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Protective Effect of *Morus nigra L.* (Mulberry) Fruit Extract on the Liver Fatty Acid Profile of Wistar Rats

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Abstract.- Antioxidants are not only produced by the body cells but can also be obtained through food. As is known, fruits and vegetables contain molecules which have antioxidant activities. These molecules protect tissues against stress and diseases. In this study, it was aimed to investigate the protective effect of the methanol extract of *Morus nigra* L. fruit on the prevention of lipid peroxidation (LPO) by analyzing lipid fraction of liver tissue and fatty acid profiles of Wistar rats by using Gas chromatography. Statistically no significant difference was observed in the hepatic lipid fractions of rats containing *M. nigra* fruit extract and the control group. However, the level of LPO were found to be very high in the group which contained Fenton reagent compared to the groups that contained *M. nigra* and antioxidant molecules ($p < 0.001$). Besides that, it was found that saturated and unsaturated fatty acid levels were higher in the groups including resveratrol and fruit extract compared to the group including Fenton reagent ($p < 0.001$). We believe that the main reason for the decrease in the amount of LPO and the protection of fatty acids is the presence of antioxidant molecules in the fruit.

Keywords: *Morus nigra* L., black mulberry, lipid peroxidation, fatty acids, Fenton reagent (Fenton R, H_2O_2 + $FeCl_2$).

INTRODUCTION

Morus belongs to the Moraceae family. *Morus* has 24 species, 100 varieties, and a sub-species. It is geographically widely dispersed, it has adapted to different agro-climatic conditions easily, it regenerates quickly, and it is suitable for a variety of cultivation and pruning methods. In Turkey, traditional products such as mulberry molasses and mulberry pulp are made using its fruits. Fruits of this plant are eaten fresh or dried and even used in marmalade, fruit juice, liquor, natural dyes, and cosmetics industry (Özan *et al*., 2008).

In the previous studies, it was observed that mulberry extracts show strong antioxidative effects against low-density lipoprotein (LDL) oxidation and have a blocking effect on LDL induced by dead macrophage cells. Therefore it has been suggested that consumption of mulberry extract as food may reduce the incidence of heart diseases such as atherosclerosis through the extract's antioxidant activity (Chen *et al*., 2005).

Morin, obtained from the fruits of *M. nigra*, is a substance with flavonoid structure and this substance has been determined to have anti-inflammatory activity on macrophages. Similarly cyclosporine (CsA), obtained from these fruits, is suggested to be used as a potential immunosuppressive agent for the treatment of autoimmune diseases and in the course of organ transplantation (Fang *et al*., 2005).

Metals are known to play important and different roles on living organisms. It is stated that the radical production increases with increasing level of Fe molecules in the body. In line with this, the hydroxyl (OH^-) radical, which has a toxic effect, is produced by two types of reactions that require primary metals. The first of these includes direct reduction of hydrogen peroxide (H_2O_2) with superoxide anion. It is catalyzed with Fe and called Haber-Weiss reaction (Kelson *et al*., 1997). The second is referred to as the Fenton reaction. Ferrous chloride ($FeCl_2$) is suggested to be effective in the increase of the amount of OH^- and OH^--like radicals that are efficacious in lipid peroxidation. Similarly, it is demonstrated that the application of $FeCl_2$ decreased the activity of Na^+, K^+-ATPase and resulted in stress condition in the cell due to the presence of ions (Anderson and Means, 1985; Willmore and Rubin, 1984; Jagetia *et al*., 2004).

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In this study, the effect of *M. Nigra* extract, which is administered *in vitro* with $FeCl_2$, on the fatty acid composition in the liver tissue of rats, was investigated. In this way, it is aimed to contribute to the determination of the biological and pharmacological effects of this fruit on biomolecules.

MATERIALS AND METHODS

Fruit extract

M. nigra fruits were used as the plant material. *M. nigra* fruits were collected in Elazig region and extracted with 80% methanol. Fresh fruits, fruits dried in the shade, and sun-dried fruits were used for that purpose. Methanol phase of the extracts was evaporated in a rotary evaporator under vacuum at 55 °C. Following this process, the remaining dry extract was dissolved in DMSO (dimethyl sulfoxide) and made available for further processing.

Procedure adopted

Male rats of Wistar strain weighing 200-250 g were randomly selected from rats fed on a standard diet in the Research Center of Experimental Animals at Firat University. They were weighed and in accordance with the ethical committee report they were decapitated. Liver tissue was taken immediately after the decapitation. After determining the weights, they were homogenized with a mixture of 250 ml 3/2 (v/v) n-hexane/isopropyl alcohol. The homogenate was filtered and its solvent was evaporated under vacuum at 45°C. The remaining lipid fraction was dissolved with 200 ml Tris + HCl + Tris Base + KCl + Tween 20 (pH = 7.4) buffer mixture (neutral buffer) and extracted. After this process, the following groups were created:

1. Control Group (n = 5): 5 ml buffer solution with lipid fraction + 2 ml neutral buffer + 1 ml DMSO
2. Fenton R group (n = 5): 5 ml buffer solution with lipid fraction + 1 ml $FeCl_2$ + 1 ml 10 mM H_2O_2
3. *M. nigra* group (n = 5): 5 ml buffer solution with lipid fraction + 1 ml $FeCl_2$ + 1 ml 10 mM H_2O_2 + 1 ml fresh fruit extract
4. *M. nigra* (shadow) group (n = 5): 5 ml buffer solution with lipid fraction + 1 ml $FeCl_2$ + 1 ml 10 mM H_2O_2, + 1 ml fruit extract dried in the shadow
5. *M. nigra* (Sun) group (n = 5): 5 ml buffer solution with lipid fraction + 1 ml $FeCl_2$ + 1 ml 10 mM H_2O_2 + 1 ml sun-dried fruit extract
6. Resveratrol group (n = 5): 5 ml buffer solution with lipid fraction + 1 ml $FeCl_2$ + 1 ml 10 mM H_2O_2 + 50 µL 20 mM resveratrol + 1 ml neutral buffer
7. Quercetin group (n = 5): 5 ml buffer solution with lipid fraction + $FeCl_2$ + 1 ml 10 mM H_2O_2 + 50 µL 20 mM quercetin + 1 ml neutral buffer

Mixtures were incubated for 24 h at 37 °C and by the end of this period, oxidation was stopped by the addition of 100 µL 4% butylated hydroxytoluene (BHT). 1 ml was taken for the measurement of thiobarbituric acid reactive substances (TBARS) products formed in the reaction environment and the following operations were performed.

Flavonoid analysis

The flavonoids in the extracts 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 (Zu *et al*., 2006). PDA detector (SPD-M10A VP) 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

M. nigra fruit samples were weighed and homogenized with n-hexane/isopropyl at 3/2 (v/v) ratio and after the hydrolysis with 5% KOH at 85 °C, extraction of phytosterols was performed with n-hexane. The amounts of ADEK vitamins and

phytosterols were analyzed at 202 nm and 326 nm using a UV detector on a HPLC device.

HPLC analysis of the amount of LPO

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 min 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 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 75% ACN/30 mM KH_2PO_4 (pH = 5) mixture was used as the mobile phase. Analysis time was set as 5 min. 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

Fatty acids were isolated by the addition of 10 ml 3/2 (v/v) hexane/isopropanol mixture on the liquid phase of the samples remaining after LPO measurement. Then, hexane phase was taken into separate test tubes and 5 ml 2% methanolic sulfuric acid was added onto it; then the mixture was left at 55°C for 12 h. 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% $KHCO_3$ solution, then the n-hexane phase was vaporized with nitrogen stream (Christie, 1992), 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

In the analyses conducted with the HPLC device, α-tocopherol, δ-tocopherol□ vitamin D-3 and vitamin K-1, stigmasterol, β-sitosterol, retinol, retinoic acid molecules, and flavonoids such as rutin, quercetin, kaempferol, naringenin, and resveratrol were detected in the methanol extract of *M. nigra* fruit extract (Tables I, II).

Table I.- Flavonoid content of *M. nigra* fruit extract (µg/g).

Flavonoids	Fresh *M. nigra* fruit	Sun-dried *M. nigra* fruit	*M. nigra* fruit dried in the shade
Rutin	36.85	41.35	110.4
Quercetin	2.7	6.9	28.6
Kaempferol	0.05	0.45	1.2
Naringenin	0.2	0.35	0.1
Resveratrol	0.03	0.2	0.2

Table II.- Lipophilic vitamin and phytosterol content of *M. nigra* fruit extract (µg/g).

Vitamins and phytosterols	Fresh *M. nigra* fruit	Sun-dried *M. nigra* fruit	*M. nigra* fruit dried in the shade
δ-tocopherol	13.01	43.11	27.1
Vitamin D-3	3.6	10.61	8.08
α-tocopherol	1.8	6.53	6.06
Vitamin K-1	5.13	26.41	18.74
Stigmasterol	15.34	60.94	44.06
β-sitosterol	55.51	153.13	107.79
Retinol (vitamin A)	0.04	0.08	0.01
Retinol acetate	0.05	0.04	0.04

In the lipid peroxidation study conducted on the liver tissue, there was no statistically significant difference between the *M. nigra* fruit extract added

groups and the control group; however the LPO level of the group with sun-dried *M. nigra* fruit extract was partly higher ($p > 0.05$) (Fig. 1). In the comparison between the group that contains Fenton reagent and the groups that contain *M. nigra* and antioxidant molecules, the level of LPO was found to be very high in the Fenton R group ($p < 0.001$) (Fig. 1).

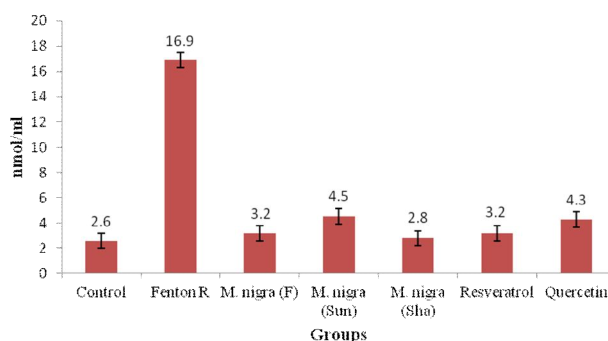

Fig. 1. The level of lipid peroxidation formed in the liver lipid fraction (nmol/ml) (*M.nigra* (F) = Fresh *M.nigra, M.nigra* (Sun) = Sun-dried *M. nigra, M. nigra* (Sha) = *M. nigra* dried in the shadow).

In the liver tissue fatty acid profile, partial reduction was observed in the amounts of palmitic (16:0) and stearic (18:0) acids in the Fenton R group in comparison to the control group whereas increases at various ratios were detected in the groups that were administered with *M. nigra* extract and antioxidant molecules ($p<0.05$, $p<0.01$, $p<0.001$) (Table III).

In correspondence to the decrease in the amount of oleic acid in the Fenton R group ($p<0.05$), significant increases were observed in *M. nigra* groups ($p<0.001$) and a partial increase was observed in the resveratrol group ($p<0.05$). It was also determined that the amount of palmitoleic acid decreased in the Fenton R group ($p <0.01$) but it was high in groups containing dried *M. nigra* extract ($p < 0.01$). Despite the fact that the amounts of linoleic acid (18:2, n-6) and arachidonic acid (20:4, n-6) decreased significantly in the Fenton R group ($p < 0.001$), the amounts of these acids in *M. nigra* extract added groups were significantly higher ($p < 0.001$). Although the amount of docosahexaenoic acid (22:6, n-3) decreased significantly in the Fenton R group ($p < 0.001$), the amount in the *M. nigra* groups was significantly higher ($p<0.05$, $p<0.01$, $p < 0.001$) (Tables III, IV).

Total saturated fatty acids, MUFA, PUFA amounts showed a decrease at different ratios in the Fenton R group, whereas the amount in *M. nigra* extract added groups was significantly higher ($p < 0.001$). Similar results were also found in the resveratrol group ($p < 0.01$) (Tables III, IV).

DISCUSSION

Vitamins and phenolic substances are the main factors which determine the antioxidant and the antiradical activities in vegetable materials and foods. Therefore, the changes in the total phenolic content and the ratios of vitamins affect the antioxidant activity.

There are considerably many studies on the phenolic and antioxidant properties of red fruits and the measurement of anthocyanin levels (Özgen *et al*., 2007; Sun *et al*., 2002; Çelik *et al*., 2008). However, studies on determination of biological and pharmacological effects of these fruits on biomolecules are still limited and detailed studies are not available. Naderi *et al*. (2004)'s study has shown that *M. nigra* fruit extracts have a protective effect against peroxidative damage to biomembranes and biomolecules. In a study conducted by Ozgen *et al*. (2009), phytochemicals and anthocyanin content were investigated together with the antioxidant properties of some of the black and red mulberry fruits grown in Turkey. Total phenolic and anthocyanin contents were compared and found to be higher in the black mulberry. In our study, black mulberry fruit extracts were also determined to have a high level of flavonoid and vitamin content.

Oki *et al*. (2006) examined the change in anthocyanin content and antioxidant activity of mulberry fruits depending on the process of ripening. They determined that anthocyanin content and antioxidant capacity increased with ripening. In a study conducted by Aydin *et al*. (2011), it was indicated that ripe black mulberry extract was more effective than the unripe fruit extract in preventing the formation of LPO. Depending on this result, they reported that polyphenolic compounds in the

Table III.- The fatty acid profiles of liver tissue lipid fraction (µg/ml)

Fatty acids	Control	Fenton R	*M. nigra* (Fresh)	Quercetin
16:0	32.95 ± 0.65	21.39 ± 0.64 [b]	79.81 ± 4.91 [d]	44.91 ± 3.12 [c]
16:1, n-7	5.07 ± 0.12	3.71 ± 0.18 [c]	5.26 ± 0.43	4.52 ± 0.45
18:0	32.41 ± 0.65	21.23 ± 0.33 [c]	60.36 ± 3.05 [d]	36.61 ± 0.90
18:1, n-9	12.70 ± 0.61	9.96 ± 0.44 [b]	29.44 ± 1.71 [d]	14.30 ± 1.20
18:2, n-6	22.90 ± 1.06	12.38 ± 1.06 [c]	59.31 ± 3.95 [d]	21.37 ± 1.40
20:4, n-6	30.45 ± 0.84	11.15 ± 0.48 [d]	49.24 ± 2.27 [b]	23.91 ± 1.43 [b]
22:6, n-3	4.79 ± 0.26	2.43 ± 0.20 [d]	6.43 ± 0.24 [b]	3.24 ± 0.17 [b]
Σ Saturated	65.36 ± 1.45	45.82 ± 1.11 [c]	140.17 ± 2.22 [d]	81.52 ± 1.33 [c]
Σ MUFA	17.77 ± 0.82	13.47 ± 0.48 [b]	34.70 ± 0.75 [c]	14.48 ± 0.84
Σ PUFA	58.11 ± 1.12	25.96 ± 0.42 [d]	114.98 ± 1.57 [d]	48.52 ± 0.64 [b]

Table IV.- The fatty acid profiles of liver tissue lipid fraction (µg/ml)

Fatty acids	Control	Fenton R	*M. nigra* (Shadow)	*M. nigra* (Sun)	Resveratrol
16:0	32.95 ± 0.65	21.39 ± 0.64 [b]	40.93 ± 3.87 [b]	54.30 ± 3.71 [c]	64.67 ± 1.95 [d]
16:1, n-7	5.07 ± 0.12	3.71 ± 0.18 [c]	8.11 ± 0.42 [b]	9.31 ± 0.19 [c]	5.27 ± 0.22 [a]
18:0	32.41 ± 0.65	21.23 ± 0.33 [c]	37.65 ± 9.68 [a]	43.62 ± 3.20 [b]	61.27 ± 2.02 [d]
18:1, n-9	12.70 ± 0.61	9.96 ± 0.44 [b]	40.12 ± 0.65 [d]	44.71 ± 1.63 [d]	18.27 ± 0.79 [b]
18:2, n-6	22.90 ± 1.06	12.38 ± 1.06 [c]	42.72 ± 2.77 [d]	46.50 ± 3.74 [b]	38.49 ± 1.32 [d]
20:4, n-6	30.45 ± 0.84	11.15 ± 0.48 [d]	52.13 ± 1.85 [d]	57.14 ± 1.78 [d]	49.13 ± 1.41 [d]
22:6, n-3	4.79 ± 0.26	2.43 ± 0.20 [d]	8.65 ± 0.31 [c]	10.31 ± 0.16 [d]	5.91 ± 0.13 [a]
Σ Saturated	65.36 ± 1.45	45.82 ± 1.11 [c]	78.58 ± 1.78 [b]	97.92 ± 1.92 [c]	125.94 ± 2.21 [d]
Σ MUFA	17.77 ± 0.82	13.47 ± 0.48 [b]	48.73 ± 0.52 [d]	54.02 ± 0.64 [d]	23.54 ± 0.56 [b]
Σ PUFA	58.11 ± 1.12	25.96 ± 0.42 [d]	103.50 ± 1.57 [d]	113.95 ± 1.82 [d]	93.53 ± 1.33 [c]

d: $p < 0.001$, c: $p < 0.01$, b: $p < 0.05$, a: $p > 0.05$

mulberry fruit had free radical scavenging properties and had a protective effect on the unsaturated fatty acids.

In a study by Chen *et al*. (2005), progression of atherosclerosis was shown to be inhibited in rabbits fed on aqueous *M. alba* extract. The extract used in this study was determined to contain 2.5% anthocyanin and 4.6% total phenol. In their study, they observed that mulberry extract reduced the level of cholesterol and triglycerides in rabbits fed on 3% lard diet. In addition, they indicated that mulberry extract did not have toxic effects on liver and kidney functions during the experimental period (Ercisli and Orhan, 2007).

Fatty acid levels of organisms exposed to certain metals or heavy metals have been reported to be affected by the exposure (Dayangaç *et al*., 2011). In parallel to this, in some studies, iron molecule was suggested to initiate lipid peroxidation (Muliawan and Kappus, 1983). In addition, it was suggested that administration of $FeCl_2$ at certain amounts caused changes in the cell activity and induced necrosis and apoptosis by increasing lipid peroxidation (Reilly *et al*., 1991; Jajte *et al*., 2002; Yao *et al*., 2005). In parallel with these studies, we also determined in our study that the addition of $FeCl_2$ increased lipid peroxidation (Fig. 1).

It has been investigated in detail that flavonoids are powerful antioxidants, free radical scavengers, and have metal binding activities (Saija *et al*., 1995; van Acker *et al*., 1996). With their powerful antioxidant effects and capability of donating electrons due to the OH^- groups in their structure, flavonoids decrease lipid peroxidation. Polyphenols of different structures may show protective effects in various ways and scavenge reactive oxygen species (Plumb *et al*., 1999; Laughton *et al*., 1991; Robak and Gryglewski *et al*., 1988). Studies on the chemical structure of flavonoids show that antioxidant activity may

depend on the degree of hydroxylation (Plumb *et al.*, 1999; Rice-Evans *et al.,* 1996). Pekkarinien *et al.* (1999) suggested that the antioxidant activity of flavonoids depended on the increase and the position of OH^- groups in the phenolic structure. Among flavonoids, myricetin and rutin were determined to have a synergistic effect in providing the antioxidant activity with α-tocopherol. Myricetin, quercetin, and rutin have been reported to have a protective effect against inactivation of α-tocopherol by radicals. Myricetin molecule has been suggested to be more effective in providing this effect. Although α-tocopherol has better proton expulsion characteristics in preventing the formation of LPO and hydroperoxide in the presence of linoleic acid, myricetin and quercetin molecules have been suggested to have better antioxidant characteristics. In another study, quercetin, which is also present in the fruit of *M. nigra* at certain amount, was indicated to have analgesic effect and also *in vitro* anti-mutagenic effect (Decker, 1995). Moreover, quercetin was determined to protect human intestinal cells against oxidative effect and decreased the level of H_2O_2 released during the metabolism (Duthie and Dopson, 1999). In a study conducted by Hayek *et al.* (1997) quercetin and catechins were reported to bind with LDL and decrease the susceptibility to oxidation aggregation. In parallel with these studies, we observed in our analyses that the fruit group with higher amount of quercetin, rutin, and kaempferol was more effective in the prevention of lipid peroxidation compared to the other fruit groups (Table I, Fig. 1).

In this study, it was observed in the analyses that *M. nigra* fruit extract had different amounts of flavonoids and antioxidant vitamins. We hypothesize that the decrease in fatty acid levels and the increase in MDA levels in the $FeCl_2$ administered group are due to the fact that this compound causes lipid degradation in living things and increases the OH^- radical by increasing oxidative stress. We suggest that the main reason for the significant decrease in MDA values of the groups containing $FeCl_2$ and mulberry fruit extract in comparison to the $FeCl_2$-only group and the increase in fatty acid values is the presence of antioxidant substances in the content of this fruit.

As a result, black mulberry fruit extract was determined to prevent oxidation of both saturated and unsaturated fatty acids *in vitro* in liver tissue fatty acid profiles, and, in parallel, to be effective in reduction of the level of LPO.

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