

## ANTIOXIDANT ACTIVITIES OF OLEUROPEIN AND THE AQUEOUS EXTRACTS OF *OLEA EUROPAEA* L. VARIETIES GROWING IN TURKEY

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### Abstract

The genus *Olea* L. (*Oleaceae*) is represented by 67 species in the world. Two varieties of *Olea europaea* L. (*Olea europaea* L. var. *europaea* and *Olea europaea* var. *sylvestris* (Miller) Lehr.) are growing in Turkey and are found mostly in the West and South Anatolia. In this study, antioxidant activity of the aqueous extracts obtained from the leaves and branches of *O. europaea* and oleuropein were investigated.

The antioxidant activities were studied by two different techniques: Qualitative DPPH\* (1,1-diphenyl-2-picrylhydrazyl radical) assay to detect the free radical scavenging activity and the thiobarbituric acid (TBA)-assay to detect liposome lipid peroxidation. The lipid peroxidation was initiated in liposomes obtained from bovine brain extracts by addition of ascorbic acid and iron source and was measured spectrophotometrically with TBA test. The highest activity was observed with the extract of *O. europaea* var. *sylvestris* leaves with an  $IC_{50}$  value of 0.29 µg/ml where propil gallat ( $IC_{50}=0.21\pm0.01$ ) was used as the positive control.

**Key words:** *Olea europaea*, Antioxidant activity, DPPH, Lipid peroxidation

### Türkiye’de Yetişen *Olea europaea* L. Varyetelerinin Sulu Ekstrelerinin ve Oleuropein’in Antioksidan Aktiviteleri

*Olea* L. (*Oleaceae*) cinsi dünyada 67 türle temsil edilmektedir. *Olea europaea* L. ’nin iki varyetesi (*Olea europaea* L. var. *europaea* ve *Olea europaea* var. *sylvestris* (Miller) Lehr.) Türkiye’de yetişmektedir ve çoğunlukla batı ve güney Anadolu’da bulunmaktadır. Bu çalışmada *O. europaea* ’nin yaprak ve dallarından elde edilen sulu ekstrelerin ve oleuropein’in antioksidan aktivitesi araştırılmıştır.

Antioksidan aktiviteler 2 farklı yöntemle çalışılmıştır: Kalitatif DPPH yöntemi (1,1-difenil-2-pikrilhidrazil radikali) ile serbest radikal süpürücü aktivite, tiyobarbitürik asit (TBA) testi ile lipozom peroksidasyonu tayin edilmiştir. Lipit peroksidasyon, sığır beyin ekstrelerinden elde edilen lipozomlara askorbik asit ve demir ilave edilerek başlatılmış ve TBA testi ile spektrofotometrik olarak ölçüm yapılmıştır. Propil gallatın ( $IC_{50}=0.21\pm0.01$ ) pozitif kontrol olarak kullanıldığı testte en yüksek aktivite ( $IC_{50}=0.29$  µg/ml) *O. europaea* var. *sylvestris* ’in yapraklarında tespit edilmiştir.

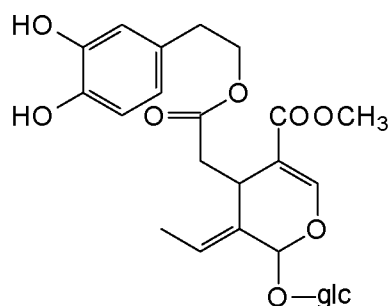
**Anahtar kelimeler:** *Olea europaea*, Antioksidan aktivite, DPPH, Lipit peroksidasyonu

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## INTRODUCTION

*Olea europaea* L. (Oleaceae) is one of the most important fruit trees and has been widely used in folk medicine in Mediterranean countries (1). Leaves of the tree became important when olive leaf extract was reported to be potent in treating fever and malaria in 1854 (2). Since then, several researchers demonstrated hypotensive (3,4), hypoglycemic, coronary dilatatory, antiarrhythmic, antiuricaemic (5), antioxidant (6), anti-complementary (7), antimicrobial (8), thyroid stimulatory (9), antiviral (10) and anti-HIV (2) activities of olive leaf extract.

Oleuropein, the active constituent of olive leaf and fruits, is a glucosidic ester of elenolic acid and hydroxytyrosol (11). It has been reported to have antioxidant, antiinflammatory (12), hypoglycemic (13), antimicrobial (14), antimycoplasmal (15), antiviral (10), anti-tumor and angiogenic (16) activities. It was found to inhibit androstenedione 6 $\beta$ -hydroxylase activity, a cytochrome P450 3A marker in human liver microsomes (17) and to prevent lipid peroxidation on rat liver microsomes (18). Oleuropein has also inhibited LDL (Low Density Lipoprotein) oxidation and it possesses vascular protection activity by inhibiting platelet aggregation induced by platelet-activating factor (19).



**Oleuropein**

In Turkey, *O. europaea* is represented by two varieties, var. *europaea* and var. *sylvestris*, and cultivated through the Western and Southern parts of the country (20). In Turkish traditional medicine 5 % infusion of the leaf is taken orally as appetizer, diuretic, antidiarrheic, antipyretic, and hypotensive. It is also used externally to clean festering sores (21).

Antioxidant activity of the extracts obtained from the leaves and wood of the olive tree has been demonstrated before (11,22). Also, antioxidative effects of olive cake extract and olive oil have been reported by different authors (23,24). However, to the best of our knowledge the antioxidant activity of the branches of the tree has not been reported up to date.

In this study, the antioxidant activities of the lyophilized extracts obtained from the leaves and the branches of the natural (*Olea europaea* L. var. *sylvestris* (Miller) Lehr.) and cultivated (*Olea europaea* L. var. *europaea*) varieties of olive tree growing in Turkey, and oleuropein, the active constituent of them, were investigated by two different techniques: Qualitative DPPH and TBA assays.

## EXPERIMENTAL

### *Plant materials*

Cultivated and natural plant materials were collected from Balıkesir-Edremit (*Olea*

*europaea* L. var. *europaea*- AEF 23334) in August 2004 and *Osmaniye* (*Olea europaea* L. var. *sylvestris* (Miller) Lehr.- AEF 23600) in July 2005. Taxonomic identity of the plants was confirmed by Prof. Dr. H. Duman, in the Department of Biological Sciences, Faculty of Art and Science, Gazi University, Ankara, Turkey. Plant materials were dried at room temperature. Voucher specimens were deposited at Herbarium of the Faculty of Pharmacy, Ankara University, Turkey.

#### *Extraction method*

3 gram of the dried powdered materials were extracted with 25 ml of distilled water for 1 hour at room temperature using an ultrasonic bath. After every 20 minutes of the extraction, the solutions were rested for 20 minutes. The resultant extracts were filtered and freeze-dried.

#### *Preparation of the extracts for DPPH assay*

10 mg of each lyophilized extract and standard oleuropein were dissolved in 1 ml of distilled water to obtain an aqueous solution at a concentration of 10 mg/ml.

#### *Preparation of the extracts and standard solutions for TBA assay*

For each lyophilized extract, 7 different test solutions of the following concentrations were prepared: 1; 0.50; 0.25; 0.125; 0.0625; 0.031; 0.0156 mg/ml, respectively.

From oleuropein (Extrasynthese; 32619) and propyl gallate (Aldrich P53306), 7 different test solutions of the following concentrations were prepared: 1; 0.20; 0.04; 0.008; 0.0016; 0.00032; 0.000064 mg/ml, respectively.

#### *Qualitative DPPH• assay*

A rapid thin-layer chromatography (TLC) screening method was used to evaluate the antioxidant activity of the freeze-dried extracts of natural and cultivated varieties of *O. europaea* due to their free-radical scavenging properties. When 0.2 % DPPH solution in ethanol is sprayed onto a TLC plate, the compounds having antioxidant properties are seen as yellow zones on a purple background (25).

5 µl from the solutions prepared were applied to the silica gel TLC plates (Merck, Darmstadt, Germany) by Wiretrol II micropipettes. Using ethyl acetate: formic acid: glacial acetic acid: distilled water (100:11:11:26) as a solvent system, the aqueous solutions were developed and sprayed with 0.2 % DPPH solution in ethanol, left at 20°C, and examined after 30 minutes of the spraying.

#### *In vitro thiobarbituric acid (TBA) assay*

The principle components of the assay, apart from the standardized liposomes (Brain extract – Sigma B 3635) source are ascorbic acid (Aldrich 255564) and FeCl<sub>3</sub> (Sigma F 1513) which is used as an iron source. Brain extract suspension was made in PBS (Phosphate Buffered Saline – Sigma P 4417).

For the test reaction of the extract, a mixture of liposomes, FeCl<sub>3</sub>, ascorbic acid and PBS was used. All of the tubes were incubated at 37 °C for 20 minutes. After that, the TBA test was performed by adding BHT (butylated hydroxytoluen – Sigma B 1378) in ethanol followed by thiobarbituric acid (TBA – Sigma T 5500) in NaOH and HCl (Merck). The tubes were heated to 90 °C for 30 minutes and then allowed to cool down completely. The chromogens were extracted by using *n*-butanol. The mixture was vortexed to ensure complete extraction of the chromogen and then centrifuged at 3500 rpm for 15 minutes at room temperature in order to separate the two layers. The absorbances of the upper layers which contain the chromogen were determined by a Shimadzu UV-1601 UV/VIS spectrophotometer at 532 nm.

Percentage inhibition of lipid peroxidation was determined by comparing the absorbance of the full reaction mixture containing no inhibitor with that of the test extract reaction mixtures which include the substance to be assessed. The absorbance readings of the extract alone and the liposomes alone were also taken into account as follows:

$$\% \text{ inhibition} = 100 \times [ (\text{FRM} - \text{B}) - (\text{ET} - \text{B} - \text{EA}) ] / (\text{FRM} - \text{B})$$

FRM is the absorbance of the full reaction mixture (liposomes and iron source plus solvent water without the test substance), B is the absorbance of the blank mixture (liposomes only), ET is the absorbance of the extract test mixture (full reaction mixture plus test substance) and EA is the absorbance of the extract alone (25). The half-maximal inhibitory concentrations (IC<sub>50</sub>) of the *O. europaea* leaf and branch samples belonging to different varieties were calculated by linear regression analysis. Propyl gallate was used as a reference compound in this assay.

## RESULTS and DISCUSSION

In the present study, the antioxidant properties of aqueous extracts of the two varieties of *O. europaea* were examined by means of two different *in vitro* systems.

In the DPPH test, yellow zones on a purple background were seen with standard oleuropein and with the extracts of branches and leaves of both natural (*O. europaea* var. *sylvestris*) and cultivated (*O. europaea* var. *europaea*) olive tree. The results of the DPPH<sup>•</sup> test demonstrate that both the leaves and the branches of *O. europaea* varieties have faintly free radical scavenging activity. However, the yellow zones were seen more prominent in *O. europaea* var. *sylvestris* than the other variety. The highest inhibition zones were seen in the standart oleuropein (Fig. 1).



**Figure 1.** TLC screening of DPPH test

**1:** Leaf extract of *O. europaea* var. *europaea*; **2:** Branch extract of *O. europaea* var. *europaea*;  
**3:** Leaf extract of *O. europaea* var. *sylvestris*; **4:** Branch extract of *O. europaea* var. *sylvestris*;  
**S:** Oleuropein.

In a previous study, Altarejos et al. reported that the ethanolic extracts of the wood of *O. europaea* showed antioxidant activities based on their scavenging abilities on DPPH radical (22). However, in the present study, the obtained results from the qualitative DPPH assay showed that the aqueous extracts of the branches of both *O. europaea* varieties had slight antioxidant activities. The reduction in the activity may be related to the extraction solvent since the ethanolic extracts contain both the polar and nonpolar compounds.

For *in vitro* antioxidant activity tests, TBA was used to determine the efficacy of the compounds to protect liposomes from lipid peroxidation. In most of the membrane systems, peroxidation leads to the formation of free malonaldehyde (MDA), which reacts with TBA in the ratio of 1:2 to give a colored product that absorbs light at 532 nm in an acidic environment. This colored product can be measured and quantified spectrophotometrically and the intensity of color is a measure of MDA concentration. When an antioxidant compound takes part in the lipid peroxidation assay reaction, the color formation and absorbance reduces due to the reduction of the extent of peroxidation (25,26).

The antioxidant activities of the extracts of two varieties and standard oleuropein on liposomes obtained from TBA test are given in Table 1. By using Propyl gallate (IC<sub>50</sub>: 0.21 µg/ml) as a positive control for evaluating the obtained data, the extracts of the leaves of *O. europaea* var. *sylvestris* and *O. europaea* var. *europaea* were found to have high activities with IC<sub>50</sub> values of 0.29 µg/ml and 0.75 µg/ml respectively. On the other hand, the IC<sub>50</sub> values of the branch extracts of *O. europaea* var. *sylvestris* and *O. europaea* var. *europaea* were found as 3.41 µg/ml and 5.00 µg/ml respectively. These results show that the aqueous extracts of the leaves have higher activities than the branches for both of the varieties. The standart oleuropein was also found to have antioxidant activity when compared with propyl gallate. Briante et al. reported that *O. europaea* leaf extracts had an inhibition effect *ca* 20-30% longer than oleuropein (6). Our results are compatible with this result.

In our previous HPLC analysis, we found that the content of oleuropein which we used as an authentic sample in the activity tests is high in natural olive tree leaves (27). The result of the HPLC analysis supports the data that we found in the activity tests. As it can be seen from the DPPH-sprayed TLC plate, there are two more compounds besides oleuropein which may be responsible for the activity and this proves the strong antioxidant effect of olive leaf extracts. The antioxidant effect of *O. europaea* leaf extracts can be caused by other water-soluble phenolic components besides oleuropein. In conclusion, on the basis of the results of this investigation, *O. europaea* var. *europaea* and *O. europaea* var. *sylvestris* leaves could represent potential sources of natural antioxidants.

**Table 1.** Antioxidant activities of the tested lyophilized extracts and oleuropein.

Test Samples	IC <sub>50</sub> value (µg/ml)± SD
Leaves of <i>O. europaea</i> var. <i>europaea</i>	0.75 ± 0.04
Leaves of <i>O. europaea</i> var. <i>sylvestris</i>	0.29 ± 0.02
Branches of <i>O. europaea</i> var. <i>europaea</i>	5.00 ± 0.14
Branches of <i>O. europaea</i> var. <i>sylvestris</i>	3.41 ± 0.09
Oleuropein	2.73 ± 0.01
Propyl gallate	0.21 ± 0.01

SD= Standard Deviation; n= 4

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