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Radical Scavenging Activity of the *Pistacia terebinthus* in Fenton Reagent Environment and Its Protective Effects on the Unsaturated Fatty Acids

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This study investigated protective effects of the *Pistacia terebinthus* extracts on the unsaturated fatty acids and the prevention of lipid peroxidation formation in the Fenton reagent environment. Antioxidative activities of the *Pistacia terebinthus* extracts were determined with the following modifications. Lipid peroxidation level in the Fenton R group significantly high compared to control group, but lipid peroxidation in the *Pistacia terebinthus* extract groups significantly decreased according to the Fenton R group ($p < 0.0001$). In addition, it was determined that *Pistacia terebinthus* extracts had scavenging effect on the DPPH radical depending on the increase in flavonoid concentration. The fatty acid levels in the Fenton R group were lower than the control group ($p < 0.001$). However, fatty acid levels in the *P. terebinthus* extract groups were higher than the Fenton R group ($p < 0.001$). Present results confirm that the *Pistacia terebinthus* extracts decrease lipid peroxidation level in the Fenton reagent environment and protects markedly unsaturated fatty acids in the environment with radical sourced oxidations.

Key Words: Lipid peroxidation, Fenton reagent, *Pistacia terebinthus*, flavonoids, Radical scavenging effects.

INTRODUCTION

Oxidative stress appears to play an important role in several human chronic diseases, including atherosclerosis and cancer[1]. Clinical studies suggest that a diet which is high in fruit and vegetables reduces the incidence of cancer, coronary diseases and hypertension[2]. The most important benefit of fruit and vegetables in terms of human health is that they contain antioxidants. Therefore, antioxidants may be useful in the prevention and treatment of these conditions. Antioxidants help counter the damaging effects of free radicals and preserve the oxidant-antioxidant balance. Free radicals cause the occurrence of some diseases, as well as enabling their progress, by causing lipid peroxidation, which is their most significant role[3].

Pistacia terebinthus L. (Anacardiaceae) is one of the 20 *Pistacia* species widely distributed in the Middle East and Southern Europe possessing many biological activities and they have a wide range of uses in food industries. *Pistacia* species

have attracted the attention of researches because of their antioxidant potential besides antimicrobial, antiinflammatory and cytotoxic activities, particularly due to flavonoids and other phenolic contents[4].

In Turkey, the species *P. terebinthus* is found growing on dry rocky slopes and hillsides or in pine forests, particularly in the southern mountain, from just above sea level to 1600 m. The fruits have been used as an appetizer in human nutrition in southern Turkey, but also in baking of special village breads and as coffee substituent either before or after roasting. In folk medicine, the fruits are used in the treatment of gastralgia (internally), rheumatism, cough (externally), eczema, diarrheic, throat infections, asthma and stomach ache. Additionally, some effect is described as a stimulant, diuretic, antitussive, astringent, antipyretic and antibacterial[4].

Metabolic disorders stemming from lipid peroxidation are related not only to age but also to environmental factors and lifestyle. For this reason, the contents of the food in human diet play an important role in reducing the risk of contracting certain diseases, as well as reducing disease-caused mortality. The purpose of this study is to investigate the antioxidant activities and phytochemical characteristics of *P. terebinthus*. Previous studies related to this subject generally dealt with the antioxidant capacities of vegetative resources and their chemical contents were not examined. In contrast, this study examined the phytochemical characteristics of *P. terebinthus* (known as menengic) samples grown in the Elazig region of Turkey and investigated their impacts on human health by determining their antioxidant activities *in vitro*.

EXPERIMENTAL

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

The samples we use in our research within the boundaries of Elazig, in the coordinates of 38°43'21" N - 39°13'19" E and at the altitude of 1270-1280 m, in the village of Gümüsbaglar, in August-September in 2008, were collected regularly in 15-day period. In the creation process, maturity of the fruits were considered. Terebinth fruits were divided into 3 groups as mature terebinth, semi mature terebinth and crude terebinth.

The fruits were immediately washed and frozen, to be further freeze-dried. Freeze-dried samples were maintained at -20 ºC prior to analysis. Only healthy looking fruits (without mechanical damage or bacterial infection) were selected for examination. The antioxidant activity and flavonoid analysis were determined in DMSO extracts.

Preparation of the vegetable oils: For the samples of fatty acids which were used in the experiment, soy and corn oil were used. 0.3 mL oil sample was prepared in 15 mL DMSO resolution.

Preparation of exracts: Fruit samples were homogenized at a ratio of 1:10 g/mL with methanol at a rate of 85 %. After filter of homogenates, it was centrifuged at 5000 rpm at a temperature of 4 ºC for 5 min. After the centrifugation, supernatant which was in the upper part, was removed and the part of rotovapor and methanol were removed at 45 ºC under vacum. The rest were dissolved in DMSO for the usage in the experiment.

Chromatographic conditions for flavonoid analysis: Chromatographic analysis was carried out using PREVAIL C_{18} reversed-phase column (15 × 4.6 mm) 5 μm diameter particles. The mobile phase was methanol/water/acetonitile (46/46/8, v/v/v) containing 1.0 % acetic acid[5]. This mobile phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated 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 RPHPLC separation at 280 nm for catechin and naringin, 254 nm for rutin, myricetin, morin and quercetin, 306 nm for resveratrol and 265 nm for kaempferol. 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 ºC.

Antioxidant assay by DPPH radical scavenging activity: The free radical scavenging effect in extracts was assessed by the decolouration of a methanolic solution of DPPH according to the method of Brand-Williams *et al.*[6]. 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 and 500 μL of extract in DMSO. The reaction mixture was stored in darkness at room temperature for 0.5 h. The absorbance of the mixture was measured spectrophotometrically at 517 nm.

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; Abs sample is the absorbance of DPPH radical + sample extract/standard.

Antioxidative activity testing in the fatty acid environment: Antioxidative activities of the *P. terebinthus* extracts were determined by the method of Shimoi *et al.*[7] with the following modifications: 1 mM $FeCl_2$ ($FeCl_2 \cdot 2H_2O$) and 3 μM hydrogen peroxide (H_2O_2) solutions were prepared freshly for every treatment, using doubly deionized water. Extracts of *P. terebinthus* were also prepared freshly using DMSO. Buffer solutions were prepared with 0.2 % Twin 20, 0.05M Tris-HCl-BASE and 0.15M KCl (pH = 7.4).

In vitro experiment for both corn and soybean oil the first group was used as a control and 0.4 mL fatty acid mixture (for corn oil; linolenic acid: 0.83 %/1 mL; 58.72 %/1 mL linoleic acid and 26.97 %/1 mL 18:1, for soybean oil: linolenic acid: 4.9 %/1 mL; 54.85 %/1 mL linoleic acid and 22.71 %/1 mL 18:1) was suspended in 5 mL buffer solution. The second group was a Fenton reagent group ($FeCl_2 + H_2O_2$, Fenton R) 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 groups were Fenton's R plus *P. terebinthus* (crude-HC) extract and 0.4 mL fatty acid mixture 40 μM $FeCl_2$ and 60 μM H_2O_2 and 1 mL fruit extracts were suspended in 5 mL buffer solution, The fourth groups were Fenton's R plus *P. terebinthus* (semi-mature-YOC) extract and 0.4 mL fatty acid mixture 40 μM $FeCl_2$ and 60 μM H_2O_2 and 1 mL fruit extracts were suspended in 5 mL buffer solution, The fifth groups were Fenton's R plus *P. terebinthus* (mature-OC) extract and 0.4 mL fatty acid mixture 40 μM $FeCl_2$ and 60 μM H_2O_2 and 1 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) butylated hydroxytoluene (BHT) DMSO solution was added to prevent further oxidation. Then, 1 mL of each mixture was taken and 1 mL 0.6 % of 2-thiobarbituric acid (TBA) 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.

The third group was Fenton's R plus *Pistacia terebinthus* (crude) extract (HC) including group, the fourth group Fenton's R plus *Pistacia terebinthus* (semi-mature) extract (YOC) including group and the fifth group Fenton's R plus *Pistacia terebinthus* (mature) extract (OC) including group.

Quantitation of oxidized products: The extent of oxidation of unsaturated fatty acids was determined by reading the fluorescence detector, which was set at (excitation) = 515 nm and (emission) = 543 nm and the amount of malonaldehyde (MDA) 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 VP software (Shimadzu, Kyoto Japan). An Inertsil ODS 3 column (15 × 4.6 mm, 5 μm) was used as the HPLC column. The column was eluted isocratically at 20 ºC with a 5 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (85:15, v/v) at a rate of 1 mL/min[8].

Stability of fatty acids: The remaining mixtures of oleate, linoleate and linolenate in the test tube were converted to methyl esters by using 2 % sulfuric acid (v/v) in methanol[9]. 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- 215 ºC, with an increment of 4 ºC/min. Injector and FID temperatures were 240 and 280 ºC, 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.

Statistical analysis: Statistical analysis was performed using SPSS software (version 13.0). 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 AND DISCUSSION

Lipid peroxidation: When the lipid peroxidation level in corn oil was analyzed, it was detected that LPO level in FR group was considerably higher than that in CG group ($p < 0.0001$). As FR group and extract groups were compared, it was observed that the LPO amount in extract groups was decreased at a significant level ($p < 0.0001$). When the CG and the extract groups were compared, it was detected that although the LPO level significantly increased in HC group ($p < 0.001$), there was a smaller increase in OC and YOC groups ($p < 0.05$) (Fig. 2).

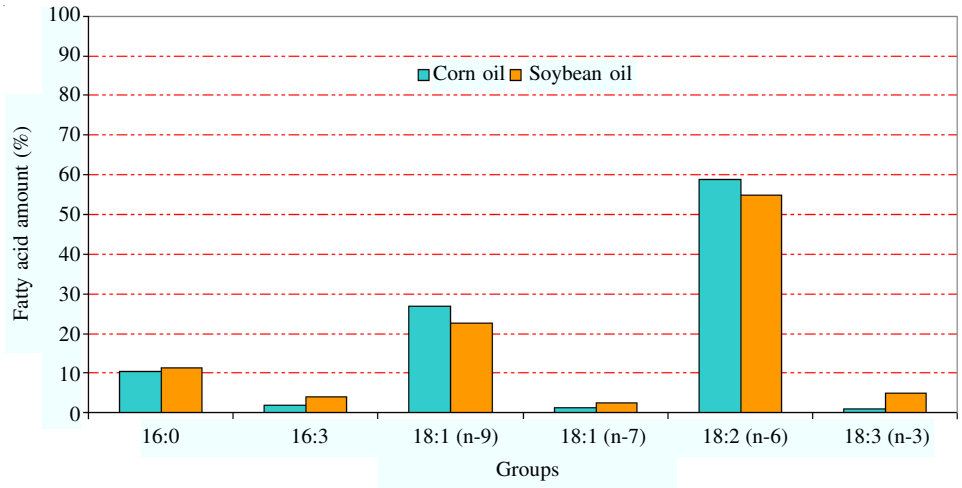

Fig. 1. Fat content of oil groups used in present study

When the LPO levels in soybean oil was compared according to CG, a considerably high increase in FR group ($p < 0.0001$) and a partial increase in groups containing fruit extracts ($p < 0.001$) were observed respectively. When the FR group and the extract groups are compared (to each other), it was observed that the LPO levels

decreased very saliently ($p < 0.0001$). When the *P. terebinthus* groups were compared to each other, while no statistically significant difference was observed between OC and YOC ($p > 0.05$), a partial difference was detected in HC group ($p < 0.05$) (Fig. 2).

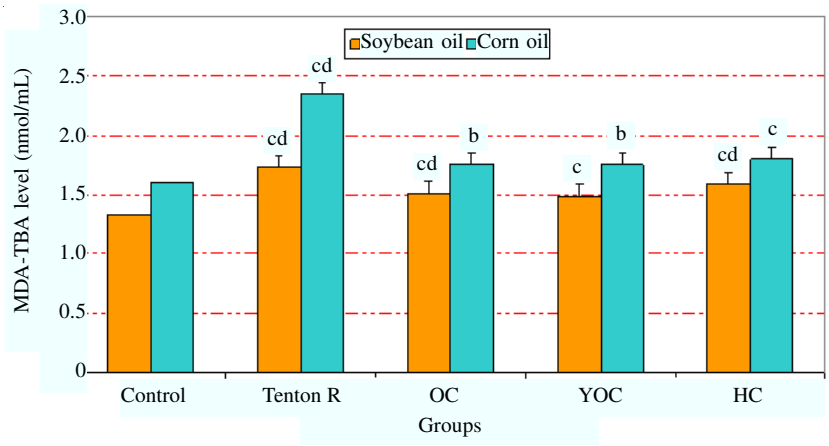

Fig. 2. *The levels of MDA-TBARS in the environments of Fenton reagent with *P. terebinthus* extracts (Fenton R: Fenton reagent)

In the present study the decrease in high level of LPO in the plant extracts that determined only in the Fenton including group, is believed to be related to its flavonoid ingredient. The flavonoid contents of *Pistacia terebinthus* included rutin, quercetin, resveratrol, myricetin, naringin and naringenin (Table-1). The flavonoids have long been suggested to act as antioxidants, owing to their radical scavenging and metal-chelating capabilities. They represent a multitude of phenolic compounds found exclusively in plants. Epidemiological studies suggest that dietary flavonoids protect the molecules of the cells against the formation of LPO in different degenerative diseases[10-12].

TABLE-1
FLAVONOIDS AND RESVERATROL CONTENT IN *Pistacia terebinthus* (µg/1 g)

Flavonoids	HC	YOC	OC
Rutin	Trace^{cd}	1775.73±1.25	1205.97±1.24
Myricetin	45.30±3.45	44.43±1.55	533.75±1.41^{cd}
Morin	Trace	Trace	Trace
Quercetin	4.04±2.07	10.55±1.02	19.00±1.47^{c}
Kaempferol	Trace	Trace	Trace
Cathechin	Trace	Trace	Trace
Naringin	892.77±3.70^{cd}	352,68±2.12	194.48±2.55
Naringenin	Trace	1.23±0.12	3.36±0.41^{c}
Resveratrol	83.10±1.24^{cd}	1.03±0.41	Trace
Total	1025.21	2185.65^{c}	1956.56

cd: $p < 0.0001$ c: $p < 0.001$ b: $p < 0.05$ a: $p > 0.05$

Food derived flavonoids, especially kaempeferol, quercetin and myricetin are widely occurring flavonoids and are reported to exhibit multiple biological functions such as antiallergenic, antiartherogenic, antiinflammatory, antimicrobial, antithrombotic, antioxidant, cardioprotective and vasodilator effects[13]. Duthie[11] stated that LPO level was decreased by the quercetin. Quercetin, with *o*-di-hydroxyl structure in ring B could directly scavenge free radicals effectively as reported by Gao *et al.*[14]. Kostiuk *et al.*[15] have reported antioxidative activity of quercetin and rutin in various systems of lipid peroxidation.

Amount of fatty acids: As a result of gas chromatography analysis, when the amounts of oleic acid, linoleic acid and linolenic acid in corn oil were compared according to the control group, it was detected that the amounts of these fatty acids decreased significantly in the group which contained Fenton reactive ($p < 0.0001$) (Fig. 3). While partial decrease was observed in OC and YOC extract groups ($p < 0.05$), it was detected that fatty acids in HC extract group were at a significantly high level with respect to the control group ($p < 0.0001$). When the fruit extracts were compared to each other, it was observed that the fatty acid levels in HC group were much higher than those of OC and YOC groups ($p < 0.0001$). However, no statistically significant difference was found between YOC and OC groups ($p > 0.05$) (Fig. 3).

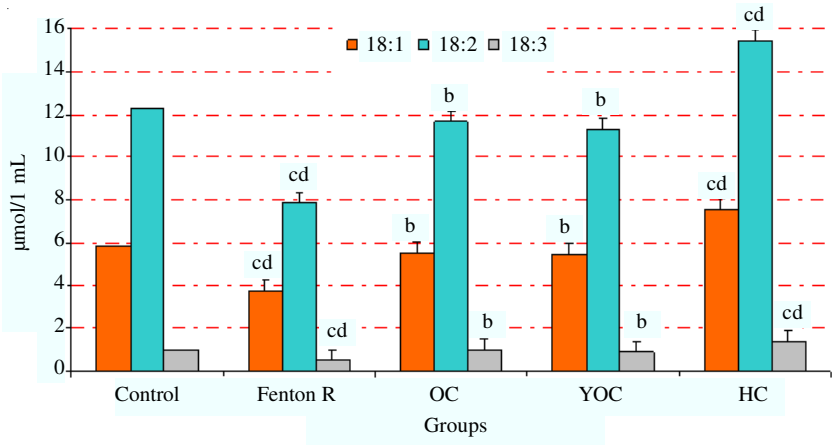

Fig. 3. Levels of linolenic acid (18:3, n-3), linoleic acid (18:2, n-6) and oleic acid (18:1, n-9) in the crop oil reaction environment (µmol/1 mL)

When the oleic acid amount in soybean oil composition was compared with respect to the control amount; it was observed that the level decreased significantly in FR, OC and HC groups ($p < 0.0001$), while the amount of oleic acid in YOC group was decreased at a less significant level ($p < 0.001$) (Fig. 4).

When the amounts of linoleic acid and linolenic acid in the medium were compared according to the control group, it was detected that fatty acids significantly decreased in all extract groups as well as the group containing Fenton reactive ($p < 0.0001$).

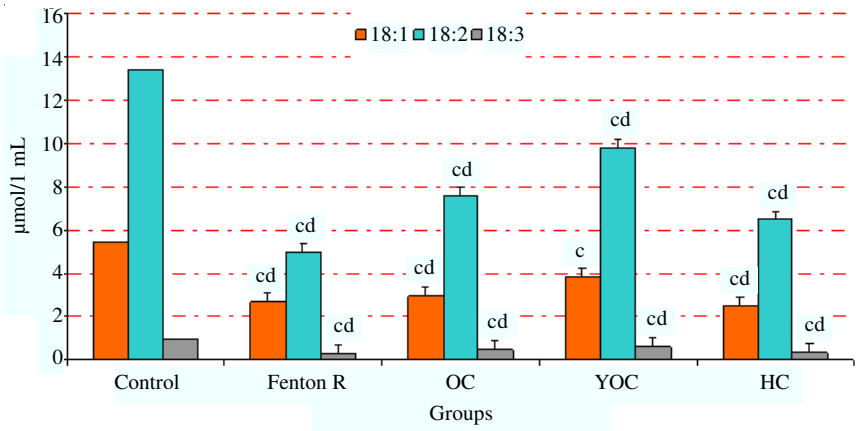

Fig. 4. Levels of linolenic acid (18:3), linoleic acid (18:2, n-6) and oleic acid (18:1, n-9) in the soybean oil reaction environment (µmol/1 mL)

When the extract groups were compared to each other in terms of preserving oleic acid, linoleic acid and linolenic acid; while a difference between OC and YOC groups was observed at a very low level, it was determined that HC group is less effective than other extract groups ($p < 0.05$ - $p < 0.001$) (Fig. 4).

In the present study, when fatty acid quantity was examined each of the three fatty acids in *Pistacia terebinthus* including extracts are high compared to Fenton reagent group. So we think that the flavonoids of extracts may protect unsaturated fatty acids from radical sourced oxidations. All favonoids acted as antioxidants on oxidation of methyl linoleate although the antioxidant response of kaemferol and rutin is weak. Quercetin and myricetin inhibites the hydro peroxide formation in methyl linoleate environment[16].

Determination of antioxidant activity: According to the results of DPPH free radical neutralization; it was detected that *P. terebinthus* extracts were significantly effective between concentrations of 25 mL and 50 mL ($p < 0.0001$), however *P. terebinthus* groups showed antagonistic effect at concentrations higher than 100 mL. When the *P. terebinthus* groups are compared to each other according to the OC group, no statistically significant difference was observed among OC, YOC and HC groups at the concentrations between 25 mL and 100 mL ($p > 0.05$). When radical neutralization activities of fruit extracts were compared at concentrations in the interval of 250-500 mL, it was determined that antioxidant activity effectiveness in YOC and HC groups were slightly higher than that of OC ($p < 0.05$) (Fig. 5).

The results of DPPH free radical neutralization in soybean oil and corn oil indicated that both two oil groups showed antioxidant property starting from 25 mL concentration and in parallel with the increasing concentrations, they increased their antioxidant activities ($p < 0.0001$). However beyond 500 mL concentration, it was detected that they showed antagonistic effect as it was the case in *P. terebinthus* extract groups (Fig. 5).

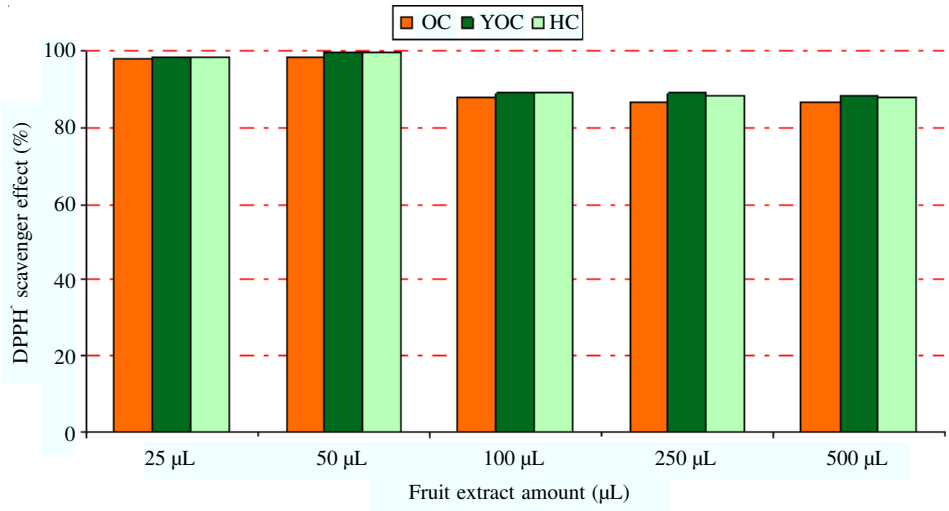

Fig. 5. DPPH• scavenger effect of *P. terebinthus* extracts (%)

It is presumed that there is a link between the decrease in the level of LPO of the plant extracts and scavenging capacity of the DPPH radical. When DPPH free radical scavenging capacity was examined, it was determined that a statistically non-significant difference exist between *Pistacia terebinthus* samples quercetin, myricetin, naringin and that they both have high level of DPPH radical scavenging ability like quercetin, myricetin and naringin.

Flavonoids of fruits: According to the conducted flavonoid analyses results, it has been detected that low amount of rutin flavonoid was available in the HC group ($p < 0.0001$) and significantly high amount of flavonoid was available in YOC and OC groups. However, it has been detected that there was no statistically significant difference between YOC and OC groups (Table-1).

When the groups were compared with respect to quercetin and myricetin amounts, it has been observed that OC group contained these flavonoids at very significant levels when compared to HC and YOC groups ($p < 0.0001$, ($p < 0.001$). While no important statistical difference between HC and YOC groups was found in terms of naringenin flavonoid, a significant difference has been detected in OC group ($p > 0.05$, ($p < 0.001$). When compared to other groups, the amount of naringin was determined in at a very significant level in HC group ($p < 0.0001$) (Table-1). Moreover, when all groups were compared in terms of resveratrol, it was detected that HC group contained this component at a very significant level ($p < 0.0001$).

When all samples were compared in terms of total flavonoid amounts, it has been detected that YOC group contained these flavonoids at significant levels compared to other groups ($p < 0.001$), no statistically important difference has been detected among other groups (Table-1).

Some previous studies suggested that the phenolic compound of *Pistacia* genus were comprised of momoterpenes[17], tetracyclic triterpenoids[18] besides other

triterpenoids[19], flavonoids[20] and other phenolics including gallic acid[21] and essential oils[22].

As a result, it is presumed that there is a strong link between *Pistacia terebinthus*'s flavonoid ingredients and its feature of preventing lipid peroxidation. Particularly, it was determined that YOC group a partial difference decreases the LPO level compared to OC and HC groups and that both plants have a high level of capacity to scavenge the radical of DPPH. Besides, if one consider the fact that unsaturated fatty acids are also protected from radical sourced oxidation, it is possible to argue that those plants have high level of antioxidant activities.

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