The Projective Effect of Pomegranate Juice on Fibrosis, Carboxylesterase Activity and Fatty Acid Profile in Rat Liver Tissues Exposed to Carbon Tetrachloride

[1]Ahmet Ozkaya*, [2]Zafer Sahin, [1]Seyda Cagri Bulbul, [3]Mirac Uckun, [4]Ertan Yologlu
[5]Ahmet Turk and [6]Yusuf Ozay
[1]Department of Chemistry and Chemical Processing Techniques, Vocational School of Technical Sciences, Adiyaman University, Adiyaman-02040, Turkey.
[2]Department of Physiology, Faculty of Medicine, Karadeniz Technical University, Trabzon-61080, Turkey.
[3]Department of Food Engineering, Faculty of Engineering, Adiyaman University, Adiyaman-02040, Turkey.
[4]Department of Science Education, Faculty of Education, Adiyaman University, Adiyaman-02040, Turkey.
[5]Department of Histology and Embryology, Faculty of Medicine, Adiyaman University, Adiyaman-02040, Turkey.
[6]Department of Medical Biology and Genetics, Faculty of Medicine, Adiyaman University, Adiyaman-02040, Turkey.
*aozkaya@adiyaman.edu.tr**

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Summary: In this study, it was aimed to investigate the effects of pomegranate juice (PJ) on liver carboxylesterases (Ces), oxidative stress parameters, liver histology, and fatty acids in rats exposed to carbon tetrachloride (CCl_4). Adult male Wistar albino rats were randomly divided into four groups as control, CCl_4, PJ, and CCl_4+PJ (n=7 in each group). We determined that the decrease in Ces activity due to CCl_4-induced liver damage was alleviated by PJ. Moreover, PJ reduced CCl_4-induced oxidative stress, liver degeneration, and apoptosis. While the CCl_4 group increased the 15:0, 16:0 fatty acid levels, it decreased the 20:4 and PUFA fatty acid levels compared to the control group. In the CCl_4+PJ group, 16:0 and ΣSFA fatty acid percentages decreased with the effect of PJ compared to the CCl_4 group, while 18:2n-6, ΣPUFA and ΣUSFA fatty acid ratios increased. We concluded that PJ has an ameliorative effect on liver damage caused by CCl_4 exposure.

Keywords: Pomegranate juice; Carbon tetrachloride; Carboxylesterase; Fatty acids; Liver; Rat.

Introduction

Hepatic fibrosis is a significant liver disease characterized by excessive extracellular matrix production by hepatic stellate cells in response to liver injury, which disrupts the liver's structure and function, and can eventually progress to cirrhosis and result in death [1]. There are still points that are not well-known in relation to the processes involved in the pathogenesis of liver fibrosis. Oxidative stress is recognized as a significant process in the pathophysiological changes observed in various liver diseases [2,3]. Free radicals formed as a result of oxidation attack the unsaturated fatty acids in cell membranes, resulting in peroxidation and the destruction of protein and DNA, leading to liver damage at varying degrees [4].

In recent years, it has been suggested that oxidative stress exerts an important role in the pathophysiology of liver fibrosis [3]. Carbon tetrachloride (CCl_4) is a member of the organic solvents that is extensively used in cleaning reagents [5]. It has been used in animal models to investigate synthetic poison-induced internal organ damage. When this lethal compound is introduced into the body by ingestion, inhalation, or skin absorption, it is disseminated throughout the body, often accumulating in the liver, cerebrum, kidney, muscle, fat, and blood [5, 6]. There are several studies demonstrating CCl_4 induced liver damage [6-8]. CCl_4 intoxication in animals is used experimentally to induce oxidative stress under various physiological conditions. Prolonged exposure to CCl_4 induces histopathological outcomes such as inflammatory leukocyte infiltration, necrosis, fibrosis, and cirrhosis, and it may also result in the development of cancer [9, 10].

Flavonoids are polyphenolic compounds that play an essential role in free radical scavenging. These polyphenolic compounds are found in vegetables, fruits, and medicinal plants. Therefore, antioxidants may be helpful in reducing liver fibrosis in oxidative stress-induced liver damage [11, 12]. Pomegranate (Punica granatum), a fruit that draws attention with its health benefits, has been grown for a long time in the Mediterranean region and consumed as fresh fruit or beverage. Pomegranate fruit, juice, and peel have a pronounced antioxidant capacity [13], with a high content of polyphenols, especially ellagitannins, dense tannins,

*To whom all correspondence should be addressed.

and anthocyanins [14]. Some of these antioxidant molecules have been shown to be bioavailable and safe [15]. It has been suggested that the consumption of pomegranate juice (PJ) produces chemopreventive, anti-atherosclerotic, anti-inflammatory, and chemotherapeutic effects in the body [16-18], and its consumption has increased tremendously [15,19]. In the body, especially in the liver, carboxylesterases (Ces) play an important role in detoxifying endogenous lipids and foreign substrates including drugs and environmental toxins. It is important for the body's protective function that the activity of these enzymes remains high, especially when exposed to environmental pollutants. Ces activity is widely distributed in mammalian tissues, with the highest levels present in hepatic tissue and the small intestine [20]. To the best of our knowledge, no study has been carried out concerning the interaction of PJ with hepatic Ces activity as well as fatty acids against CCl_4 treatment. Therefore, determining the effects of PJ on these enzymes and related factors in the liver is important to reveal its antioxidant activity and liver protective role. For this reason, in our study, it was aimed to investigate the effects of PJ on liver Ces enzyme activity, oxidative stress-related parameters, and liver fatty acids in rats exposed to CCl_4.

Experimental

Animals

A total of 28 adult Wistar albino male rats (260±10 g body weight) were obtained from Adiyaman University Experimental Animal Production Application and Research Center (ADYU DEHAM, Adiyaman, Turkey). The animals were housed under a standard light/dark cycle (12 hours of light and 12 hours of dark), at a regular temperature (22-25°C) with free access to fresh water and standard pelleted food (lipid 2.8%, protein 23.1%, cellulose 5%, ash 7.1%, humidity 12.8%). The experimental procedures were approved by the Ethics Committee of Adiyaman University (Document No: 2019/25), and the rats were treated in strict compliance with the international laws on the use and care of experimental animals. Four weeks before the experiment began, rats were placed in cages for adaptation purposes. No treatment was applied to the rats.

Experimental Procedure

The experimental animals were randomly divided into four groups as control, CCl_4, PJ, and CCl_4+ PJ (n = 7 in each group). The control group was treated with the solvent of CCl_4 twice a week for 6 weeks [21], followed by saline treatment (4 ml/kg) for the next 30 days. CCl_4 was prepared in 50% olive oil and injected intraperitoneally at a dose of 0.2 ml/100 g twice a week for 6 weeks. For 30 days, PJ was administered to the PJ group by orogastric gavage at a dose of 4 ml/kg [22]. The PJ was prepared by passing fresh pomegranate seeds through a juicer. In the CCl_4+PJ group, CCl_4 was injected intraperitoneally at a dose of 0.2 ml/100 g twice a week for 6 weeks, and for the next 30 days, PJ was administered at a dose of 4 ml/kg by orogastric gavage. The animals were sacrificed at the end of the experiments, and their hepatic tissue was obtained. The liver samples were stored at −80°C until the assays were performed.

Measurement of Ces activity

p-nitrophenol (PNPA, 26 mM) prepared in 96% ethanol was used as a substrate for the measurement of Ces enzyme activity. The reaction solution contained 5 µl of the sample and 250 µl of the solution with 0.05 M Trizma at pH: 7.4. This mixture was pre-incubated at 25°C. In the last step of the process, 5 µl of PNPA was added so that the final concentration of the solution would be 0.5 mM, and the absorbance changes were read at 405 nm on the Thermo-3001 Microplate Attachment UV/VIS spectrophotometer [23].

Determination of malondialdehyde (MDA), reduced glutathione (GSH), and glutathione S-transferase (GST)

MDA analysis was performed with the Thermo-3001 UV/VIS device (Thermo Fisher Scientific, Finland) with a Microplate Attachment according to the method developed by Placer [24]. A solution of 0.375% TBA (thiobarbituric acid) and 15% TCA (Trichloroacetic acid) in 0.25 N HCl was used. The MDA concentrations in the liver tissue samples were calculated by reading absorbance values at 532 nm.

Reduced GSH levels were analyzed using the Sedlak and Lindsay method [25]. Hepatic tissues were precipitated with 50% TCA and centrifuged at 1000xg for 5 min. 0.5 ml of the obtained supernatants were taken, and 2 ml of Tris-EDTA buffer (0.2 M, pH: 8.9) and 0.1 ml of 0.01 M 5.5'-dithio-bis-2-nitrobenzoic acid were added. The mixture was incubated at room temperature for 5 min. Then, absorbance values were measured at 412 nm with the Thermo-3001 UV/VIS device.

GST enzyme activity was tested using the Thermo-3001 UV/VIS device according to the method specified by Habig et al. [26]. In the analysis, 20 mM 1-chloro-2,4 dinitrobenzene (CDNB), prepared in 96% ethanol, was used as a substrate. Reduced GSH was used as a cofactor. 100 µl of 0.1 M pH: 6.5 phosphate buffer, 100 µl of 0.002M GSH solution, and 10 µl of CDNB solution were pipetted into 96 microplate wells. The samples were analyzed at 344 nm.

Extraction of Lipids and Preparation of Fatty Acid Methyl Esters

Lipid extraction was performed according to the Hara-Radin method [27]. The liver tissue samples (1 g)

were taken into a homogenization tube, and 5 ml of a 3:2 (v/v) hexane-isopropanol mixture was added. The samples were homogenized for 30 seconds. The samples were taken into centrifuge tubes and centrifuged at 4500 rpm for 10 minutes. After centrifugation, the supernatant was taken into tubes for methylation. Then, 5 ml of 2% methanolic sulfuric acid was added to the tubes, and the mixture was left to methylate in a 50ºC water bath for 15 hours [28]. The tubes were removed from the water bath and cooled to room temperature. Extraction was performed by adding 5 ml of 5% NaCl and 5 ml of hexane. The hexane phase was pipetted and treated with 5 ml of 2% $KHCO_3$ (potassium hydrogen carbonate). Again, 2 ml of hexane was added to the fatty acid methyl esters. The hexane phase samples containing the fatty acids taken into the vial were read on the Shimadzu QP 2010 Ultra Gas Chromatography device (GCMS-QP2010 Ultra System, Japan) with a Restek Rtx 2330 column.

Histopathological examination

For the histopathological examinations of the liver tissue samples, a portion of the liver was fixed in 10% formalin for 12 hours and then embedded in paraffin. Sections of 4 µm were taken from all tissue samples. The sections were stained with Masson's trichrome, evaluated semi-quantitatively under a light microscope (Leica DM500 attached Leica DFC295 Digital Image Analyze System), and photographed [29]. Centrilobular vein (CLV) scoring for fibrosis was made as 0: normal vessel, 1: appearance of moderately thickened CLV (star-shaped vessel wall), 2: markedly thickened CLV (ring-shaped vessel wall with numerous fibrous extensions between hepatocytes), and 3: cirrhosis.

Immunohistochemical analyses

The streptavidin-biotin-peroxidase complex method was applied for measuring Caspase-3. Sections of 4-6 µm thickness were taken from the tissues blocked with this method and deparaffinized. The primary antibody Caspase-3 (Rabbit polyclonal IgG, Abcam, ab2302, London, UK) diluted by 1/200 was used with the Thermo Scientific™ TP-015-HA commercial kit. Positive and negative control protocols were performed as recommended by the manufacturers. After AEC Chromogen was applied, staining with Mayer Hematoxylin was performed, the samples were examined under a Leica DM500 microscope (Leica DM500 attached Leica DFC295 Digital Image Analysis System), evaluated, and photographed. Based on the extent of immunoreactivity based on staining (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: no, +0.5: very little, +1: little, +2: moderate, +3: severe), a histoscore was established (Histoscore = prevalence x severity) [30].

Statistical analysis

All results are expressed as mean ± SEM. The statistical comparison of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey–HSD test. The histopathological results were analyzed using the non-parametric Kruskal–Wallis test. Pairwise comparisons between groups exhibiting significant values were made by Mann–Whitney U test. The level of statistical significance was accepted as $p<0.05$.

Result and Discussion

Hepatic Ces activities are shown in Fig 1. Compared to the control group (1273.47±46.32 nmol/min/mg protein), the liver Ces activity of the CCl_4 group (728.25±48.77 nmol/min/mg protein) was determined to be significantly lower ($p <0.001$). It was found that the liver Ces activities of the PJ (1346.85±121.03 nmol/min/mg protein) and CCl_4+PJ (1478.54±102.41 nmol/min/mg protein) groups were on statistically similar levels as the control group. According to CCl_4 group Ces enzyme activity, an increase was detected in PJ and CCl_4+PJ groups ($p<0.001$).

Hepatic MDA, reduced GSH, GST, and GR levels are presented in Table-1. Liver MDA concentrations were found to be significantly higher in the CCl_4 group compared to the control group ($p <0.05$). The MDA concentrations of the CCl_4+PJ group were on statistically similar levels as the control group. It was observed that the MDA level of the CCl_4+PJ group decreased compared to the CCl_4 group ($p<0.05$). Reduced GSH levels were found to be lower in the CCl_4 group ($p<0.05$, $p<0.001$) and higher in the PJ group compared to the control group ($p<0.001$). Moreover, it was determined that the reduced GSH values of the CCl_4+PJ group were on a statistically similar level as the control group. There were no significant differences in terms of GST and GR activities among the groups.

Table-1: The responses of oxidative stress-related biochemical parameters in rat liver tissues, (n=7).

Biochemical Parameteres	Control	CCl_4	PJ	CCl_4+PJ
MDA (nmol/mg protein)	0.09±0.01	0.13±0.01[a]	0.09±0.01[x]	0.09±0.01[x]
Reduced GSH (µmol/mg protein)	0.11±0.01	0.08±0.01[a]	0.15±0.01[az]	0.11±0.01[x]
GST (nmol/min/mg protein)	40.26±1.14	36.91±2.18	37.05±2.18	36.22±0.01
GR (nmol/min/mg protein)	2.5±0.22	2.61±0.41	2.83±0.26	2.87±0.51

[a] $p < 0.05$, [b] $p <0.01$ compared with the control group.
[x] $p < 0.05$, [y] $p <0.01$, [z] $p<0.001$ compared with the CCl_4 group.

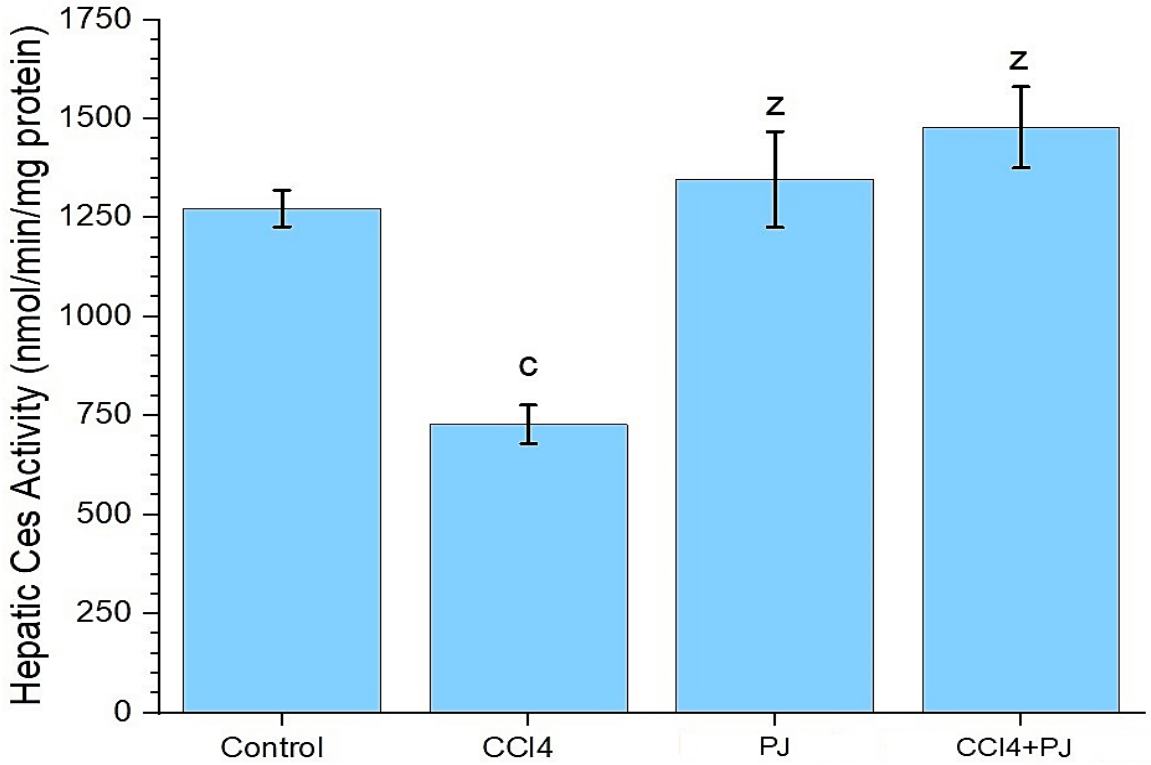

Fig. 1: Ces activity levels in the liver. c: $p < 0.001$ compared to control group. Abbreviations: Ces: Carboxylesterase, CCl_4: Carbon tetrachloride.

Table-2: Alterations of percentages (%) of fatty acids in rat liver tissues.

Fatty acids	Control	CCl_4	PJ	CCl_4+PJ
4:0 (Butyric acid)	0.082±0.007	0.107±0.107	0.078±0.007	0.082±0.082
6:0 (Caproic acid)	0.070±0.003	0.080±0.004	0.059±0.002	0.062±0.004
14:0 (Myristic acid)	0.299±0.017	0.405±0.052	0.152±0.011	0.377±0.078
15:0 (Pentadecylic acid)	0.317±0.013	0.499±0.088^{a}	0.210±0.007^{z}	0.342±0.013
16:0 (Palmitic acid)	20,544±0.276	23,212±0.471^{c}	18,221±0.139^{bz}	20,689±0.618^{z}
17:0 (Margaric acid)	0.751±0.037	0.974±0.135	0.665±0.031^{x}	0.707±0.042
18:0 (Stearic acid)	21.035±0.406	19.126±0.926	22.139±0.235^{x}	19.088±0.799
20:0 (Arachidic acid)	0.086±0.007	0.089±0.005	0.100±0.005	0.089±0.003
22:0 (Behenic acid)	0.181±0.008	0.265±0.029^{b}	0.153±0.010^{z}	0.186±0.008^{x}
23:0 (Tricosylic acid)	0.171±0.010	0.209±0.027	0.166±0.007	0.177±0.012
24:0 (Lignoceric acid)	0.264±0.004	0.159±0.016	0.308±0.010	0.293±0.013
ΣSFA	43.800±0.750	45.247±0.851	42.251±0.645^{x}	42.092±0.782^{x}
16:1n-7 (Palmitoleic acid)	0.426±0.039	0.698±0.103	0.246±0.023	0.857±0.307
18:1n-9t (Oleic acid-trans)	0,062±0.005	0.036±0.005^{b}	0.028±0.001^{c}	0.049±0.004
18:1n-9c (Oleic acid-cis)	5.521±0.279	8.415±0.677^{a}	4.515±0.221^{z}	8.624±0.850^{b}
20:1n-9 (Eicosenoic acid)	0.221±0.012	0.289±0.057	0.203±0.018	0.160±0.003^{x}
24:1n-9 (Nervonic acid)	0.092±0.004	0.197±0.015^{c}	0.105±0.005^{z}	0.115±0.004^{z}
ΣMUFA	6.322±0.204	9.635±0.411^{c}	5.097±0.301^{z}	9.805±0.385^{c}
18:2n-6c (Linoleic acid^{e})	16.700±0.382	15.934±0.626	16.644±0.358	19.009±0.340^{bz}
18:3n-6 (α-Linolenic acid^{e})	0.175±0.013	0.167±0.016	0.185±0.012	0.195±0.008
18:3n-3 (γ-linolenic acid)	0.392±0.032	0.279±0.032	0.411±0.013	0.484±0.052^{y}
20:2n-6 (Eicosadienoic acid)	0.524±0.030	0.495±0.026	0.703±0.067^{ax}	0.570±0.039
20:3n-6 (Dihomo-γ-linolenic acid)	0.720±0.030	0.949±0.079^{a}	0.547±0.028^{z}	0.755±0.047
20:4n-6 (Arachidonic acid^{pe})	25.831±0.347	20.831±0.529^{c}	28.626±0.513^{az}	22.210±1.096^{b}
20:5n-3 (Eicosapentaenoic acid^{pe})	0.174±0.014	0.111±0.015^{a}	0.159±0.010	0.142±0.011
22:6n-3 (Docosahexaenoic acid^{pe})	5.362±0.205	6.352±0.558	5.377±0.147	4.738±0.213^{y}
ΣPUFA	49.878±0.874	45.118±0.753^{b}	52.652±0.901^{z}	48.103±0.812^{y}
ΣUSFA	56.200±0.985	54.753±0.651	57.749±0.847^{x}	57.908±0.953^{x}

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; ΣUSFA: total unsaturated fatty acids; n-3: omega-3 fatty acids; n-6: omega-6 fatty acids.

e: Essential fatty acids, pe: Partially essential fatty acid. Data are expressed as mean ± SEM (n=7 for each group).

^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ compared with the control group.

^{x} $p < 0.05$, ^{y} $p < 0.01$, ^{z} $p < 0.001$ compared with the CCl_4 group.

The percentages of fatty acids in the liver are presented in Table-2. Regarding butyric acid, caproic acid, and myristic acid, there was no significant difference between the control group and the experimental groups. In only the CCl_4 group, the percentage of pentadecylic acid was significantly higher than the control group ($p<0.05$). The percentage of palmitic acid in the CCl_4 group was significantly higher ($p<0.001$), and this percentage was significantly lower in the PJ group ($p<0.01$) compared to the control group. There was no significant difference in terms of the percentages of margaric acid, stearic acid, or arachidic acid between the control group and the experimental groups. In only the CCl_4 group, the percentage of behenic acid was significantly higher than that in the control group ($p<0.01$). Regarding tricosylic acid, lignoceric acid, and palmitoleic acid, there was no significant difference between the control group and the experimental groups. Although not statistically significant, the percentage of the total saturated fatty acids (ΣSFA) was found to be relatively higher in the CCl_4 group in comparison to the control group. However, a decrease was observed in the PJ and CCl_4+PJ groups compared to the ΣSFA percentage of the CCl_4 group ($p<0.05$). In the CCl_4 and PJ groups, the percentages of oleic acid-trans were significantly lower than those in the control group ($p<0.01$ and $p<0.001$, respectively). In only the CCl_4 group, the percentage of oleic acid-cis was significantly higher than that in the control group ($p<0.01$). There was no significant change in the percentages of eicosenoic acid between the control group and the experimental groups. In only the CCl_4 group, the percentages of nervonic acid and total monounsaturated fatty acids (ΣMUFA) were significantly higher than those in the control group ($p<0.001$). Regarding α-linolenic acid, there was no significant difference between the control group and the experimental groups. The 18:2n-6 level of the CCl_4+PJ group increased compared to the control and CCl_4 groups ($p<0.01$, $p<0.001$). In addition, the 18:3n-3 fatty acid level of the CCl_4+PJ group increased compared to the CCl_4 group ($p<0.01$). The percentages of eicosadienoic acid in the PJ group were significantly higher than those in the control and CCl_4 groups ($p<0.05$). In only the CCl_4 group, the percentage of dihomo-γ-linolenic acid was significantly higher than that in the control and PJ groups ($p<0.05$, $p<0.001$). The percentage ratio of 20:4n-6 fatty acids in the CCl_4 group was decreased compared to the control group ($p<0.001$). However, in the CCl_4+PJ group, the percentage ratio of 20:4n-6 fatty acids was found to increase relative to the CCl_4 group ($p>0.05$). The percentage ratio of 20:4n-6 fatty acids in the PJ group increased compared to all other groups ($p<0.05$, $p<0.001$). In only the CCl_4 group, the percentage of eicosapentaenoic acid was significantly lower than that in the control group ($p<0.05$). There was no significant difference in the percentage of docosahexaenoic acid between the control group and the experimental groups. In only the CCl_4 group, the percentage of total polyunsaturated fatty acids (ΣPUFA) was significantly lower than all the other groups ($p<0.01$, $p<0.001$). The percentage of total unsaturated fatty acids (ΣUSFA) in the CCl_4 group decreased compared to the CCl_4+PJ and PJ groups ($p<0.05$).

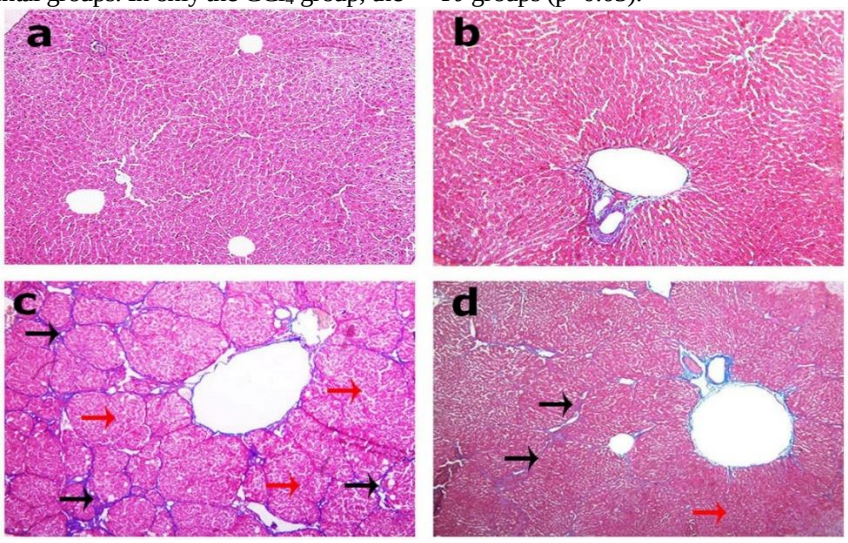

Fig. 2: Histopathological evaluation results in liver tissue. Normal-appearing liver tissue of the control group (A). Normal-appearing liver tissue belonging to the PJ group (B). Degeneration, necrosis (black arrow) and increased liver tissue fibrosis (black arrow) in the CCI_4 group (C). Significant reduction in degeneration and necrosis (black arrow) and liver tissue fibrosis (black arrow) in the CCl_4+PJ group (D). Abbreviations: Ces: Carboxylesterase, CCl_4: Carbon tetrachloride.

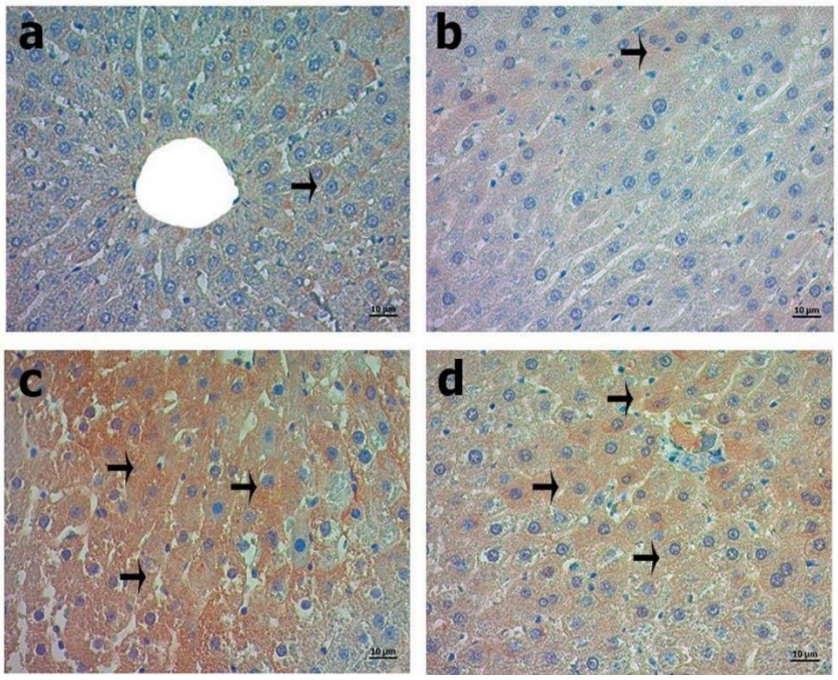

Fig. 3: Immunohistochemical staining for Caspase-3 (black arrow) in liver tissue. a- Control group, b- PJ group, c- Increase in Caspase-3 immunoreactivity of CCl_4 group, d- Decrease in Caspase-3 immunoreactivity of CCl_4+PJ group. Abbreviations: Ces: Carboxylesterase, CCl_4: Carbon tetrachloride.

The liver tissues of the control (Fig 2a) and PJ (Fig 2b) groups had normal histological appearance. Fibrosis, hepatocyte degeneration, and necrosis were observed significantly more in the CCl_4 group (Fig 2c) compared to the control group ($p<0.05$). Relative to the CCl_4 group, a significant decrease in fibrosis (black arrow) and a decrease in degeneration and necrosis (red arrow) were observed in the CCl_4+PJ group (Fig 2d) ($p<0.05$). As a result of the immunohistochemical staining for caspase-3 immunoreactivity under light microscopy, caspase 3 immunoreactivity was observed in hepatocytes (black arrow) in the liver tissue samples. Regarding hepatic tissue caspase-3 immunoreactivity, the control group (Fig 3a) and PJ group (Fig 3b) were similar. Compared to the control group, caspase-3 immunoreactivity was found to be significantly higher in the CCl_4 group (Fig 3c, $p<0.05$). Moreover, caspase-3 immunoreactivity was significantly lower in the CCl_4+PJ group than the CCl_4 group (Fig 3d).

Environmental pollution is contaminated by pollutants from natural and anthropogenic sources. The toxic chemicals used by humans have negative effects on many biochemical parameters in the cell [31]. In our study, the effects of PJ on Ces activity and oxidative stress and fatty acids in the liver against liver injury induced in rats by CCl_4 were evaluated. According to our findings, liver Ces activity was significantly decreased due to CCl_4 exposure. The Ces activity value of the CCl_4+PJ group was on approximately the same level as the control group. Therefore, we suggest that the decrease in Ces activity due to liver injury induced by CCl_4 was attenuated by PJ. The liver is an important organ that is responsible for detoxification. Due to this feature, Ces, a group of detoxifying enzymes, is found at high levels in the liver [20]. The maintenance of Ces activity in the liver is important for the detoxification of lipids, drugs, and foreign substrates, including environmental toxins. Therefore, it may be stated that the decrease in Ces activity as a result of CCl_4 exposure was associated with toxic injury to the liver. Studies on the effects of CCl_4 on liver Ces activity are very few. One study suggested that microsome, cytosol, and serum malathion Ces activities were not altered after a single intraperitoneal administration of carbon tetrachloride in rats [32]. However, only acute effects were evaluated in that study. In another study on the topic, it was determined that serum and hepatic Ces activities changed depending on hepatotoxic paracetamol and carbon tetrachloride doses in mice. Moreover, serum esterase activity was reported to be unchanged or marginally reduced depending on the duration of treatment and the substrate that was used [33]. In our previous study, we found that aluminium, which is a toxic substance, reduces the activity of Ces enzymes [34]. In addition, it has been reported that xenobiotics,

which causes environmental pollution, reduces Ces enzyme activity in fish [35]. According to these results of our study, we thought that the decrease in Ces activity due to liver injury induced by CCl_4 was alleviated by PJ.

In our experiments, we determined that liver MDA concentrations were significantly higher in the CCl_4 group. However, the MDA concentrations of the CCl_4+PJ group were almost the same as those in the control group. In comparison to the control group, liver GSH levels were lower in the CCl_4 group and higher in the PJ group. Moreover, the reduced GSH level of the CCl_4+PJ group was almost the same as that in the control group. There was no significant difference among the groups based on their GST and GR activities. According to our findings, exposure to CCl_4 created oxidative stress in the liver, and PJ had a reducing effect on this damage. The content of PJ we used in our experiments was determined as phenolic acid 490.75 mg/kg, anthocyanin 137.1 mg/L, ellagic acid 175 mg/100 g, total flavonoids 63 mg/kg and total antioxidants 1530 mg/kg in our previous study [36]. The bioactivities of polyphenols have been associated with their antioxidant properties. That is, it is the ability to protect against damage caused by reactive oxygen species [37]. According to the examination results of our histopathological images, liver degeneration and fibrosis developed in relation to oxidative and toxic damage induced by CCl_4 exposure. As an important finding, we determined that the degree of CCl_4-induced liver damage and fibrosis was reduced by the PJ application. Another important finding in our study was that apoptosis due to the increase in liver caspase-3 activity induced by CCl_4 exposure was reduced by PJ. It is well-known that CCl_4 causes liver injury [6-8]. Necrosis, fibrosis, cirrhosis, and even cancer may develop due to oxidative stress and toxic damage in the liver [9,10]. CCl_4-induced liver injury is associated with triggering the apoptosis process, as well as necrosis [38,39]. We considered that oxidative stress and increased apoptosis may play an important role in loss of function due to liver injury. In our study, it was an important finding that PJ reduced oxidative stress, as well as liver degeneration and apoptosis, induced by CCl_4.

It is known that pomegranate or its juice increases chemopreventive, anti-inflammatory, and especially anti-atherosclerotic activity in the body [16-18]. The PJ contains antioxidants that positively affect cholesterol and fat metabolism. It has been proposed that the effects of PJ on cholesterol are similar to statins [40-42]. Statins are a class of medication involved in reducing blood cholesterol levels by inhibiting the cholesterol synthesizing enzyme, 3 hydroxyl 3 methyl glutaryl coenzyme A (HMG-CoA) reductase [43], decreasing mevalonate formation involved in the synthesis of cholesterol [44], increasing endothelial function, and improving the activity of antioxidants [45]. Atorvastatin, a member of the statins class, is used to reduce the biosynthesis of cholesterol by inhibiting the precursor enzyme HMG-CoA reductase [46]. There is evidence that PJ and atorvastatin have a synergistic effect on cholesterol accumulation in the body and consequent changes in fat metabolism [40-42]. The interaction of PJ with HMG-CoA is an important result because these data may provide a new treatment option for metabolic disorders such as hyperlipidemia and atherosclerosis. In this study, we determined for the first time that CCl_4 increased the percentage of myristic acid, pentadecylic acid, and behenic acid, which are members of the SFA class, and PJ treatment prevented this increase.

There are significant differences in fatty acid profiles in patients with various disease states [47]. In humans, the levels of MUFAs such as oleic acid tend to increase with age, after the age of 18. Some other PUFAs, particularly arachidonic acids, also decline with age in the elderly [48]. Therefore, the levels of these fatty acids can change over the years depending on metabolic and environmental factors. Moreover, oxidative stress can affect the fatty acid composition of the liver and the entire body. In such cases, natural compounds can regulate the percentages of hepatic total saturated and unsaturated fatty acids altered due to oxidative damage [49]. Patients with advanced liver fibrosis generally tend to have increased palmitic acid, stearic acid, oleic acid, and ΣMUFA levels, while long-chain fatty acids tend to decrease in their body [50]. Recently, it was reported that an increasing trend of hepatic USFA in the body is associated with decreased fibrosis severity in nonalcoholic fatty liver disease [51]. In our experiments in this study, we determined that compared to the control group, the percentages of palmitic acid and oleic acid-cis were significantly higher in the CCl_4 group and lower in the PJ group. Moreover, the percentages of nervonic acid-cis and ΣMUFA were significantly higher only in the CCl_4 group. Regarding long-chain fatty acids, the percentage of ΣPUFA in the liver was significantly lower only in the CCl_4 group. Additionally, the percentage of ΣPUFA was higher in the PJ group. Albeit not statistically significantly, the percentage of ΣUSFA was lower in the CCl_4 group. However, we observed that the ΣUSFA level of the CCl_4+PJ group increased with the effect of PJ. There was a significant alteration in the essential fatty acid, linoleic acid, between the control group and the experimental

groups, and a significant increase was detected in the CCl_4+PJ group. Among the partially essential fatty acids, the percentage of arachidonic acid was significantly lower in the CCl_4 group and significantly higher in the PJ group. Besides, the percentage of eicosadienoic acid was higher in the PJ group. Another partially essential fatty acid is docosahexaenoic acid, which showed no change in its level in our study. In an important study, it was reported that palmitic and oleic acids increased significantly, and linoleic and arachidonic acids decreased in the livers and plasmas of rats given CCl_4 [52]. Our findings, in line with the relevant literature, showed that liver injury and fibrosis due to CCl_4 exposure led to significant changes in the liver's individual ΣSFA, ΣMUFA, and ΣUSFA levels. It was an important finding of ours that these changes were reversed in the group that was administered PJ with CCl_4. These results demonstrated that PJ had beneficial effects against liver fibrosis.

In the latest study conducted by Pekmez et al. on pomegranate juice, they found that lead acetate increased MDA levels, decreased GSH levels, and decreased GST and Ces enzyme activities. [53]. It was stated that these negative effects of lead acetate on rat disease were corrected by the addition of pomegranate juice. It was also determined that lead acetate corrected the protective effects of changes in rat heart rate. Our previous study reported that CCl_4 caused cellular degeneration and adverse effects on biochemical enzymatic/non-enzymatic parameters in rat brain tissue [54].

Conclusion

In conclusion, we determined that liver Ces activity decreased due to CCl_4 exposure, but such a decrease was not observed in the CCl_4+PJ group. To our knowledge, this is the first study which found that the decrease in Ces activity due to CCl_4-induced liver damage was alleviated by administering PJ. Additionally, as an important result, we observed that PJ reduced CCl_4-induced oxidative stress, liver degeneration, and apoptosis. We determined for the first time that CCl_4 increased the percentages of pentadecylic acid and behenic acid, which are members of the SFA class, and PJ treatment prevented this increase. It was also found that CCl_4-exposed rat liver tissues tended to increase the proportions of individual saturated fatty acids. Liver injury and fibrosis due to CCl_4 exposure led to significant changes in the liver's individual levels of ΣSFA, ΣMUFA, and ΣUSFA. For the first time, in our study, these changes were observed to be improved in the group treated with CCl_4+PJ. All results obtained in our experiments indicated that PJ has the potential to have a curative effect on liver fibrosis by improving altered Ces activity, oxidative stress, and fatty acids in the liver induced by CCl_4 exposure.

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