African Journal of Biotechnology Vol. 10(71), pp. 16037-16044, 14 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.261
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effectiveness of matured *Morus nigra L.* (black mulberry) fruit extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and hydroxyl (OH•) radicals as compared to less matured fruit extract

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Accepted 7 October, 2011

In this study, we investigated the antioxidant effect of the *Morus nigra* L. (black mulberry) fruit extracts in 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and hydroxyl (OH•) radicals environments gathered from the district of Elazig. Fruits were extracted with 80% methanol, and methanolic extract's scavenging effect of DPPH• radical was examined depending on the quantity. It was observed that less matured fruit extract's (SMF) radical scavenging activity increased from the 50 to 250 µL, but decreased after 500 µL (p<0,001). As for matured fruits (MF), the most scavenging activity were observed in 50 and 100 µL extracts on DPPH• and after 250 µL, it decreased (p<0,001). Methanol phases of extracts by evaporation were dissolved in dimethyl sulfoxide (DMSO) in the environment in which with Fenton reaction, OH• radical is constituted; preventing the formation of lipopolysaccharide (LPO) and protective effect on unsaturated fatty acid were examined. In the Fenton reagent environment, preventing effect on the formation of LPO, it was determined that MF extract was more effectual than SMF extract (p<0,001). Accordingly, it was established that *M. nigra* fruit extract has protecting effect on unsaturated fatty acids (p<0,001). We considered that fruit extracts having scavenging effects on DPPH• radical and diminishing the formation of LPO that was originated from OH• radicals resulted from flavonoids in extracts. In the flavonoid analyses carried out with high performance liquid chromatography (HPLC) equipment, it was observed that fruit extracts has polyphenolic compounds such as rutin, resveratol, myricetin and naringerin.

Key words: *Morus nigra* L. (black mulberry), matured fruit extract (MF), less matured fruit extract (SMF), Fenton reagent (H_2O_2+$FeCl_2$), DPPH•, flavonoids, lipid peroxidation, unsaturated fatty acid.

INTRODUCTION

Morus (Mulberry) belongs to Moraceae family. There are 24 species, 100 varieties and one subspecies of *Morus* genus. Mulberry tree, for its leaves that are the food of silkworm, is commonly grown in northern Europe and India. Its fruits can be eaten both raw and desiccated. There are three kind of mulberry; red, white and black. White mulberry is grown in West Asia; red mulberry is grown in North America and black mulberry (*M nigra* L.), which originated from Iran, is mainly grown in Southern Europe and in Southwest Asia (Tutin, 1996). *M. nigra* is also grown widespread in Turkey (Yaltirik, 1982). It has noteworthy population in the regions of Mediterranean and northeast region of Turkey, especially in the valley of Coruh. It is especially grown for its edible, delicious fruits. In 2005, the production of mulberry in Turkey was recorded as 78.000 tones. The fruits of *M. nigra* are extensively consumed in Anatolia. The fruits of this plant

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Abbreviations: DPPH•, 2,2-Diphenyl-1-picrylhydrazyl; **OH•,** hydroxyl radical; **MF,** matured fruits extract; **SMF,** semi-matured fruit extract; **LPO,** lipopolysaccharide; **DMSO,** dimethyl sulfoxide; **HPLC,** high performance liquid chromatography.

are picked after they are matured. Morin, a form of flavonoid that is highly found in black mulberry, has been suggested of having anti-inflammatory activity on macrophages. Also, the Cyclosporine (CsA) material that is isolated from fruits can be used in treatment of autoimmune illnesses and during organ transplantation as potential immunosuppressive agent (Fang et al., 2005). When black and white mulberries are compared, it was observed that total phenolic and flavonoid substances highly existed in black mulberry. The studies about the phenolic and antioxidant features of the red fruits and on the quantity of anthocyanin are excessive (Özgen et al., 2007; Sun et al., 2002; Çelik et al., 2008). However, studies on the antioxidant and phytochemical features of the mulberries and characterization are limited. First of all, Gerasopoulos and Stavroulakis (1997), Ercisli and Orhan (2007) and Güneş and Çekiç (2004) have investigated the fruit quality and chemical characters of some Morus species; but these studies are mostly concentrated on phytochemical and antioxidant features and genotypes of some local morus species.

Lee et al. (2004) found that mulberries have cyanidin based anthocyanins, especially cyanidin-3-glucoside and cyanidin-3-rutinoside. However, the studies about stating these fruits' biologic and pharmacologic effects bio molecules are still in limited degree and detailed studies do not exist. In the studies of Naderi et al. (2004), it was shown that *M. nigra* fruit extracts have a protecting effect against peroxidative damage. Also, in the study carried out by Özgen et al. (2007) on some black and red mulberries that are grown in Turkey, phytochemical and anthocyanin content and antioxidant features are studied. The fruits' total phenolics were justified with the tests of total anthocyanin quantity, titration acidities, trolox-equivalent antioxidant capacity (TBAC) and ferric reducing antioxidant power. It was shown that black mulberries have higher total phenolic, total anthocyanin quantity, titration acidities in accordance with red mulberries.

It is claimed that syrup arranged from mulberries has therapeutic characters (Anonymous, 1986). Also, it was stated that mulberries that are produced in Armenia are used as a coloring material for pasteurized milks (Aganova, 1989). Moreover, it is also used in the production of alcoholic drinks like white wine (Alian and Musenge, 1977). Wills et al. (1987) studied the ingredients of mulberry and gave the approximate ingredients of every 100 g portion that are eaten as; 89.3% water, 2.2% protein, 0.2% lipid, 2% glucose, 2.3% fructose, 2.2% diet (fiber), 0.19% malic acid, 0.59% citric acid, 0.8% ash, 121 kJ energy, 10 mg vitamin C, 0.01 mg thiamine, 0.01 mg riboflavin, 0.7 mg niacin, 0.01 mg β-carotene, 310 mg K, 6 mg Na, 20 mg Ca, 0.3 mg Fe and 0.2 mg Zn.

In this study, we aimed at examining *M. nigra* L (black mulberries) picked up as matured and semi-matured from the region of Elazig, by investigating their antioxidant effect on $DPPH^{\bullet}$ radical and on OH radical in environments where they exist. We also aimed to prevent lipid peroxidation (LPO) and to show whether these fruits have a protective effect on the unsaturated fatty acid or not. With this intention, black mulberries were examined by being gathered in the form of matured and semi-matured.

MATERIALS AND METHODS

Chemicals

Oleic acid (18:1, n-9), linoleic acid (18:2, n-6), linolenic acid (18:3, n-3), Tween 20, Tris-base and hydrochloride, quercetin, myricetin, resveratrol, catechin, naringin, naringenin, kaempferol and HPLC grade methanol, acetonitrile, n-hexane, isopropyl alcohol, $FeCl_2$ $2H_2O$, H_2O_2, KH_2PO_4, butylated hydroxytoluene (BHT), n-Butanol, α,α-diphenyl-β-picrylhydrazyl, (DPPH), dimethyl sulfoxide (DMSO), 2-thiobarbituric acid (TBA) and ethyl alcohol were purchased from Sigma-Aldrich.

Fruit material

The fruits of *M. nigra* L were collected from Elazig Province, Turkey, in June 2009. The fruits were immediately frozen to freeze-dried. Lyophilized samples were maintained at -20°C prior to analysis.

Preparation of fruit extracts

The frozen fruit (50 g) was homogenized with 250 ml 80% v/v methanol. Homogenates were centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was then concentrated by drying under reduced pressure 25 at 50°C using a rotary evaporator. Each extract was re-suspended in DMSO to give a stock solution, and stored at -20°C until further analysis.

Antioxidative activity testing in the fatty acid environment

Antioxidative activities of the mulberry fruit extracts were determined by the method of Shimoi et al. (1994) with the following modifications: 1 mM $FeCl_2$ ($FeCl_2$ $2H_2O$) and 3 mM hydrogen peroxide (H_2O_2) solutions were prepared freshly for every treatment, using doubly deionized water. Extracts of mulberry fruit were also prepared freshly by dissolving 3.97 mM methyl oleate (18:1, n-9), 10.44 mM linoleate (18:2, n-6, LA) and 2.30 mM linolenate (18:3, n-3, LNA) in DMSO. Buffer solutions were prepared with 0.2% Tween 20, 0.05 M Tris-HCl and 0.15 M KCl (pH = 7.4). During *in vitro* experiment, the first group was used as a control, the second group was Fenton reagent group, ($FeCl_2$+H_2O_2, Fenton R), and the third group was Fenton R plus MF extract, while the fourth group was Fenton R plus SMF extract. The first group was prepared and 0.4 ml fatty acid mixture (LNA: 3.26 µM/1 ml; 14.82 µM/1 ml LA, and 4.99 µM/1 ml 18:1) was suspended in 5 ml buffer solution. The second group was a Fenton reagent group, and 0.4 ml fatty acid mixture, 40 µM $FeCl_2$ and 60 µM H_2O_2 were suspended in 5 ml buffer solution. The third and fourth groups were Fenton reagent and black mulberry fruit extract, and 0.4 ml fatty acid mixture, 40 µM $FeCl_2$ and 60 µM H_2O_2 and 2 ml fruit extracts were suspended in 5 ml buffer solution.

All of the mixtures were incubated at 37°C for 24 h. After incubation, 100 µL of 4% (w/v) BHT solution was added to prevent further oxidation. Then, 1ml of each mixture was taken and 1 ml 0.6% TBA solution was added to the reaction mixture and incubated at 90°C for 40 min. Samples were allowed to cool to

room temperature and the pigment produced was extracted with 3 ml of *n*-butanol. Samples were then centrifuged at 6,000 rpm for 5 min and the concentration of the upper butanol layer was measured using a HPLC- fluorescence detector.

Quantitation of LPO level *in vitro* environment

The products of peroxidation of fatty acids *in vitro* environment were determined by reading the fluorescence detector set at λ (excitation) = 515 nm and λ (emission) = 543 nm. Formation of the malonaldehyde *in vitro* environment expressed as thiobarbituric acid-reactive substances (TBARS) was calculated from a calibration curve using 1,1,3,3-tetraethoxypropane as the standard. The MDA-TBA complex was analyzed using the HPLC equipment. The equipment consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto Japan). Inertsil ODS-3 column (15 × 4.6 mm, 5 µM) was used as the HPLC column. 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 (De Las Heras et al., 2003)

Quantitation of the remaining fatty acids *in vitro* environment

Remaining mixtures of oleate, linoleate and linolenate in the test tube were converted to methyl esters using 2% sulfuric acid (v/v) in methanol (Christie, 1990). Fatty acid methyl ester forms were extracted with n-hexane. Analysis was performed in a Shimadzu GC-17A V3 instrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm i.d. Permabond fused-silica capillary column (Macherey - Nagel, Germany). The oven temperature was programmed between 160 to 215°C and 4°C / min. Injector and FID temperatures were 240 and 280°C, respectively. The nitrogen carrier gas flow was 1 ml/min. The methyl esters of oleate, linoleate and linolenate were identified by comparison with authentic external standard mixtures analyzed under the same conditions. *Class GC 10* software version 2.01 was used to process the data. The results were expressed as µmol/ml.

Chromatographic conditions for flavonoid analysis

Chromatographic analysis was carried out using PREVAIL C18 reversed-phase column (15 × 4.6 mm) 5 µM diameter particles. The mobile phase was methanol/water/acetonitrile (46/46/8, v/v/v) containing 1.0% acetic acid (Zu et al., 2006). This mobile phase was filtered through a 0.45 µM membrane filter (Millipore), then de-aerated ultrasonically prior to use. Catechin (CA), naringin (NA), rutin (RU), resveratrol (RES), myricetin (MYR), morin (MOR), naringenin (NAR), quercetin (QU) and kaempferol (KA) were quantified by DAD following HPLC separation at 280 nm for CA and NA, 254 nm for RU, MYR, MOR and QU, 306 nm for RES, and 265 nm for KA. Flow rate and injection volume were 1.0 ml/min and l0 µL, respectively. The chromatographic peaks of the analyses were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using the external Standard method. All chromatographic operations were carried out at a temperature of 25°C.

Antioxidant assay by DPPH radical scavenging activity

The free radical scavenging effect of black mulberry extracts was assessed by the discoloration of a methanolic solution of DPPH• according to the method of Brand-Willliams et al. (1995). A solution of 25 mg/L DPPH in methanol was prepared and 4.0 ml of this solution was mixed with 25, 50, 100, 250, 500 and 1000 µL of extract in DMSO. The reaction mixture was stored in darkness at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm in a spectrophotometer (Hsu et al, 2006). The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control)] × 100 Where, Abs control is the absorbance of DPPH radical + methanol and Abs sample is the absorbance of DPPH radical + sample extract /standard.

Statistical analysis

Statistical analysis was performed using SPSS software (ver 15.0). The experimental results were reported as mean ± SEM (standard error of means). Analysis of variance (ANOVA) and an LSD (least significant difference) test were used to compare the experimental groups with the controls.

RESULTS

Lipid peroxidation level

When the level of MDA-TBARS was compared with other groups as to the control group, It was observed that only in the group containing Fenton reagent and that containing Fenton R + SMF, were the level of TBARS distinctively high ($p<0,0001$). As for the group that contained Fenton R + MF, a drop was observed ($p<0.02$) (Figure 1). When only the two groups that contain Fenton reagent and the groups of Fenton R + SMF with Fenton R + MF were compared, it was examined that the level of MDA-TBARS had a distinctive drop ($p<0,0001$). But when the two group that contain SMF extract and MF extract was compared, it was determined that SMF had lower effect in preventing the formation of MDA than MF ($p<0.0001$) (Figure 1). In our study, it was found that resveratrol had the highest LPO decreasing effect. When the LPO decreasing effects of rutin, quercetin and resveratrol were compared to the antioxidant activity, it was in the order of resveratrol>quercetin>rutin (Figure 2).

DPPH scavenging effect of *M. nigra* extracts

When the scavenging activity of DPPH radical from mature mulberry fruit extracts' (MF) was examined, it was observed that its activity rose from 50 to 250 µL, but after 500 µL, its activity diminished. It was found that the highest scavenging activity level of DPPH radical was in the concentration of 100 to 250 µL ($p<0.0001$). It was stated that although there were no difference in the concentrations of 50 and 1000 µL ($p>0.05$), after 2000 µL; however, there was a decrease (Figure 3).

In the semi-mature mulberry extracts (SMF), although it showed maximum scavenging effect in 50-100 µL ($p < 0,0001$), in the trials after 250 µL (500, 1000 and

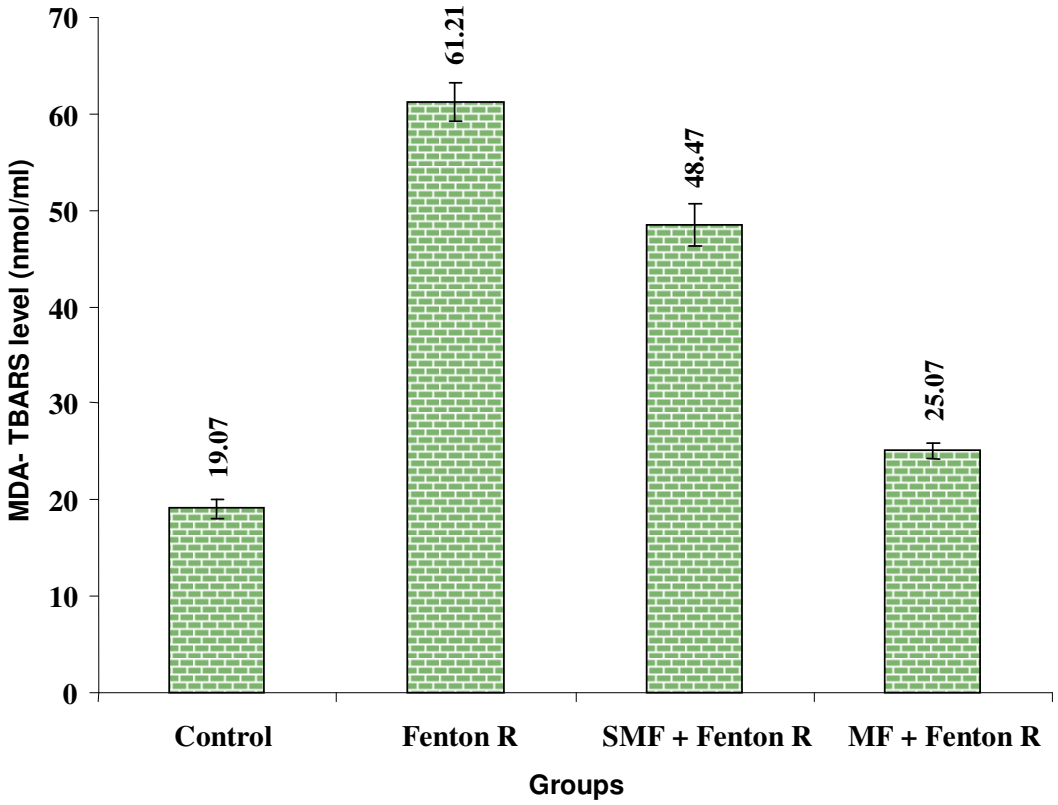

Figure 1. The transformation in the TBARS level in groups in which mulberry fruit extract and Fenton reagent was added (nmol/ml).

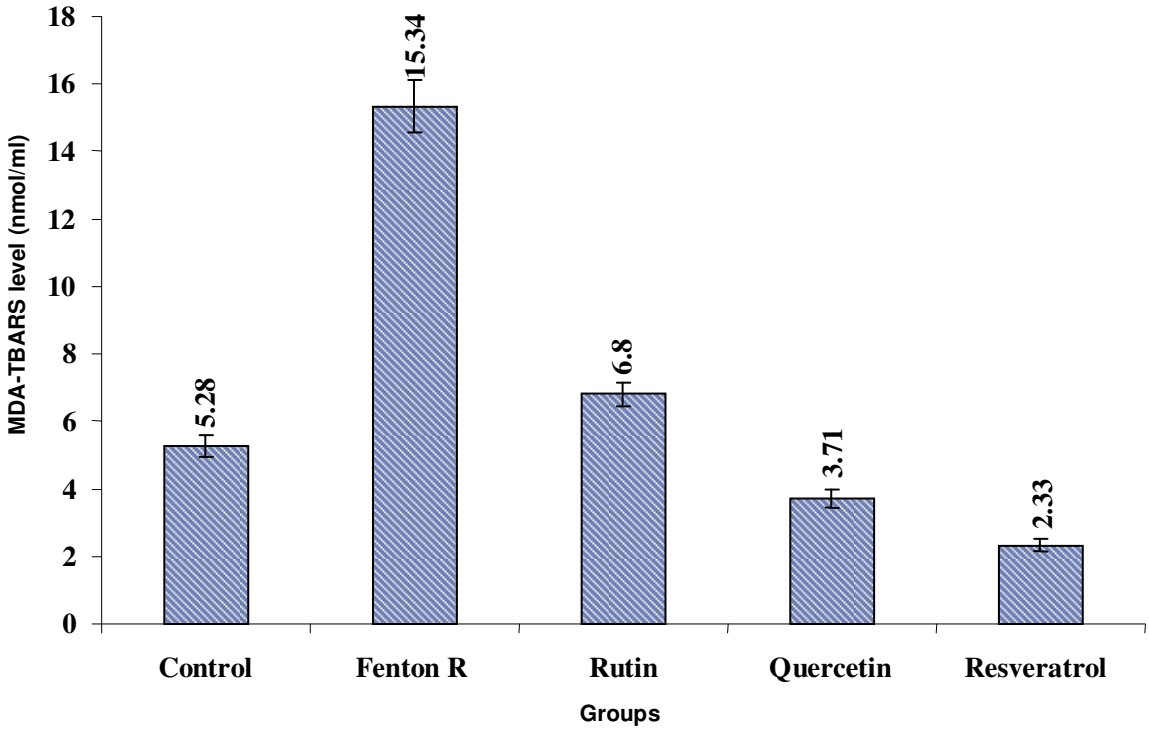

Figure 2. Inhibition activity of LPO formation *in vitro* environment of rutin, quercetin and resveratrol used in the level of 10 µM

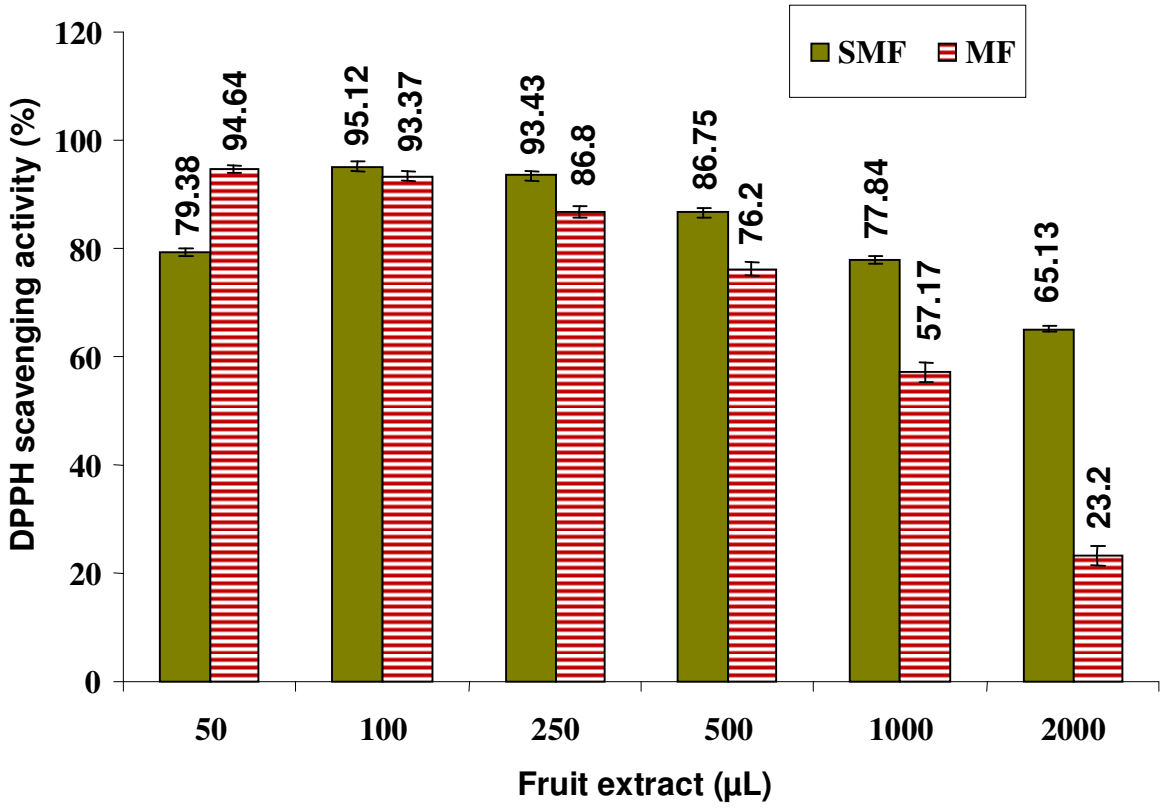

Figure 3. Scavenging effects of matured (MF) and semi-matured fruit (SMF) extracts on the DPPH free radical.

2000), it was stated that scavenging effect gradually decreased, and it was observed that 2000 µL was at the lowest level in accordance with 50 and 100 µL ($p<0,0001$) (Figure 3).

The amount of fatty acids in the reaction environment

When the amount of the fatty acids that was added to the environment was measured at the end of the reaction, the amount of the oleic acids was higher in the groups of SMF + Fenton R and MF + Fenton R, than the group that contain Fenton R ($p<0.02$, $p<0.01$). When the groups of SMF + Fenton R and MF + Fenton R were compared, it was observed that there were no statistical difference between their rate ($p>0.05$) (Figure 4).

It was stated that the amount of LA was more significantly protected in the groups of SMF + Fenton R and MF + Fenton R than in Fenton R ($p<0.01$, $p<0,001$). Moreover, it was confirmed that the amount of LNA was higher in the groups of SMF + Fenton R and MF + Fenton R than the group of Fenton R ($p<0.001$, $p<0.0001$). A difference was however not observed between the groups of SMF + Fenton R and MF + Fenton R in protecting the amount of LA and LNA ($p<0.05$) (Figure 4).

DISCUSSION

Mulberry fruits are valuable food supplies that are consumed in various ways by humans. The fruits which are the member of *Morus spare* are slightly in acidic level and have a quite dark color. It was mentioned that the origin of this color is anthocyanins that has an immense role in food industry (Jia et al., 1999). Some researchers, in their previous studies, have reported that they have antioxidant and free radical scavenging activity in considerable level (Jia et al., 1999; Naderi et al., 2004; Suh et al., 2003, 2004). It has been detected that in mulberry fruit, besides anthocyanins, there are other polyphenolic components (Hakkinen et al., 1999). It has been found that the mulberry fruit that was used in our study contains rutin at the highest rate between its methanolic extracts, at lower rate myricetin and merely kaempferol, quercetin and resveratrol (Table 1). It is known that naturally found flavonoids have extensively scavenging effect on oxygen radicals *in vivo* and *vitro* (Kravchenko et al., 2003; Seyoum et al., 2006).

It is surveyed that in the studies we carried out *in vitro* environment on both MF extract and SMF extract, lipid peroxidation level decreased (Figure 1). It could be said that the decreasing of the lipid peroxidation event in the environment in which extracts are added is because of

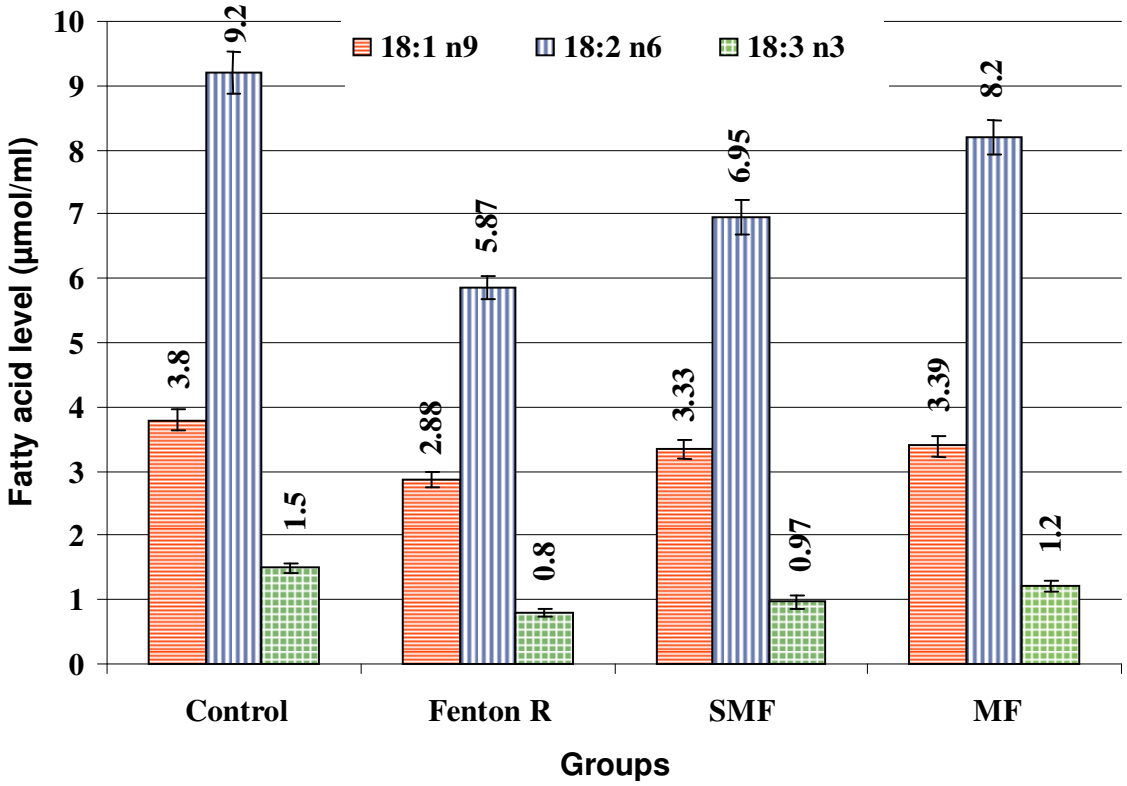

Figure 4. The levels of linolenic (LNA: 18:3, n-3), linoleic (LA: 18:2, n-6) and oleic acid (18:1, n-9) in the reaction environment.

Table 1. In the methanolic extract of MF and SMF, some polyphenolic components that were found in result of HPLC analysis birimi (ug/g)

Flavonoid	MF	SMF
Rutin	76.87	47.80
Resveratrol	0.03	0.133
Myricetin	2.60	1.43
Naringenin	3.33	0.46
Quercetin	0.02	0.2
Kaempferol	0.07	0.07
Total flavonoid	82.92	50.09

MF, matured fruit ; SMF, semi-matured fruit.

scavenging characteristic of the polyphenolic components. Our indications have showed that the decreasing LPO level in the environment in which MF extract is added as to the one that SMF is added is related with the amount of components (Figure 1, Table 1). In a study carried out by Yang et al. (2008) in which rutin antioxidant characteristics were observed, it was detected that the formation of lipid peroxidation is effectively prevented by rutin. Also, in the lipid peroxidation study carried out with 10 µM rutin, quercetin and resveratrol, it was observed that the LPO level in the group in which rutin was added has decreased distinctively in accordance with the only group that Fenton reactive was added (Figure 2). This result is in agreement with that of Yang et al. (2008). In the same study, it was experienced that both quercetin and resveratrol effectively diminished the formation of LPO. In the results of many researches, it has been reported that resveratrol especially decreases the formation of LPO (Signorelli and Ghidoni, 2005; Delmas et al., 2005).

In a study carried out by Pekkarinen et al. (1999), it was suggested that myricetin had a higher effect in

preventing the formation of lipid peroxidation than quercetin. Moreover, it was determined that over hydroperoxide formation, the antioxidant activity was in the order of myricetin >quercetin>tocopherol> (+)catechin> kaempferol = rutin. In a study by Velioğlu et al. (1998) about plant ingredients, they suggested that there is a relation between plant species' antioxidant activities and phenolic components. In a study by Ercişli and Orhan (2007), they stressed that there is a correlation between *M. nigra* fruits' total phenolic ingredients and antioxidant activity. Also, in the study about the scavenging effect of DPPH radical that has a stable form in our findings, it could be seen that MF extract has a more different scavenging effect than SMF extract (Figure 3). It was mentioned that the scavenging activity of the DPPH radical is about the amount of phenolic components, too (Seyoum et al., 2006).

DPPH radical is quite commonly used to unearth the radical scavenging capacity of the extracts or molecules (Mensor et al., 2001; Turkoglu et al., 2010a, b). DPPH is a radical in the color of violet and a stable substance in room temperature environment. In the existence of antioxidant molecules, DPPH is degraded and the violet color becomes colorless. There is a strong relationship between the polyphenolic components of the plant extracts ingredients and the scavenging effect of DPPH radical. According to polyphenolic ingredients, the scavenging activity rises to the maximum level in a stable concentration, however, after a stable concentration, scavenging activity of the radical either rises or declines. In our findings, this result could be examined. It is confirmed that though MF extract has maximum scavenging activity in 50 µl polyphenolic, SMF extract has the same activity in 100 µL (Figure 3) and it is observed that the radical scavenging activity decreases. It was detected that in the flavonoid analysis carried out with HPLC device, there is rutin at highest rate inside the extract of *M. nigra* fruits followed by Naringenin and myricetin (Figure 1). In a study by Yang et al. (2008) in which the antioxidant characteristic of rutin searched *in vitro* environment in 0.05 mg/ml concentration, scavenging activity of DPPH radical was detected as 90. 4% . In our findings, it was observed that the fatty aids that were added to the reaction environment to prevent oxidation were effective according to the group that Fenton R was added. When SMF and MF extract are compared, it was observed that MF extract was more effective. In our findings, it was confirmed that both the complete amount of flavonoid and the amount of myricetin are high in MF extract (Table 1). When the amount of myricetin is considered, our findings are in line with the findings of Pekkarinen et al. (1999). Finally, it can be said that when many fruits and vegetables that serve as food approaches maturity, functional molecules that are used in living system for synthesis operation is completed. Besides, as mentioned in many researches, it was also confirmed that antioxidants that are taken in high rates may show prooxidant effect.

ACKNOWLEDGEMENT

This study constitutes a part of the 1824 numbered project that is supported by FÜBAB (Firat University Scientific Research Projects Unity). We appreciate the Firat University for support.

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