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The effects of *Juglans regia* L. (walnut) extract on certain biochemical parameters and in the prevention of tissue damage in brain, kidney, and liver in *CCl₄* applied Wistar rats

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Abstract: Objective: The purpose of this study is to demonstrate protective effects of walnut samples on *CCl₄*-induced tissue damage in vivo.

Methods: Walnut fruits were extracted and then subjected to vitamin and flavonoid analyses. The extracts obtained were injected intraperitoneally every other day to Wistar male rats given carbon tetrachloride (*CCl₄*) and the animals were decapitated at the end of the study period. The brain, kidney, and liver tissues were removed and lipid peroxidation (LPO) measurements were done in the lipid fraction generated. The fatty acids in the lipid extract were analyzed by gas chromatography after converting them into methyl esters. In addition, the amounts of glutathione, protein, and vitamins were analyzed.

Results: Given the results achieved, it was found that the levels of fatty acids increased in the brain and kidney tissues after *CCl₄* administration (p<0.001). In the groups given walnut extract against *CCl₄*, it was found that the glutathione (GSH) level increased and malondialdehyde (MDA) level reduced in all tissues (p<0.05, p<0.01). Given lipophilic vitamin levels, it was found that α-tocopherol level increased in the brain and liver tissues in the group receiving additional walnut in comparison with the controls (p<0.05), and cholesterol level increased in the tissues, except kidney, in all groups in comparison with the control group (p<0.05, p<0.001).

Conclusion: Our data indicates that walnut extract has protective effects against LPO formation in the brain, kidney and liver tissues.

Keywords: *Juglans regia* L., lipid peroxidation (LPO), antioxidant, MDA, *CCl₄*

Özet: Amaç: Bu çalışmaya *CCl₄* uygulanan sıçanlarda *CCl₄*'e bağlı olarak ortaya çıkan doku hasarlarını karşı ceviz ekstraktının koruyucu etkilerinin ortaya çıkarılması amaçlanmıştır.

Metod: Ceviz meyveleri ekstrakte edildikten sonra vitamin ve flavonoid analizi yapıldı. Elde edilen ekstreler karabon-tetaklorür (*CCl₄*) uygulanan Wistar ırkına ait erkek ratlara gün aşını intraperitoneal yolla verildikten sonra deney süreleri sonunda dekapite edildi. Beyin, böbrek ve karaciğer dokusu alındıktan sonra oluşan lipit fraksiyonunda lipit peroksidadyon (LPO) ölçümü yapıldı. Lipit ekstrakti içindeki yağ asitleri metil esterlerine dönüştürülükten sonra gaz kromatografisi ile analiz edildi. Ayrıca glutatyon, protein ve vitamin miktarlarından incelendi.

Bulgular: Elde edilen sonuçlara göre; *CCl₄* uygulaması sonucu beynin ve böbrek dokularında yağ asidi düzeylerinin yükseldiği saptandi (p<0.001). *CCl₄* uygulamasına karşı ceviz ekstrakti verilen gruplarda bütün dokulara
glutatyon (GSH) düzeyinin yükseldiği ve malondialdehit (MDA) düzeyinin de azaldığı belirlendi (p<0.05, p<0.01).

Lipofilik vitamin düzeyleri incelendiğinde, α-tokoferol düzeyinin kontrole göre ceviz eklenen grupta beyin ve karaciğer dokusunda arttığı (p<0.05), kolesterol düzeyinin ise böbrek dokusu hariç dokularda kontrol grubuna göre bütün gruplarda yükseltiği tespit edildi (p<0.05, p<0.001).

Sonuç: Verilerimiz ceviz ekstraktının beyin, böbrek ve karaciğer dokularında LPO oluşumuna karşı koruyucu etkisi olduğunu gösterdi.

Anahtar Kelimeler: Juglans regia L., lipit peroksidasyonu (LPO), antioksidan, malondialdehit (MDA), karbontetraklorür (CCl₄)

Introduction

The walnut plant belongs to the genus Juglans from Juglandaceae family of the order Juglandales. Walnut is a fruit that has been known and cultivated since ancient times. Its plantation is spread over all regions of Türkiye [1].

Walnut is a valuable source of nutrients with cholesterol-free contents, substitutes for animal proteins, and high concentrations of unsaturated fatty acids. It is pretty rich in linoleic acid (18:3, n-3) and linolenic acid (18:2, n-6), polyunsaturated fatty acids that are essential for a healthy life. The ratio of linoleic acid to linolenic acid is 4:1 and this ratio is considered as the excellent balance. Linolenic acid prevents arteries from inflammation, sclerosis, obstruction and thrombosis through reducing blood pressure. In addition, it protects the heart by reducing cholesterol level and makes the body stronger against certain cancer types. Walnut that has a pretty high nutritional value is also beneficial for the brain. Before the birth, nerve cells proliferate very rapidly (approximately 250 000 per minute), however, proliferation of nerve cells stops thoroughly after birth and the cells begin to die gradually. New cells do not compensate the dead nerve cells, however, the capacity of the existing nerve cells can be enhanced by the consumption of beneficial fruits, such as walnut [2].

With the studies conducted, it was found that there were significant differences between distinct walnut varieties in terms of oil content, fatty acids, and tocopherol depending on environmental conditions [3]. In addition, it was reported that walnut contained high concentrations of α-tocopherol, a compound belonging to vitamin E family, which have strong anti-oxidant effect preventing the process of lipid oxidation [3,4]. It was also reported that protein varieties [5], plant sterols [6], folate, tannins and polyphenols [7,8] were efficacious compounds in healthy nutrition. CCl₄ is a substance that is used widely to generate experimental liver cirrhosis and its pro-oxidant activity is well known [9,10]. CCl₄ is metabolized to haloalkane free-radicals (CCl₃ and CCl₃O₂ •) by mixed function cytochrome oxidase complex that uses (NADPH)-cytochrome P450 electron transport chain in the hepatic smooth endoplasmic reticulum [11]. These substances generated cause hepatotoxic CCl₄, initiating lipid peroxidation in the membranes [12,13]. CCl₄ enters into the body via inhaled air, water, food intake and contact to skin. It spreads over the entire body with higher concentrations in the liver, kidneys, muscles, adipose tissue, and blood.

The phenolic compounds, vitamins, and carotenoids contained in fruits and vegetables possess anti-oxidant activity and are highlighted as efficacious compounds to prevent from the diseases relating to oxidative stress. It is widely accepted that anti-oxidants play a protective role against nutritional origin or other hazards. In this research, phytochemical ingredients of walnut samples cultivated in Elazığ province, and it was aimed to demonstrate anti-oxidant effects of this content on CCl₄-induced tissue damage in vivo.

Materials and Methods

Herbal material

The fruits of Juglans regia L. were used as herbal materials. The fruits of Juglans regia L. were collected from Elazığ region and the residues of flavonoid in the fruit were extracted with 85% methanol. The methanol extracts were centrifuged and its flavonoid content was analyzed according to Zu et al’s method [14]. Then, extracts were evaporated in the rotatory evaporator at 55°C under vacuum. After this process, the remaining dried extract was dissolved in dimethylsulfoxide (DMSO). The mixture of DMSO was used in animal experiment.

Flavonoid analysis of fruit extract

The flavonoids in the methanolic extracts of fruit were analyzed by using PREVAIL C18 (15x4.6 mm, 5μm) HPLC column. Methanol/water/acetonitrile mixture (46/46/8,
v/v/v) containing 1% acetic acid as the mobile phase was used [14]. PDA detector (SPD-M10A VP, Shimadzu, and Kyoto, Japan) was used for the analysis of flavonoids. Standardly, catechin (CA), naringin (NA), rutin (RU), resveratrol (RES), myricetin (MIR), morin (MOR), naringenin (NAR), quercetin (QU), and kaempferol (KA) mixture was used. Amounts of flavonoids were calculated using the external standard method on the CLASS-VP software (Shimadzu, Kyoto, Japan). Results were expressed as µg/g.

**Extraction and analysis of ADEK and phytosterols of fruit extract**

*M. nigra* fruit samples were weighed and homogenized with n-hexane/isopropyl at 3/2 (v/v) ratio [15] and after the hydrolysis with 5% KOH at 85°C, extraction of phytosterols was performed with n-hexane [16]. The amounts of ADEK vitamins and phytosterols were analyzed at 202 nm and 326 nm using a UV detector on a HPLC device. [16–18].

**Animal experiment**

The experimental study were provided by the Experimental Research Center at Firat University School of Medicine (FÜDAM) with no: 30 ethics committee permits (26.03.2009). Wistar albino male rats were used. These rats were divided into four groups. These groups and the concentrations of substance given to these groups were as follows:

**Experiment groups**

1. **Control group** (n=6): The rats in this group were administered intraperitoneally 0.4 mL/kg of olive oil.
2. **CCl₄ group** (n=6): a mixture of 0.4 mL/kg of 20% CCl₄ and olive oil
3. **CCl₄+Walnut group** (n=6): 0.4 mL/kg of CCl₄ group+2 mL/kg of walnut extract

   The dose of the extract walnut was determined in accordance to the research we did before [19]. The dose of CCl₄ was found to be 0.4 mL/kg body weight in an equal olive oil as a result of our experiments [20].

   Injection molding process, by making other day for 45 days was performed. The extracts were given by way interaperitoneal. To observe the effects of flavonoids on CCl₄ as individual routine flavonoid and CCl₄ group was formed. After the end of the experiment, procedures were performed according to the following rules and tissues were taken.

According to the ethics committee report, the animals were given anesthesia and sacrificed by withdrawing blood from the heart, then they were decapitated. Promptly after decapitation, blood, liver, kidney and brain tissues were collected. These tissue samples were promptly rinse with 0.9% serum physiologic, so that, the samples were cleaned from the blood and were stored at -20°C until biochemical analyses.

**Analysis of tissue ADEK vitamins and cholesterol levels by HPLC device**

Tissue samples were homogenized with 10 mL n-hexane/isopropyl at 3/2 (v/v) ratio [15] and centrifuged in +4°C at the 2800 rcf. To analysis of unsaponifiable lipophilic molecules, 5 mL of supernatant was put into 25-mL test tubes with caps, then the tubes were added 5% KOH solution. After vortexing, the tubes were kept at 85°C for 15 minutes. The tubes were removed and cooled to room temperature; then, the tubes were added 5 mL of distilled water and stirred. The unsaponifiable lipophilic molecules were extracted with 2x5 mL of hexane. The hexane phase was evaporated by nitrogen stream; then it was dissolved in 1 mL of acetonitrile/methanol (50%+50%, v/v) mixture, put into auto-sampler vials and analyzed [16–18]. The analysis was done on Shimadzu HPLC device. Calculations were done using Class VP 6.27 program (Shimadzu, Kyota Japan).

**Determination of GSH and total protein levels**

To quantify the glutathione and total protein levels in the tissues, sufficient amounts of tissue samples collected from the animals were homogenized in 10 mL Tris-HCl and EDTA (pH:7) buffer, then separated from tissue pellet by centrifugation at 9072 rcf for 10 min at +4°C. After centrifugation, the supernatant were divided into two aliquots, one of which was used for GSH analysis. For this purpose, the proteins in the supernatant were precipitated by adding 1 ml of 5% metaphosphoric acid reactive. This mixture was centrifuged at 2268 rcf for 5 min, so that the pellet was precipitated and the supernatant was removed into another tube. The supernatant was added 1 mL 150 µL DTNB and 2 mL of 0.3 M Na₂HPO₄ solution, the color changed to yellow was read at 412 nm against blank [21]. The aliquot of supernatant remaining from the glutathione analysis was used for total protein analysis. For this purpose, the supernatant was separated and subjected to Lowry method [22].
HPLC Analysis of the Amount of LPO

LPO levels in tissues was measured by making modifications in the Ohkawa et al (1978) method [23]. For this analysis, from Tris-HCl and EDTA buffer tissue homejanat 1 ml was taken from each group prepared; 0.6% 2-thiobarbituric acid (TBA) solution and 2 ml distilled water were added onto the mixtures; and then the samples were vortexed. Then they were kept at 90°C for 60 minutes and the pink color formed as a result of the reaction was extracted with 3 ml n-butanol. The samples were centrifuged and the density of the supernatant part obtained by the end of centrifugation was measured by a fluorescence detector (at λ (excitation) =515 nm and λ (emission) =543 nm) on the HPLC device. Shimadzu fully automatic HPLC equipment was used in the analyses. Inertsil ODS-3 C18 HPLC column (150x4.6 µm) was used for measurement and The column was eluted isocratically at 20°C with a 5 mM sodium phosphate buffer (pH=7.0) and acetonitrile (85:15, v/v) at a rate of 1 ml/min [23]. Analysis time was set as 5 minutes. Standardly, 1,1,3,3-Tetraethoxy-propane (TEP) was used. Results were reported as nmol/µl.

Isolation of fatty Acids and gas chromatographic analysis of fatty acid methyl esters

After unsaponifiable molecules analysis, to analysis of fatty acids, 5 mL of supernatant was put into 25-mL test tubes with caps, then the tubes were added 5 ml 2% methanolic sulfuric acid was added onto it; then the mixture was left at 55°C for 12 hours. At the end of this time, 5 ml of 5% sodium chloride was added and the fatty acid methyl esters were extracted with 5 ml of n-hexane. The mixture was treated with 5 ml of 2% KHCO₃ solution, then the n-hexane phase was vaporized with nitrogen stream [24], fatty acid methyl ester residues were dissolved in 1 ml heptane and taken to autosampler vials. The analysis of fatty acid methyl ester was conducted with a Shimadzu GC 17 device (Kyoto, Japan). Machery-Nagel (Germany) capillary column of 25 m length, 0.25 µm inner diameter, and Permobond 25 µm thickness was used for this analysis. Nitrogen gas was used as the carrier gas. During analysis, mixtures of standard fatty acid methyl esters were injected and the retention time was determined for each fatty acid. After this process, mixtures of fatty acid methyl esters of the samples were analyzed.

Statistical analysis

SPSS 15.0 program was used for statistical analysis. Comparison between the control group and experimental groups was done with the analysis of variance (ANOVA) and LSD tests. The results were given as mean±SEM. For determining the differences between the groups, p>0.05, p<0.05, p<0.01, and p<0.001 values were used.

Results

Flavonoid and vitamin contents of walnut fruit extract

With the analyses done on HPLC device, it was found that in methanol extract of Juglans regia L. fruit, there were α-tocopherol, δ-tocopherol, vitamins D-3 and K-1, stigmasterol, β-sitosterol, retinol molecules and flavonoids such as rutin, myricetin, morin, kaempferol, naringin and naringenin (Table 1, 2).

Effect of walnut fruit extract on changes in GSH, total protein and MDA concentrations in the brain, kidneys and liver

It was observed that in the brain tissue, GSH concentration remarkably reduced in CCl₄ group compared with the controls (p<0.001). MDA level was prominently high in both groups compared with the controls (p<0.001). Total protein

| Flavonoids     | Walnut fruit µg/g. |
|----------------|-------------------|
| Rutin          | 54                |
| Myricetin      | 281.5             |
| Morin          | 4.65              |
| Kaempferol     | 0.15              |
| Naringin       | 49.95             |
| Naringenin     | 0.85              |

| ADEK vitamins and phytosterols | Walnut samples µg/g. |
|-------------------------------|----------------------|
| α-Tocopherol                  | 0.63                 |
| D-3                           | 0.19                 |
| δ-Tocopherol                  | 8.06                 |
| Ergosterol                    | 0.41                 |
| K-1                           | 0.16                 |
| Stigmasterol                  | 15.99                |
| β-sitosterol                  | 64.26                |
| Retinol (vitamin A)           | 0.01                 |
level decreased in both groups compared with the controls (p<0.05) Conversely, GSH concentration increased in CCl₄+Walnut group compared with the CCl₄ group, MDA level was found to be declines partially (p<0.001, p>0.05) (Table 3).

In the kidneys, it was observed that MDA level increased in CCl₄ group compared with the controls (p<0.05) whilst it was found declines in the fruit extract group (p<0.05, p<0.01). It was found that total protein level increased in CCl₄ group (p<0.01). Otherwise, MDA level decreased in CCl₄+Walnut group compared with the CCl₄ group (p<0.05). (Table 4).

In the liver, it was found that GSH concentration decreased CCl₄ group compared with the controls (p<0.001). MDA levels were found to be increased remarkably in both group compared with the controls (p<0.01, p<0.001). Total protein level declined in CCl₄ group (p<0.05, p<0.01). Compared with the CCl₄ group, GSH and total protein levels were found increased, whilst MDA level was declined in CCl₄+Walnut group (p<0.001, p<0.001, p<0.05) (Table 5).

**Effects of walnut fruit extract on composition of fatty acids in the brain, kidneys and liver**

In the brain, it was found that the concentrations of 16:0, 18:0 and 18:1 increased in all groups compared to the controls (p<0.001). It was also found that the total fatty acid concentrations were significantly higher in both group than the controls (p<0.05, p<0.001). It was also found partially decline and increase in almost all fatty acids in CCl₄+Walnut group compared with the CCl₄ group (p<0.05) (Table 6).

Given the effect of walnut fruit extract on the fatty acid profile in the kidneys, no significant difference was observed between CCl₄ group and the control group with regard to 16:0 concentration (p>0.05), but it increased in fruit extract groups (p<0.001). Given the levels of 16:1, n-7 and 18:1, n-9, 18:2, n-6, it was found that these fatty acids increased prominently in both study group compared with the control group (p<0.001). It was found that concentrations of 20:4, n-6 and 22:6, n-3 declined in CCl₄ and CCl₄+Walnut groups (p<0.05, p<0.01). Conversely, it was found that concentrations of 16:0, 16:1, n-7, 18:1, n-9 and

### Table 3: The changes of GSH, total protein and MDA levels in the brain.

| Groups          | GSH (μg/g)       | Total protein (mg/g tissue) | MDA (nmol/g tissue) |
|-----------------|------------------|-----------------------------|---------------------|
| Control         | 240.33±1.27      | 67.04±2.11                  | 17.79±0.53          |
| CCl₄            | 150.11±1.36d     | 49.78±2.03b                 | 34.83±1.22d         |
| CCl₄+Walnut     | 240.67±3.38a     | 54.17±2.18                  | 31.94±0.98d         |

\(^d: p<0.001; ^c: p<0.01; ^b: p<0.05; ^a: p>0.05.\)

### Table 4: Changes in GSH, total protein and MDA levels in the kidneys.

| Groups          | GSH (μg/g)       | Total protein (mg/g tissue) | MDA (nmol/g tissue) |
|-----------------|------------------|-----------------------------|---------------------|
| Control         | 232.67±3.07      | 145.26±5.01                 | 5.64±0.35           |
| CCl₄            | 207.75±2.09b     | 165.88±4.45                 | 8.64±0.25s          |
| CCl₄+Walnut     | 193.19±1.75d     | 138.53±3.12                 | 4.76±0.28s          |

\(^d: p<0.001; ^c: p<0.01; ^b: p<0.05; ^a: p>0.05.\)

### Table 5: Changes in GSH, total protein and MDA levels in the kidneys.

| Groups          | GSH (μg/g tissue) | Total Protein (mg/g tissue) | MDA (nmol/g tissue) |
|-----------------|-------------------|-----------------------------|---------------------|
| Control         | 284.85±5.82       | 106.13±4.11                 | 19.05±0.79          |
| CCl₄            | 180.23±8.94d      | 88.93±5.09                  | 38.52±0.96d         |
| CCl₄+Walnut     | 273.73±6.13a      | 127.29±3.16                 | 27.33±1.19a         |

\(^d: p<0.001; ^c: p<0.01; ^b: p<0.05; ^a: p>0.05.\)

### Table 6: Effects of walnut fruit extract on composition of fatty acids in the brain (mg/1g).

| Fatty acids | Control | CCl₄ | CCl₄+Walnut |
|------------|---------|------|------------|
| 16:0       | 0.52±0.02 | 1.00±0.05   | 1.11±0.05   |
| 18:0       | 0.46±0.02 | 0.94±0.07   | 0.84±0.03   |
| ∑ Saturated| 0.98±0.04 | 1.94±0.12   | 1.95±0.08   |
| 18:1, n-9  | 0.58±0.02 | 1.04±0.07   | 1.11±0.06   |
| 18:2, n-6  | 0.03±0.002| 0.05±0.003  | 0.05±0.002  |
| 20:4, n-6  | 0.49±0.02 | 0.92±0.04   | 0.61±0.02   |
| 22:6, n-3  | 0.95±0.05 | 2.23±0.03   | 1.28±0.07   |
| ∑MUFA      | 0.58±0.02 | 1.09±0.07   | 1.19±0.06   |
| ∑PUFA      | 1.47±0.07 | 3.20±0.07   | 1.94±0.09   |
| ∑Total     | 3.03±0.32 | 6.50±1.34   | 5.08±0.17   |

\(^d: p<0.001; ^c: p<0.01; ^b: p<0.05; ^a: p>0.05.\)
18:2, n-6 increased in CCl₄+Walnut group compared with the CCl₂ group (p<0.001, p<0.01) (Table 7).

In the liver tissue, it was found a decline in 16:0 level in CCl₂ group compared to the control group (p<0.001), there was a remarkable increase in 18:0 concentration in CCl₂ group (p<0.01). There was a prominent decline in 18:2, n-6 level in both group (p<0.001). It was found that concentrations of 22:6, n-3 decreased in all groups at certain levels compared with the controls (p<0.001). Otherwise, it was found that concentrations 16:0, 18:0, 16:1, 18:1, and 18:2, n-6 increased in CCl₂+Walnut group at prominently levels compared with the CCl₂ group (p<0.001, p<0.01) (Table 8).

**Effects of walnut fruit extract on vitamin and cholesterol profile in the brain, kidneys and liver**

It was found that in the brain, concentrations of vitamins K-1 and K-2 increased in both group at certain levels compared with the controls (p<0.05, p<0.001, p<0.001), whilst vitamin D-2 declined significantly in all groups (p<0.001). It was observed that campesterol concentration increased prominently in CCl₂+Walnut group (p<0.001). Compared with the CCl₂ group, it was observed that α-tocopherol, Campesterol and Ergosterol concentration increased prominently in CCl₂+Walnut group (p<0.001) (Table 9).

In the kidneys, it was found that vitamin K-1 and K-2 levels increased in CCl₂ groups in comparison with the control group (p>0.05, p<0.001), and concentrations of vitamin D-3 increased in CCl₂+Walnut group (p>0.05, p<0.001). Levels of α-Tocopherol increased significantly in CCl₂ group compared to the controls (p<0.01, p<0.001). Ergosterol levels increased at certain levels in both group (p<0.01, p<0.001) Conversely, it was found that level of vitamin D3 and ergosterol increased in CCl₂+Walnut group compared with the CCl₂ group (p<0.001) (Table 10).

| Table 7: Effects of walnut fruit extract on composition of fatty acids in the kidneys (mg/1g). |
|---------------------------------|-----------------|-----------------|-----------------|
| Fatty acids         | Control       | CCl₂            | CCl₂+Walnut     |
| 16:0                | 0.81±0.06     | 1.07±0.06a      | 3.01±0.26d      |
| 18:0                | 0.57±0.02     | 0.43±0.02a      | 0.69±0.05b      |
| ∑Saturated         | 1.38±0.08     | 1.50±0.08       | 3.70±0.31       |
| 16:1, n-7           | 0.12±0.02     | 0.24±0.01a      | 0.34±0.04a      |
| 18:1, n-9           | 0.45±0.02     | 0.93±0.04a      | 1.87±0.18a      |
| 18:2, n-6           | 0.74±0.02     | 0.98±0.06a      | 1.97±0.14a      |
| 20:4, n-6           | 0.58±0.02     | 0.33±0.02a      | 2.20±0.02       |
| 22:6, n-3           | 0.08±0.01     | 0.07±0.01       | 0.39±0.05a      |
| ∑MUFA               | 3.35±1.03     | 4.05±1.17a      | 8.27±6.64a      |
| ∑PUFA               |                 |                 |                 |
| ∑Fatty acid         |                 |                 |                 |

| Table 8: Effects of walnut fruit extract on composition of fatty acids in the liver (mg/1g). |
|---------------------------------|-----------------|-----------------|-----------------|
| Fatty acids         | Control       | CCl₂            | CCl₂+Walnut     |
| 16:0                | 7.35±0.25     | 5.61±0.23a      | 7.97±0.47b      |
| 18:0                | 7.47±0.21     | 6.35±0.29a      | 7.59±0.44a      |
| ∑Saturated         | 14.82±0.46    | 11.96±0.52      | 15.56±0.91      |
| 16:1, n-7           | 0.73±0.03     | 0.64±0.04a      | 0.83±0.03a      |
| 18:1, n-9           | 2.12±0.05     | 1.88±0.15a      | 2.22±0.08a      |
| 18:2, n-6           | 7.70±0.16     | 3.79±0.38b      | 5.18±0.27f      |
| 20:4, n-6           | 4.89±0.16     | 4.15±0.30b      | 4.71±0.31b      |
| 22:5, n-3           | 0.25±0.01     | 0.16±0.01a      | 0.21±0.01b      |
| 22:6, n-3           | 1.92±0.05     | 1.18±0.09a      | 1.69±0.09a      |
| ∑MUFA               | 2.85±0.08     | 2.52±0.19       | 3.05±0.11       |
| ∑PUFA               | 14.76±0.38    | 9.28±0.78       | 11.79±0.68      |
| ∑Fatty acid         | 31.43±2.45    | 22.76±4.57a     | 31.40±4.74a     |

*; p<0.001; #: p<0.01; $; p<0.05; #: p>0.05.
In the liver, it was found that vitamin K-1 concentration declined in CCl₄ group compared with the controls (p<0.001, p>0.05), but increased at certain levels in walnut group (p<0.01, p<0.001). Vitamin K-2 level increased remarkably (p<0.001), while vitamin D-2 level declined significantly in both group (p<0.001). It was found that retinol and cholesterol levels increased remarkably in both group in comparison with the control group (p<0.001). It was found that levels of vitamin K1, D2, α-tocopherol and Campesterol increased certain levels in CCl₄+Walnut group compared with the CCl₄ group (p<0.001) (Table 11).

Discussion

CCl₄ is a hepatotoxic substance, which is well known and commonly used to create hepatotoxicity. CCl₄-induced hepatotoxicity, especially in rats, is a classical animal model for free-radicals-mediated liver damage studies; it causes damage to the liver of animals, which is similar to human liver disorder [25]. In addition, the studies conducted demonstrated that CCl₄ does not only target the liver, but does also cause lipid peroxidation in other organs, e.g. heart, kidneys, and brain [26]. Rats are often used to create an animal model for experimental studies to demonstrate hepatic injury and damages occurring in other tissues due to CCl₄-mediated oxidative damage [27,28]. CCl₄ is a typical toxic agent and its toxic effect occurs via production of free radicals. Since CCl₄ is fat-soluble, it can easily pass across the cell membrane. When CCl₄ is administered, it distributes to the organs, e.g. the liver, kidneys, and heart, where it is stored [29].

In studies conducted with different methodologies and with varying doses of CCl₄, it was reported that concentrations of lipid peroxidation products increased in the liver and kidney tissues, but GSH level declined significantly [30–34]. In a study performed, it was researched the protective effect of the almond oil in CCl₄-induced hepatic damage and it was found that cholesterol levels declined remarkably in the group given CCl₄ together with almond

| ADEK vitamins | Control | CCl₄ | CCl₄+Walnut |
|---------------|---------|-----|-----------|
| K-1           | 2.34±0.13 | 3.04±0.18<sup>c</sup> | 3.04±0.20<sup>b</sup> |
| K-2           | 9.48±0.40 | 13.19±0.94<sup>d</sup> | 11.84±0.52<sup>a</sup> |
| D-2           | 8.73±0.39 | 0.11±0.004<sup>d</sup> | 0.42±0.03<sup>d</sup> |
| D-3           | 1.05±0.04 | 1.13±0.07<sup>a</sup> | 1.29±0.11<sup>a</sup> |
| α-tocopherol  | 5.63±0.31 | 6.51±0.36<sup>a</sup> | 11.33±0.68<sup>d</sup> |
| δ-tocopherol  | 0.14±0.01 | 0.14±0.01 | 0.26±0.01<sup>a</sup> |
| Campesterol   | 9.79±0.51 | 1.96±0.11<sup>d</sup> | 28.15±1.13<sup>c</sup> |
| β-sitosterol  | 1.53±0.19 | 0.29±0.02<sup>a</sup> | 0.20±0.01<sup>a</sup> |
| Ergosterol    | 0.63±0.04 | 1.46±0.09<sup>d</sup> | 8.70±0.28<sup>cd</sup> |
| Retinol       | 0.22±0.014 | 0.17±0.01<sup>c</sup> | 0.11±0.01<sup>c</sup> |
| Cholesterol (mg/g) | 0.79±0.02<sup>c</sup> | 1.18±0.02<sup>c</sup> | 1.01±0.02<sup>c</sup> |

<sup>d</sup>: p<0.001; <sup>c</sup>: p<0.01; <sup>b</sup>: p<0.05; <sup>a</sup>: p>0.05.

| ADEK vitamins | Control | CCl₄ | CCl₄+Walnut |
|---------------|---------|-----|-----------|
| K-1           | 0.79±0.03 | 1.02±0.04<sup>c</sup> | 0.75±0.03<sup>a</sup> |
| K-2           | 21.82±0.99 | 35.80±2.13<sup>d</sup> | 15.35±1.29<sup>d</sup> |
| D-3           | 0.54±0.03 | 0.48±0.03<sup>a</sup> | 2.09±0.12<sup>d</sup> |
| α-tocopherol  | 20.50±1.15 | 25.81±0.98<sup>c</sup> | 10.98±0.67<sup>d</sup> |
| δ-tocopherol  | 0.21±0.01 | 0.19±0.01 | 0.06±0.01 |
| Campesterol   | 105.30±3.74 | 111.65±4.58<sup>c</sup> | 85.12±3.51<sup>d</sup> |
| β-sitosterol  | 12.17±0.76 | 13.76±0.62<sup>a</sup> | 8.13±0.37<sup>c</sup> |
| Ergosterol    | 0.69±0.03 | 1.25±0.06<sup>c</sup> | 4.71±0.22<sup>cd</sup> |
| Retinol       | 3.22±0.18 | 2.98±0.14<sup>a</sup> | 1.66±0.10<sup>a</sup> |
| Cholesterol (mg/g) | 1.09±0.05 | 1.22±0.04<sup>d</sup> | 1.06±0.06<sup>d</sup> |

<sup>d</sup>: p<0.001; <sup>c</sup>: p<0.01; <sup>b</sup>: p<0.05; <sup>a</sup>: p>0.05.
In the same study, lipid peroxidation values were also measured and MDA value of the group added almond oil was significantly reduced compared to the group given CCl₄ alone [35].

In our study, comparing effects of fruit extracts on composition of vitamin and cholesterol in wistar rats, we observed certain increase in vitamin K₁, K₂, α-tocopherol, campesterol, ergosterol and cholesterol in the brain, whilst levels of vitamin D₃ and ergosterol levels in kidney tissues in the walnut group compared to the controls. In the liver tissue, it was observed that concentration vitamin K₁, K₂, α-tocopherol, β-sitosterol Retinol, Cholesterol increase in the walnut group compared to controls. The results that we achieved suggest that the fruit extracts affect the different tissues at varying levels, and therefore lipophilic vitamins and cholesterol levels are affected. It was observed that this diversity also occurred due to the contents of the walnut.

Glutathione accounts for the most important step of the defense against oxidative stress. GSH level increases as a consequence of adaptation mechanisms activated in situations, where oxidative stress is weak. However, in situations where oxidative stress is powerful, GSH level declines due to weakened adaptation mechanisms and increased formation of GSSG [36]. Considering our results, it can be thought that reduced GSH level in the groups given CCl₄ results from the local anti-oxidant defense mechanism getting incompetent because of CCl₄ administration.

In our study, we achieved the results being consistent with the studies mentioned above in the study groups given fruit extract with regard to MDA and GSH levels. As a result of our researches, it was observed that GSH concentration declined in the kidney and liver tissues of the groups added CCl₄. In addition, it was also observed that MDA level increased in the brain, kidney and liver tissues, indicating augmented lipid peroxidation.

In a study about walnuts, anti-oxidant effects of Juglans sinensis (Dode walnut) species against HgCl₂-induced renal failure. As a result of this study, it was found that MDA levels declined significantly in the group added walnut extract [37]. In another study, the effects of liquid extract of walnut against cyclophosphamide (CP)-induced biochemical toxicity. In that study, there were changes in P450 composition and in glutathione S-transferase, glutathione peroxidase and catalase activities in both the liver and the kidneys in the group given walnut extract in addition to cyclophosphamide. Comparing the group given walnut extract with the group given cyclophosphamide, it was found that in the kidneys, superoxide dismutase (SOD) and reduced glutathione concentrations were restored. In addition, it was observed that in the group given walnut extract, GSH level and glutathione peroxidase activity increased in both tissues, catalase activity increased in only the liver, and there were no significant changes in GST and SOD activities. In that study, lipid peroxidation values increased significantly in both tissues in the group given cyclophosphamide compared with the controls, whilst, it was found a significant decline in lipid peroxidation values in the group given walnut extract in addition to cyclophosphamide. It was demonstrated with that study that the use of walnut extract might be helpful to eliminate the toxic effects of cyclophosphamide throughout chemotherapy [38]. In our study, we achieved the results being consistent with these trials in the tissues examined.

Comparing the effects of fruit extracts on composition of fatty acids in Wistar rats in our study, we observed a more remarkable increase in 16:0, 18:0, 18:1, 18:2, 20:4, 22:6 fatty acids in the brain and kidney tissues of the rats in the groups given fruit extract in addition to CCl₄ compared to the controls.

Our results demonstrate that walnut extract has protective effects against LPO formation in the brain, kidney and liver tissues. We think that this is because of the phy-

| ADEK vitamins | Control | CCl₄ | CCl₄ + Walnut |
|---------------|---------|------|--------------|
| K₁            | 2.63±0.17 | 1.56±0.14⁺ | 3.40±0.10⁺ |
| K₂            | 0.64±0.05 | 7.04±0.37⁺ | 5.05±0.32⁺ |
| D₂            | 5.25±0.24 | 0.96±0.05⁺ | 3.14±0.17⁺ |
| α-tocopherol  | 18.25±0.59 | 17.17±0.67⁺ | 26.75±1.22⁺ |
| Campesterol   | 96.29±2.27 | 53.00±3.76⁺ | 91.90±2.59⁺ |
| β-sitosterol  | 41.83±1.85 | 46.79±2.12⁺ | 54.65±2.85⁺ |
| Ergosterol    | 4.83±0.33  | 5.79±0.33⁺ | 5.75±0.34⁺ |
| Retinol       | 0.48±0.03  | 0.58±0.02⁺ | 0.81±0.03⁺ |
| Cholesterol (mg/g) | 1.48±0.08 | 2.26±0.08⁺ | 2.60±0.12⁺ |

⁺: p<0.001; ⁺: p<0.01; ³: p<0.05; ⁴: p>0.05.
tochemical compounds contained by the fruits. In this trial, it was found that walnut extract has distinct effects on glutathione and total protein levels in different tissues.

Since the synthetic anti-oxidants being used today possess adverse effects, it is of increasing importance to find out and derive natural antioxidants from herbal sources, to reveal their effects, and to use them. We hope that the results achieved in the present study with this fruit consumed often in daily life shed some light on further studies about anti-oxidant effects of this fruit.

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