this investigation is to evaluate the protective effects of fermented red radish containing total phenolic contents and antioxidant properties by assaying serum ALT activity, triglyceride, total cholesterol, LDL cholesterol and liver malondialdehyde (MDA), nitric oxide (NO) levels, as well as active caspase-3, TNF-a and Iba-1 immunoreactivity in hepatotoxicity in rats.

Materials and Methods

Animal husbandry and maintenance: Experiments were performed using 72 200–250 g weighing adult male Wistar albino rats. The experiments were carried out in accordance with the Guidelines for Animal Experimentation approved by Erciyes University, Experimental Animal Ethics Committee (permit no: 18/047), and the experimental procedures were performed in Erciyes University Experimental Research and Application Center, Kayseri, Turkey. The animals were kept in a special room at a constant temperature 22°C ± 2°C and humidity (% 50±5) with 12-h light/dark cycles and had free access to diet (Optima rat and mouse provender) and tap water.

Experimental design: The rats were randomly divided into six groups (n= 12 to each group). The first group was used as a control which was given only intraperitoneal 0.9% NaCl (0.2 mL/kg/BW); while FRR at a dose of 250 mg/kg or 500 mg/kg was given daily by intragastric administration to group II and group III, respectively for seven consecutive days. Carbon tetrachloride (CCl_4) diluted 1:1 ratio in corn oil and was given (1.5 mL/kg) to the rats in the CCl4 group (group IV) and two therapeutic groups (group V and group VI) by intraperitoneal injection. Groups V and VI received FRR at a dose of 250 mg/kg or 500 mg/kg, respectively for seven consecutive days prior to CCl_4 injection.

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. A part of the liver tissue was preserved at -80°C for MDA and NO analysis. The remaining liver tissue was fixed % 10 neutral formaldehyde solution for histopathological examination. The blood was centrifuged at 1300 g for 10 mins and serum was frozen at -20°C until testing.

Following fixation in neutral formalin solution, liver tissue specimens were thoroughly rinsed overnight under tap water. Then, all tissue samples were dehydrated in graded alcohol, cleared in xylene and embedded in paraffin wax and sectioned (thickness, 4-5 µm), for histopathological evaluation. After staining with hematoxylin and eosin sections were examined with a light microscope. After that, all sections were semi-quantitatively evaluated for hemorrhage, steatosis, inflammation and necrosis and were graded as 1 (mild, <% 33 of liver cells), 2 (moderate, % 33 to % 66 of liver cells), and 3 (severe, >% 66 of liver cells) and the values obtained in each group were calculated for averages with percentages.

Immunohistochemical examination: in order to determine apoptosis, necrosis and macrophage activity in liver tissue, the Avidin Biotin Peroxidase Complex (ABC) technique was applied according to the standard procedure prescribed in the commercial kit (Zymed, Histostain Plus Kit, California, USA). Anti-caspase-3 (active) (Novus NB100-56113) (dilution ratio 1/2000), TNF-α (Abcam AB6671) (dilution ratio 1/50) and Iba-1 (Abcam AB107159) (dilution ratio 1/1000) were used as primary antibodies. As a negative control, PBS was applied to the liver tissues and, as a positive control, primary antibodies were applied to the control tissues recommended by the manufacturers.

Biochemical analysis: Serum ALT activity was determined with a spectrophotometer (Shimadzu UV model 1208) using commercial kits (Biolabo 80127, France). Liver tissue samples were homogenized according to MDA measurement procedures and separated into supernatants. Levels of MDA (Cayman,

USA, cat no: 10009055) and NO (Sunred Bio, Shanghai, cat no: 201-11-0704) in the liver were determined with ELISA (Bio-Tek, ELx50, USA) using commercial kits. The other serum biochemical parameters (total cholesterol, low-density lipoprotein cholesterol and triglyceride) were assayed using an autoanalyzer (Glucose Auto and Stat, GA-1122) in Gulser Dr. Mustafa Gundogdu Central Laboratory, Erciyes University.

Fermentation of red radish: Red radish obtained from a local farm in Osmaniye, Turkey, was cleaned, and sterilized at 95 °C for 15 mins and was ground using a food mixer. The mixture was then mixed with distilled water (1:1 suspension) and autoclaved for 15 mins at 121°C. The seed culture of *Lactobacillus plantarum* (provided by Kesmen Z, Department of Food Technology, School of Food Engineering, University of Erciyes, Kayseri, Turkey) was incubated in De Man, Rogosa and Sharpe (MRS) agar for 24 h at 37 °C, and propagated in MRS broth under the same conditions. The organism (approx. 1 ×10^7 CFU/mL) was then used to give concentrations of % 1.0 in red radish suspension for 48 h in a shaking incubator. The fermentation was stopped by heating at 95°C for 15 mins. At the end, the FRR was freeze-dried under high pressure at the Technological Research and Application Center, Erciyes University, packaged in aseptic zip lock pouches and stored at 4°C until use (Kim *et al*., 2017).

Total antioxidant capacity (TAC): The TAC was measured with ELISA using a commercial kit (Rel Assay Kit Diagnostics, Turkey) developed by Erel (2004). As a calibrator, Trolox, which is the water-soluble analogue of vitamin E, was used so the results are expressed in terms of millimolar Trolox equivalent per liter.

Total phenolic content (TPC): The TPC of the extracts was determined by Folin-Ciocalteu colorimetric method with some modifications (Sagdic *et al*., 2013). After 60 mins incubation at room temperature, absorbance was measured at 750 nm. The calibration curve was generated with gallic acid and total phenolic content was expressed as gallic acid equivalents (mg GAE/mL extract).

DPPH radical scavenging activity: The scavenging activity of samples for the radical 2,2-diphenyl- 1-picrylhydrazyl (DPPH) was measured as described by Orhan *et al*., (2007) with some modifications. DPPH radical solution was prepared with % 80 ethanol and % 20 distilled water. 30 mL sample and 270 mL DPPH solution were mixed and shaken vigorously for five mins and left to stand for 55 mins at room temperature in the dark and then absorbance was measured at 520 nm by microreader (Multiscan FC, Thermo Fisher, USA) (Orhan *et al*. (2007).

Statistical analysis: The significance of the difference between the experimental and control groups in terms of liver tissue damage score was performed with the Kruskal–Wallis test. Statistical analyses were carried out using SPSS 20.00. The difference between groups was determined by one-way analysis of variance (ANOVA). When the F value was found to be significant, Duncan's Multiple Range Test was applied. All values were expressed as mean values ± standard error of means. A value of $P< 0.05$ was considered significant.

Results

In the control groups (250 and 500 mg/kg red radish), no clinical signs were observed, whereas in rats in CCl_4 (Group VI), FRR + CCl_4 (Group IV and Group V) groups, weakness, hunched posture, excessive salivation, ptosis, ataxia and corneal opacity were observed.

Histopathologic and Immunohistochemical findings: Systemic necropsies of rats in the control groups revealed no macroscopic lesions and had a normal structure in histology (Fig 1. A-C). Group IV did not show any macroscopic lesions except for color changes from dark red to gray-white in the liver of rats. Histopathological examination revealed large necrotic areas in all parenchyma mainly in the periphery of the central vein (zone-3), (Fig. 1 D). Intensive macrovesicular lipid vacuoles (steatosis) were observed in most hepatocytes, including microvesicles in the cytoplasm of hepatocytes in the necrotic areas (Fig. 1 E). The hepatocytes around the vena centralis had transformed into a pink homogeneous mass due to necrotic changes.

Lymphocyte-rich mononuclear inflammatory cells and erythrocyte infiltration were detected in between the vacuolated and necrotic hepatocytes. There was also diffuse proliferation of Kupffer cells in the paranchyma and mitotic figures in hepatocytes. Histopathological lesions in the liver tissue in FRR and CCl_4 rats were similar with CCl_4 group in terms of large necrotic areas (zone 3) (Fig. 2 A, B), diffuse macro-microvesicular lipid vacuoles in hepatocytes (steatosis) (Fig. 2 C, D), lymphocyte-rich mononuclear cell infiltration, Kupffer cell proliferation, as well as large haemorrhage areas and mitosis in hepatocyte.

While TNF-α expression was negative, some caspase 3 positive hepatocytes and Iba-1-positive Kupffer cells were detected in the hepatic sinusoid of the rat livers in control groups (Fig 3 A-F). Active caspase 3, Iba-1 and TNF-α were positive in Group IV (Fig. 4 A- F). Caspase 3 positive hepatocytes around the central veins and lipid vacuoles (Figure 4 A, B) and Iba-1 positive macrophages particularly Kupffer cells (Fig. 4 C, D) in cell infiltration areas around necrosis were shown using immunochemical methods. TNF-α staining was also positive in hepatocyte cytoplasm in necrotic areas (Fig. 4 E, F). Immunohistochemical staining for active caspase 3 (Fig. 5 A, B), Iba-1 (Fig. 5 C, D), and TNF-α (Fig. 5 E, F) was positive in Group V and Group VI. The severity of lesions and positivity of immunostaining between CCl_4 groups were similar.

Liver damage scoring method: Liver damage parameters were scored for hemorrhage, steatosis, inflammation and necrosis. Histopathological sections of the liver tissues of the control groups were found to be zero, while no significant difference was observed between CCl_4 groups (Table 1).

Biochemical parameters: There were no statistical differences between the control groups (0, 250 and 500 mg/kg red radish) in serum ALT activity, triglyceride, total cholesterol and LDL cholesterol levels ($P > 0.05$). Highest ALT activity and serum lipid levels (Tg, T-chol, LDL-chol) were determined only in the CCl_4 treated group and these increases were significantly decreased ($P < 0.001$) with red radish application to the CCl_4 administered groups. However, serum ALT enzyme activity and triglyceride levels did not approach normal values (Table 2).

Liver MDA and NO levels: There was no significant difference in liver MDA levels between the control groups. While no significant difference was observed between the control group and the 250 mg/kg red radish group by liver NO level, only the 500 mg/kg red radish treated group had significantly lower NO levels compared to control and 250 mg/kg red radish treated groups. The highest tissue MDA and NO levels were determined only in the CCl_4 administered group. It was observed that MDA ($P < 0.05$) and NO ($P < 0.001$) levels were significantly decreased by administration of red radish at the of 250 and 500 mg/kg to CCl_4 treated groups (Table 2).

In the present study, there was no difference in the total phenolic content of FRR and non-fermented red radish. The TAC values in FRR (7.2857± 0.030 mmol Trolox equiv./L) were increased compared with non-fermented red radish (0.2857±0.023 mmol Trolox equiv./L). Similarly, the DPPH radical scavenging activity was found to be higher in FRR (% 27.00±2.32) compared with non-fermented red radish (% 19.86±3.01). Therefore, we used the FRR, which had the higher antioxidant capacity and better DPPH radical-scavenging activity (Table 3).

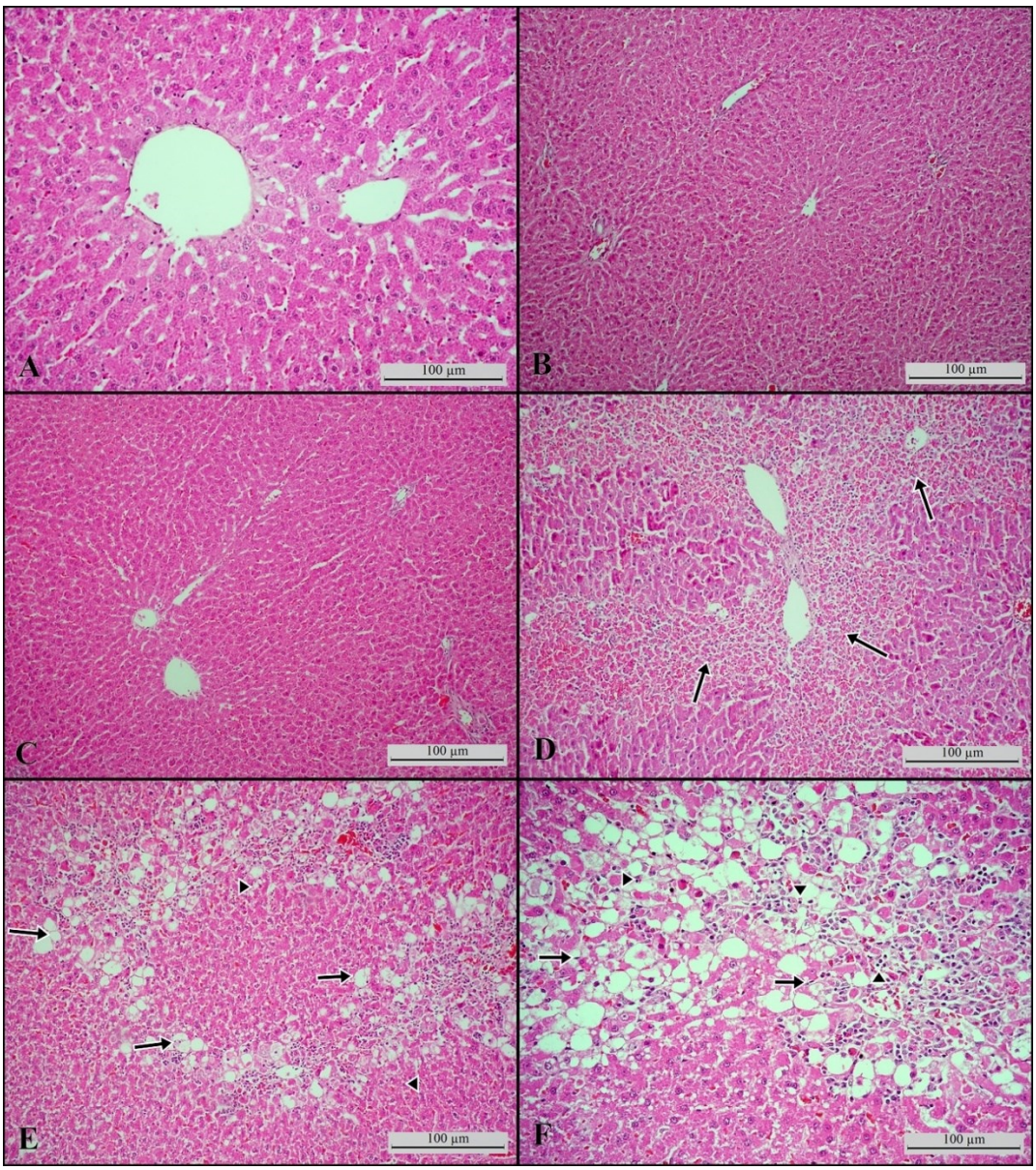

Figure 1 Normal appearance of the livers of the control (A) (100 µm), FRR (250 mg/kg) treated (B) (200 µm) and FRR (500 mg/kg) treated (C) (200 µm) groups. Large necrosis areas (arrow) in the parenchyma of the rat liver given CCl_4 (Group IV, D) 200 µm. The appearance of micro (arrowheads) and macro (arrows) vesicular fat vacuoles in all parenchyma (E) 200 µm, Kuppfer cell hyperplasia in all parenchyma (arrows) and lymphocyte-rich mononuclear cells (arrowhead) in the periphery of necrotic and fatty areas of rat liver in CCl_4 treated group (F), 100 µm, Liver, HXE.

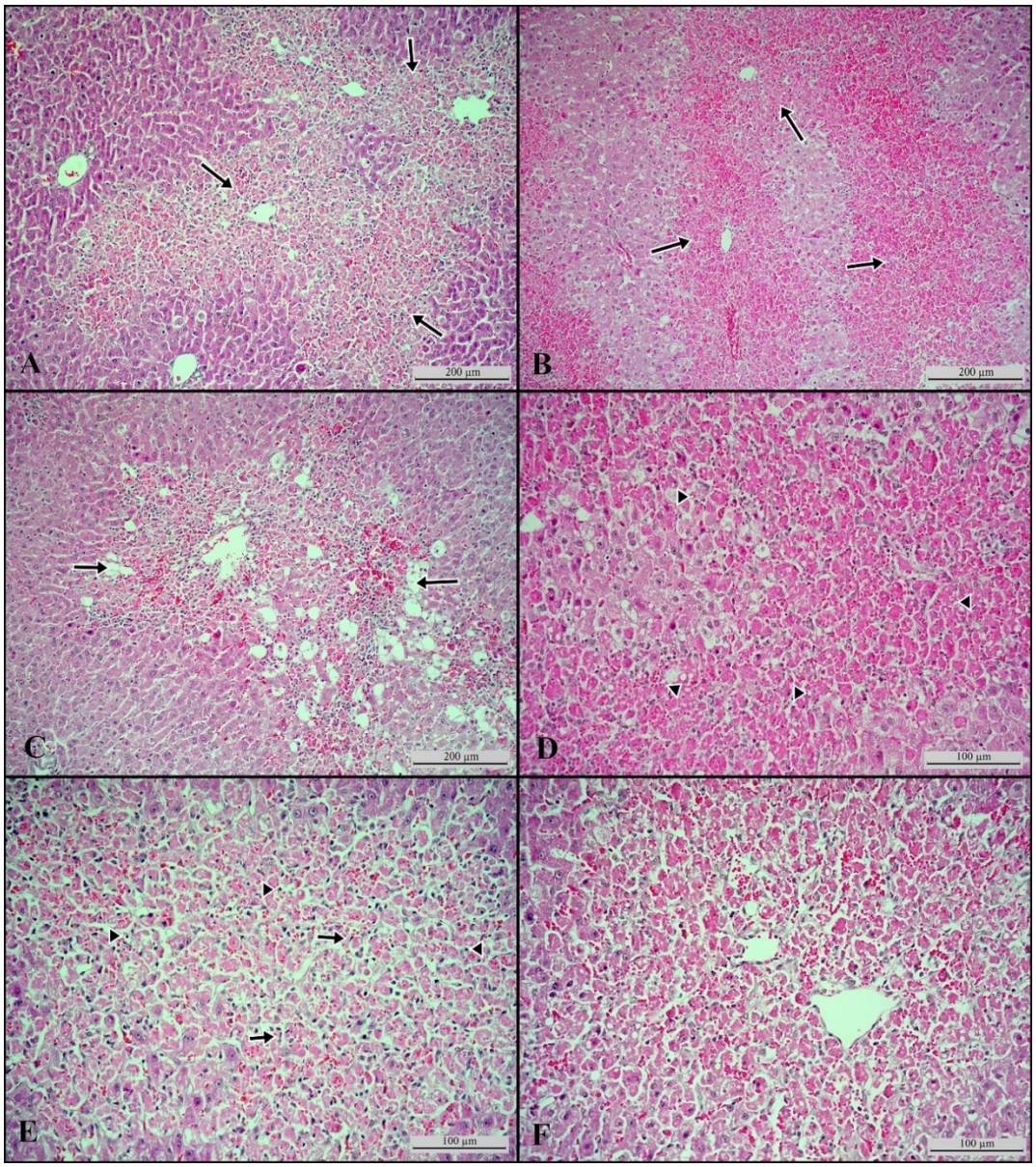

Figure 2 The appearance of large necrosis areas (arrows) in the parenchyma, more prominently in the periphery of vena centralis (zone-3) in CCl_4+250 mg/kg (A) and CCl_4+500 mg/kg (B) treated rat liver tissue (200 µm). The appearance of steatosis formed by macrovescular (arrow) and microvescular (arrowhead) lipid vacuoles of rat liver tissue given CCl_4+250 mg/kg (C, 200 µm) and CCl_4+500 mg/kg (D, 100 µm), respectively. Lymphocytic cell infiltration (arrowhead) and Kuppfer cells (arrows) in the areas of necrosis in rat liver given by CCl_4+250 mg/kg (E, 100 µm). Hemorrhage between lymphoid cells in necrotic foci of rat liver treated with CCl_4+500 mg/kg (F, 100 µm), HXE.

Table 1 Scoring system for hepatic damage in CCl_4 treated groups (n=12; $P < 0.05$).

	CCl_4 Median (%25-%75)	**CCl_4+ FRR 250 Median (%25-%75)**	**CCl_4+ FRR 500 Median (%25-%75)**	**P**
Inflammation	2 (1.0-2.0)	2.0 (2.0-2.0)	2.0 (2.0-2.0)	$P < 0.05$
Steatosis	2.0 (1.0-2.25)	2.0 (1.0-2.0)	2.0 (1.0-2.0)	$P < 0.05$
Necrosis	3.0 (1.75-3.00)	3.0 (2.0-3.0)	3.0 (2.5-3.0)	$P < 0.05$
Hemorrhage	1.0 (1.0-1.25)	2.0 (1.0-2.0)	1.0 (1.0-2.0)	$P < 0.05$

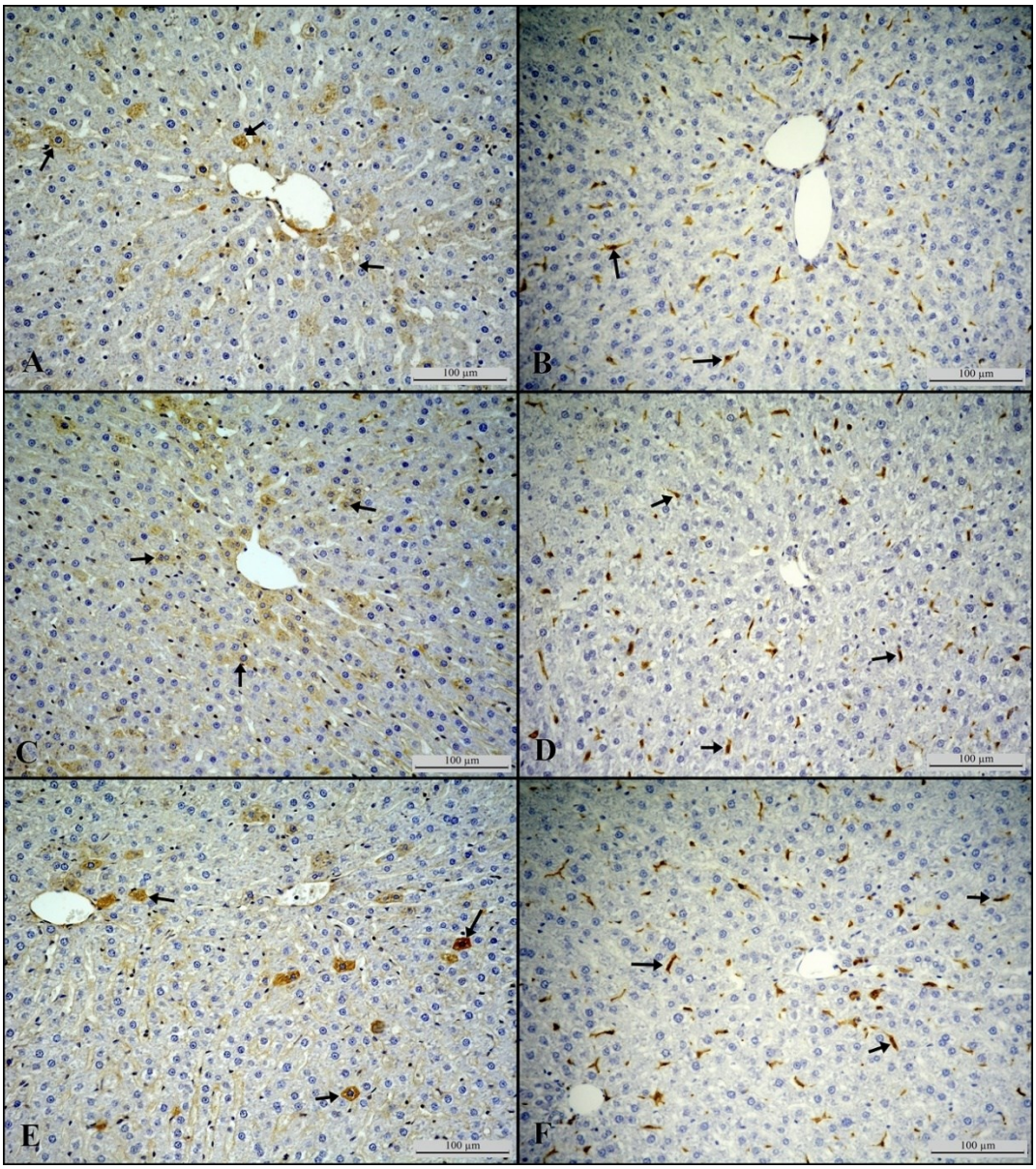

Figure 3 Some caspase-3 positive hepatocytes which were exposed to apoptosis in control (A), 250 mg/kg FKT (C) and 500 mg/kg FKT (E) groups (arrows) and Iba-1-positive Kupffer cells in the rat liver of control (B), 250 mg/kg FKT (D) and 500 mg/kg FKT (F) groups (arrowheads), immunohistochemical staining, 100 µm.

Table 2 Effects of FRR in serum ALT activity, triglyceride, total cholesterol, LDL cholesterol, liver MDA and NO levels of rats in control and CCl_4 treated groups.

	Control (n=12)	FRR 250 (n=12)	FRR 500 (n=12)	CCl_4 (n=12)	CCl_4+FRR 250 (n=12)	CCl_4+FRR 500 (n=12)	P
ALT (U/L)	48.32±2.92^{c}	47.89±2.80^{c}	43.22±2.95^{c}	387.53±18.88^{a}	311.32±11.03^{b}	302.65±38.01^{b}	***
Triglyceride (mg/dL)	82±3.12^{c}	80.43±6.01^{c}	82.43±4.43^{c}	156.43±3.60^{a}	123±3.65^{b}	127.29±3.32^{b}	***
Total Cholesterol (mg/dL)	53.43±1.65^{bc}	62.29±1.92^{b}	50.57±4.02^{c}	82.57±4.17^{a}	55.71±2.18^{bc}	59.86±2.72^{b}	***
LDL cholesterol (mg/dL)	8.43±0.90^{b}	10.14±0.55^{b}	9.29±1.27^{b}	13.43±0.78^{a}	7.57±0.65^{b}	8.43±0.61^{b}	***
MDA (µmoL/L)	18,21±1.18^{b}	19.61±0.68^{ab}	18.50±1.69^{b}	26.01±4.49^{a}	16.209±2.16^{b}	13.88±1.27b^{b}	*
NO (µM)	15.56±0.95^{b}	16.29±0.77^{b}	11.21±0.86^{c}	20.94±1.81^{a}	10.14±0.75^{c}	10.68±0.75^{c}	***

(n:12, $^{a\text{-}c}$: the difference between groups in the same line with different letters is statistically significant, *:$P < 0.05$; ***:$P < 0.001$)

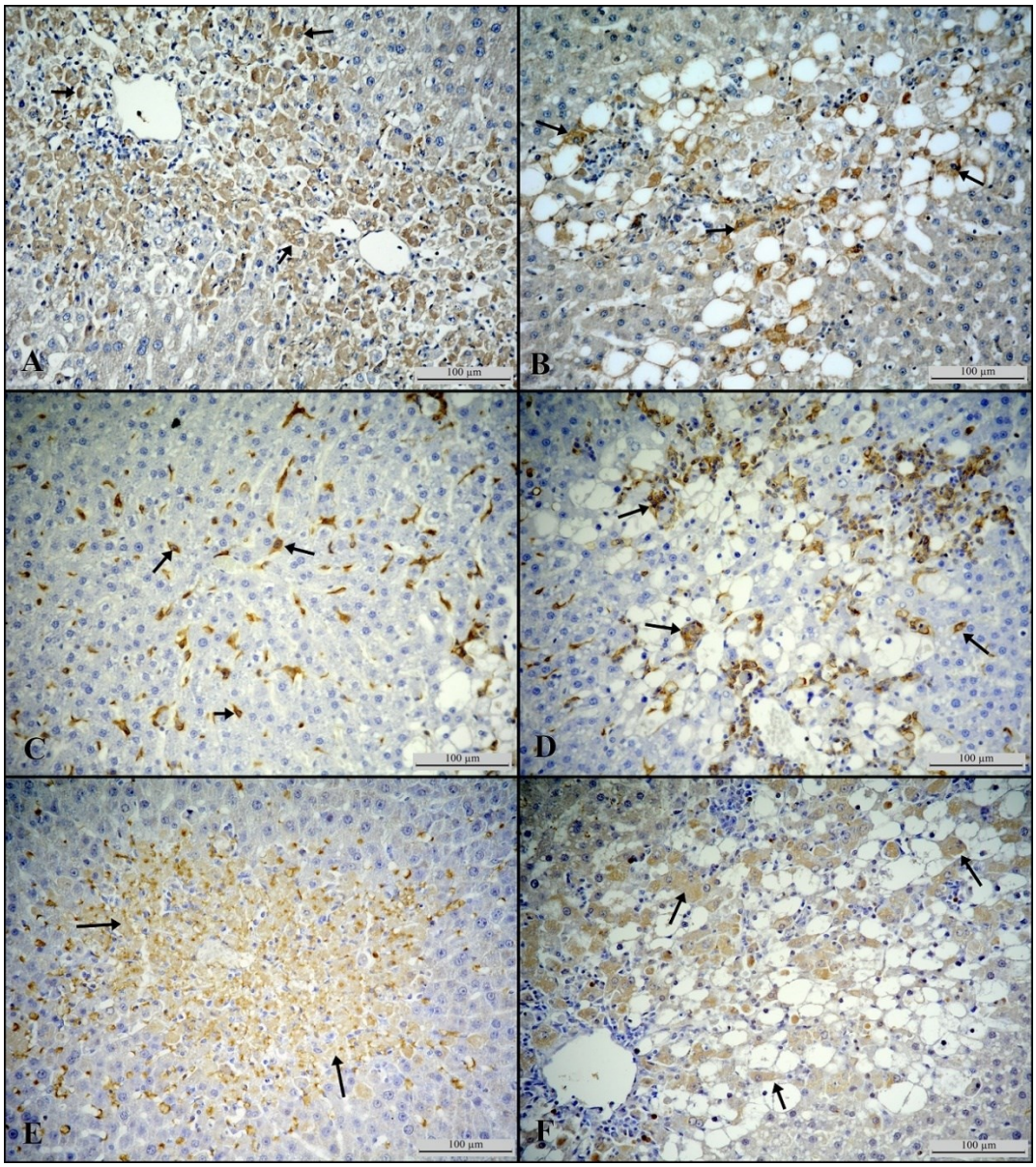

Figure 4 Immunohistochemical staining of active caspas-3 (A, B), Iba-1 (C, D) and TNF-α (E, F) in the areas of necrosis and steatosis of only CCl_4 treated rat liver (arrows), 100 µm.

Table 3 Total antioxidant capacity, DPPH activity and total phenolic content of red radish

	TAC (mmol Trolox equiv./L)	DPPH radical scavenging activity (%)	Total Phenolic Contents (mg GAE/g)
Non-Fermented Red Radish	0.2857±0.023	19.86±3.01	2.20±8.16
Fermented Red Radish	7.2857± 0.030	27.00±2.32	2.28±11.24

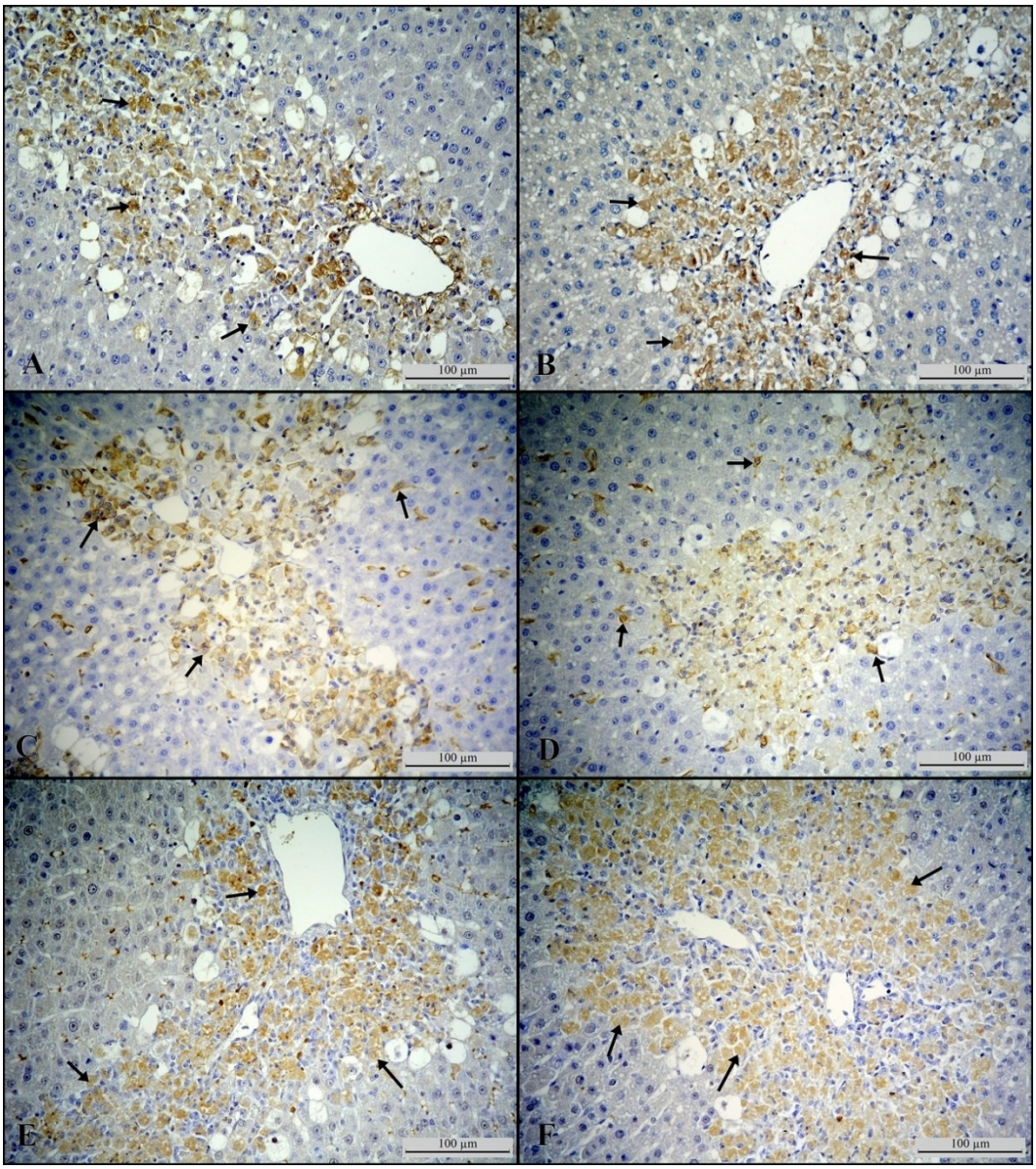

Figure 5 Immunohistochemical staining of active caspase-3 in CCl_4 + 250 mg/kg (A) and CCl_4 + 500 mg/kg (B) groups; Iba-1 in CCl_4 + 250 mg/kg (C) and CCl_4 + 500 mg/kg (D) groups and TNF-α in CCl_4 + 250 mg/kg (E) and CCl_4 + 500 mg/kg (F) groups in the areas of necrosis and steatosis in rat liver, (arrows), 100 µm.

Discussion

Carbon tetrachloride is converted into trichloromethyl (CCl_3) and trichloromethyl peroxyl (CCl_3O_2) free radicals, through the cytochrome P450 enzyme system in the endoplasmic reticulum in hepatocytes. These free radicals react with unsaturated fatty acids in the cell membrane to induce lipid peroxidation or to damage the cell membranes by binding to proteins and fats to cause liver damage (Shyur *et al.*, 2008; Weber *et al.*, 2003).

Researchers have reported that administration of carbon tetrachloride in rats to induce liver damage leads to necrosis in centrilobular hepatocytes, vacuolar degeneration in the cytoplasm, congestion in vena centralis and sinusoidal areas, interstitial edema due to deterioration of the remark cords, inflammatory cell infiltration and fatty liver (Arhoghro *et al.*, 2009; Khorshid *et al.*, 2008; Ma *et al.*, 2014; Ravikumar and Gnanadesigan, 2011). In the present study, considering the studies of previous researchers, CCl_4 was administered intraperitoneally at a dose of 1.5 mL/kg to Groups IV, V and VI. A large necrosis area that could not be clearly classified in the parenchyma or centrilobular area of the liver, lymphocyte rich inflammatory cell infiltration and the observation of sharp-edged round oil vacuoles with different sizes in hepatocytes, especially more intense in the centrilobular region and also parenchyma coincided with the findings of these investigators (Arhoghro *et al.*, 2009; Khorshid *et al.*, 2008; Ma *et al.*, 2014; Ravikumar and Gnanadesigan, 2011).

Radish plant including red radish contains various components such as fiber, protein and minerals (Gutiérrez and Perez, 2004). Red radish is rich in anthocyanins, including a combination of p-coumaric acid, ferulic acid and malonic acid (Tamura *et al.*, 2010). Anthocyanins are water-soluble natural pigments of the flavonoid class which act as potent antioxidants (Holton and Cornish, 1995). It was reported that anthocyanins were the strongest antioxidant source in 150 flavonoids (Elliott *et al.*, 1992). In previous studies,

red radish leaves and roots have been shown to have antioxidant and free radical scavenging activities (Beevi *et al.,* 2010), while vitamin C and sulphur components have hepatoprotective effects (Baek *et al.,* 2008; Salah-Abbès *et al.,* 2009).

The number of studies using red radish in order to improve liver damage in rats using carbon tetrachloride (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Kim *et al.,* 2013; Rafatullah *et al.,* 2008; Syed *et al.,* 2014; Sadeek, 2011) and other toxic substances (Anwar and Ahmad, 2006; Chaturvedi and Machacha, 2007; Salah-Abbès *et al.,* 2009) is quite limited. Researchers have been reported to have improved lesions of liver tissue of different types of red radish, including juice (Rafatullah *et al.,* 2008), extract (Syed *et al.,* 2014) and anthocyanin fractions (Dash *et al.,* 2013), to improve liver damage caused by CCl_4 in rats. Similarly, red radish extract (Chaturvedi and Machacha, 2007) has been shown to have ameliorative effects on liver lesions against different toxic substances. In the present study, unlike the findings of the researchers (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Rafatullah *et al.,* 2008; Syed *et al.,* 2014) red radish had no ameliorative effect. According to this study, it is concluded that antioxidant phytochemicals such as flavonoids, terpenoids and polyphenols in the radish plant do not improve tissue damage against CCl_4-induced liver damage.

In the present study, contrary to the findings of the researchers (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Rafatullah *et al.,* 2008; Syed *et al.,* 2014), red radish had no ameliorative effect on micro-macrovesicular steatosis, lymphocytes rich mononuclear cell infiltration; necrosis areas, mostly in zone-3, Kupffer cell hyperplasia and haemorrhage areas were similar to the CCl_4 group. According to this study, it can be concluded that antioxidant phytochemicals such as flavonoids, terpenoids and polyphenols in the radish plant do not improve tissue damage against CCl_4-induced liver damage.

Carbon tetrachloride destroys the mitochondrial phospholipid bilayer in hepatocytes and induces caspase 3 dependent apoptosis (Domitrović *et al.,* 2013; Tao *et al.,* 2012). In vitro and in vivo studies have shown that hepatocyte apoptosis is determined immunohistochemically with caspase activity in CCl_4 induced liver damage (Domitrović *et al.,* 2013; Hassan *et al.,* 2012; Tao *et al.,* 2012). Researchers have reported increased caspase 3 activity in these studies. In the present study, in CCl_4 administered groups, caspase 3 was evaluated and was consistent with the study results of the mentioned researchers (Domitrović *et al.,* 2013; Hassan *et al.,* 2012). It is suggested that CCl_4 induced free radical formation, by decreasing endogenous antioxidant enzymes, induces hepatocyte apoptosis by caspase 3 in CCl_4 toxicity (Jiang *et al.,* 2012).

Although there is no study on the effect of red radish and products on liver injury induced by CCl_4 on caspase 3 activity, there are a limited number of studies (Beevi *et al.,* 2010; Wang *et al.,* 2014) about the effect of the chemopreventive properties of radish (4-methylsulfinyl-3-butenyl isothiocyanate) on caspase 3 activity. In these studies, it has been reported that radish isolates induce apoptosis in cancer cells. As we have seen in the literature, since there were no similar studies with our study, we could not evaluate our findings as needed. In the present study, pretreatment with fermented red radish at 250 mg/kg and 500 mg/kg followed by CCl_4 exposure showed similar caspase-3 activity in the CCl_4 group while it was expected to decrease in number. These results may support the researchers (Jiang *et al.,* 2012) who have reported CCl_4 trigger hepatocyte apoptosis with caspase 3 activation, causing the formation of excess reactive oxygen derivatives and the depletion of endogenous antioxidant enzymes. According to this study's results, antioxidant phytochemicals such as flavonoids, terpenoids and polyphenols in the radish plant did not improve tissue damage and with the presence of necrosis, it can be concluded that radish has no effect on apoptosis in hepatocytes.

Free radicals formed by CCl_4 administration cause activation of Kupffer cells by increasing intracellular calcium in hepatocytes (Edwards *et al.,* 1993; Jiang *et al.,* 2012; Luckey and Petersen, 2001). Some mediators such as interferons, interleukins and TNF-α are released from activated Kupffer cells (Edwards *et al.,* 1993; Jiang *et al.,* 2012). TNF-α is a pleotropic cytokine produced by Kupffer cells that are mainly associated with various physiological and pathophysiological conditions (Chu *et al.,* 2016; Domitrović and Jakovac, 2010). It has been reported that toxic substances used to induce liver damage in rats cause activation and increase in Kupffer cells (Ahn *et al.,* 2018; Kim *et al.,* 2017; Kim *et al.,* 2018). In these studies activation of Kupffer cells around vena centralis was detected by Iba-1 antibody in the CCl_4 induced rats.

Some researchers, who used fermented white radish (Kim *et al.,* 2017) and black radish extract (Moon *et al.,* 2015) in order to improve liver damage in CCl_4-injury, reported that activation of increased Kupffer cells significantly decreased with radish treatment. In the present study, activation of Kupffer cells was detected with Iba-1 antibody in the periphery of necrotic regions and near the hemorrhagic areas and lipid vacuoles in the CCl_4 treated groups (Groups IV, V, VI). The increase in the activation of Kupffer cells and the severity of tissue damage were similar in Groups V and VI compared with Group IV.

Studies have shown that CCl_4 administration has caused an increase in serum TNF-α levels (Luckey and Petersen, 2001; Louis *et al.,* 1998; Qin and Tian 2011) and liver TNF-α immunoreactivity (Domitrović and Jakovac, 2010). Salah-Abbès et al., (2015) indicated that Tunisian radish extract improved the level of serum TNF-α against cadmium chloride intoxication and You et al., (2015) reported that radish isolates reduced elevated serum TNF-α levels and the number of lipid droplets in the hepatocytes due to excessive diet. There has been no study to determine the activity of TNF-α immunoreactivity using red radish and products to improve liver damage caused by CCl_4, in this study, there was no difference in tissue damage and TNF- α level between radish-treated groups and the CCl_4 group.

A limited number of studies have been conducted to evaluate biochemical data and lipid peroxidation indicators (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Kim *et al.,* 2013; Rafatullah *et al.,* 2008; Syed *et al.,* 2014;

Sadeek, 2011) by giving red radish extract or products to acute liver injury induced by carbon tetrachloride in rats. In accordance with the findings of these researchers, serum ALT enzyme activity, which was increased due to liver damage, decreased statistically with red radish application in the present study. However, the activity of this enzyme, which decreases with the addition of red radish, may be due to its not approaching normal values, possibly due to the duration of the experiment and/or the dose of red radish applied to animals.

Sadeek (2011) found that fresh radish juice significantly reduced serum lipids (total cholesterol, triglyceride, LDL-cholesterol) on CCl_4 induced hepatotoxicity in rats. In the present study, high triglyceride levels caused by liver degeneration decreased statistically with red radish application but did not approach normal values. This may be explained by the large amount of fat released into the blood from degenerated hepatocytes. In addition, serum total cholesterol and LDL-cholesterol levels significantly decreased due to liver damage. These decreases in the serum lipids due to oral administration of red radish were in agreement with the results of Sadeek (2011) who reported that biologically active compounds of radish have an indirect lipid lowering effect and antioxidant properties.

Some researchers (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Kim *et al.,* 2013; Syed *et al.,* 2014; Sadeek, 2011) have suggested that radish and its products significantly reduce the increased plasma lipid peroxidation levels, which may be due to antioxidant phytochemicals such as flavonoids, terpenoids and polyphenols in the radish plant. In the present study, in accordance with the findings of some investigators (Dash *et al.,* 2013; Kalantari *et al.,* 2009; Kim *et al.,* 2013; Syed *et al.,* 2014; Sadeek, 2011), the increase of lipid peroxidation indicators due to liver damage decreased with red radish application and this may be due to the antiperoxidative effect of the fermented red radish.

In conclusion, pretreatment with fermented red radish at 250 mg/kg and 500 mg/kg followed by CCl_4 exposure has no ameliorative effect on the histopathological changes in the liver. It is considered that the application of fermented red radish may have a partially antioxidative effect by decreasing biochemical and lipid peroxidation levels to the control group and this may be due to the synergistic interaction of *L. plantarum* with antioxidant phytochemicals in red radish but this amelioration is not effective in correcting the damage in liver tissue. It is also concluded that chronic and new investigations need to be performed to determine the ameliorative effects of fermented red radish on tissues using doses to give the best results without any side effects.

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