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The effect of olive leaves and their harvest time on radical scavenging activity and oxidative stability of refined olive oil

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RESEARCH ARTICLE

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Abstract

The antioxidant role of olive leaves obtained from Adana Topagi, Adana Yerli, Domat and Gemlik cultivars during olive ripening were investigated and compared to commercial olive leaf extract (OLE) as well as butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) as synthetic antioxidants. The results showed that total phenol contents of OLEs reduced 44 to 63% during ripening. OLEs, irrespective of cultivar and harvest date were found to be good sources of radical scavengers ($P<0.05$). The radical scavenging activity of OLEs at 100 or 200 µg/g were higher than BHT ($P<0.05$) and similar or lower than BHA ($P\geq0.05$). The addition of Domat and Adana Topagi OLEs at 100 or 200 µg/g to refined olive oil increased oxidative stability and caused to extracts to show better activity than commercial OLE but lower or similar activity than that of BHT and BHA ($P\leq0.05$) during different harvest times. BHA and BHT were the most effective antioxidants employed to delay the bulk oil oxidation but Domat and Adana Topagi olive leaves may have food additive value to slow down the progress of lipid oxidation and increase oxidative stability in refined olive oil. Due to concerns regarding the safety and toxicity of synthetic antioxidants, olive leaves may prove useful as safe, natural, functional ingredients with health promoting properties to food industry. Enrichment of oils or oil containing foods with the extracts from olive leaves and therefore, improving quality and healthiness of the target oils suggests a future possible use of them as a natural antioxidant and as an ingredient at an industrial scale since they seem to be useful for lipid stabilisation.

Keywords: enrichment, DPPH, olive leaf, phenolic compounds, radical scavenging

1. Introduction

Lipid oxidation has been one of the main interests of the scientific community for centuries. The oil industry has to pay special attention to oxidation problems which may occur during processing and storage. In order to overcome the stability problems of oils and fats synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, tertiary-butyl hydroquinone have been used as food additives. Synthetic antioxidants are very effective, inexpensive and stable under usual processing and storage conditions but they have certain disadvantages, including possible toxicological effects (Bouaziz *et al.*, 2008; Kiritsakis *et al.*, 2009). Because of their toxic and carcinogenic effects, their use is being restricted. Thereby, interest in finding natural antioxidants, without undesirable side effects, has increased greatly (Barreira *et al.*, 2008; Bouaziz *et al.*, 2008). Researchers are continuously seeking those natural antioxidants that will sufficiently protect fats and oils from oxidation (Kiritsakis *et al.*, 2009).

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in Mediterranean countries, where they cover 8 million ha, accounting for almost 98% of the world crop. This demonstrates the great economic and social importance of this crop and the possible benefits to be derived from utilisation of any of its by products. Olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives) and they accumulate during pruning of the olive trees. Olive leaves are considered as a cheap raw material which can be used as useful source of high-added-value product (Boudhrioua *et al.*, 2009; Ferreira *et al.*, 2007). Exploitation

of antioxidant properties of leaf extracts concentrated on some applications such as oil supplementation (Bouaziz *et al.*, 2008, 2010; De Medina *et al.*, 2011; Farag *et al.*, 2003; Paiva-Martins *et al.*, 2007; Rafiee *et al.*, 2012) during storage or heating of refined olive oil, sunflower and soybean oil. The enrichment with 1 kg of olive leaves extract is sufficient to fortify 50-320 l of refined olive oil to a similar stability as a virgin olive oil (Paiva-Martins *et al.*, 2007). The enrichment of processed foods with phenolic compounds both protects against oxidation and also benefits human health (Bouaziz *et al.*, 2008; Kontogianni and Gerothanassis, 2012). Previous studies demonstrated the great potential of olive by-product extracts as antioxidants for the food industry and proved that olive leaf extracts (OLEs) are excellent antioxidants and may serve as substitutes for synthetic antioxidants (Kiritsakis *et al.*, 2009; Rafiee *et al.*, 2012; Suárez *et al.*, 2011). Olive-oil-producing countries have to consider seriously commercialisation of olive leaf in various forms (Papoti and Tsimidou, 2009a). OLE is a dark brown, bitter-tasting liquid derived from the leaves of the olive tree (*O. europeae L.*; *Oleaceae*). Like many natural products, variation due to differences such as georaphical location, plant nutrition and cultivar can influence the composition of the olive leaves extract (Sudjana *et al.*, 2009).

The present study was carried out to evaluate antioxidant role of olive leaves extracts obtained from cultivars of Adana Topagi, Adana Yerli, Domat and Gemlik during different harvest times by 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and by their activity in stabilizing refined olive oil against oxidative deterioration, and to compare their effectiveness to synthetic antioxidants, butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) as well as commercial OLE.

2. Materials and methods

Materials

Olive leaves from Adana Topagi, Adana Yerli, Domat and Gemlik were collected from olive collection of the University of Cukurova in Adana, Turkey from the 15th of September to the 15th of December 2009 (Table 1). The olive leaves were collected from each side of the olive trees and were put in refrigerator bags (two samples for each) and kept at cold room -25 °C until they were used. DPPH, Folin-Ciocalteu reagent were purchased from Sigma (Steinheim, Germany), BHT and BHA were purchased from Merck (Darmstadt, Germany, USA), while the commercial OLE was purchased from Turkey. All chemical reagents were of analytical grade purchased from either Sigma or Merck. Refined olive oil, a product of Taris was purchased from a local market.

Table 1. Harvest dates (HD) for olive leaves.

	Harvest date
HD1	15 September 2009
HD2	15 October 2009
HD3	16 November 2009
HD4	15 December 2009

Methods

The extraction of phenolic compounds from olives was performed according to the modified methods of (Jemai *et al.*, 2009; Kiritsakis *et al.*, 2009). Leaves were left to dry on shadow for 3 weeks. 200 g of dried leaves from each cultivar were mechanically milled and placed in flasks. A 1,200 ml quantity of ethanol (6:1, v/v) was added to each flask and left for 24 h at room temperature followed by filtration using Whatman® filters (47 mm × 0.45 µm; Whatman, Steinheim, Germany). The filtrates were evaporated in a rotary evaporator at 40-60 °C. The aqueous solution was used to evaluate the total phenols and antioxidant capacity of the hydrophilic fraction and kept at tinted glass bottles at -25 °C until it is used. The total phenol content (TPC) of the olive leaves were determined by a subsequent reaction with Folin-Ciocalteau reagent at 725 nm using UV-Vis spectrophotometer (Shimadzu 1200; Shimadzu, Kyoto, Japan) according to the method proposed by (Ferreira *et al.*, 2007) and expressed in mg/kg, as caffeic acid equivalents (Gutfinger, 1981).

The radical-scavenging activity of samples was evaluated by the DPPH assay. The free radical-scavenging activity (RSA) was measured, modified method of (Ferreira *et al.*, 2007). Briefly, an aliquot 100 µl of methanol extract from the studied olives and olive oils was added to 2.9 ml of DPPH solution (6×10^{-5} M in methanol) and the mixture was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 515 nm against a blank solution by using the Schimadzu, 1200 UV-Vis spectrophotometer. Triplicate measurements for each extract were made Radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula:

$$RSA = 100 \times (A_o - A)/A_o \quad (1)$$

Where A_0 is the absorbance of DPPH solution without any antioxidant, and A is the absorbance of DPPH solution after reaction with antioxidant at 515 nm.

The antioxidant activity of each test sample was expressed in terms of concentration required to inhibit 50% DPPH radical formation (EC_{50} mg/ml) and calculated from the log-dose inhibition curve. The scavenging activity OLEs were measured using concentrations ranging from 2 to 300 µg/g. The commercial OLE, BHT and BHA were tested at similar concentrations for comparison.

AOCS oven storage test for accelerated aging of oils (method Cg 5-97; 7) conducted at 60 °C in the dark can be used to evaluate oxidative stability of oils (Decker *et al.*, 2005). Refined olive oils (2×25 g) containing 100 and 200 µg/g olive leaves from four different olive cultivars, commercial OLE or BHT and BHA in a 50-ml beaker covered with aluminium foil were allowed to spontaneously oxidise in the dark at 60 °C in the oven. The temperature of 60 °C used simulates the real heating conditions (Nissiotis and Tasioula-Margari, 2002). The progress of oxidation of oil samples was monitored in terms of peroxide value (PV) and conjugated diene (CD) values according to AOCS official method Cd-8b and Ti 1a-6, respectively (Firestone, 1989).

The results were shown as mean values. The results were evaluated by analysis of variance (ANOVA) by using SPSS 13 for windows (SPSS Inc., Chicago, IL, USA). According to the results of the ANOVA test Duncan's multiple range test was used to determine the significance at $P<0.05$ levels.

3. Results and discussion

Total phenol content

Colour development using a Folin-Ciocalteu reagent (Folin-Ciocalteu assay) is the generally preferred method for measuring phenolics (Katsube *et al.*, 2004). Papoti and Tsimidou (2009b) recently tested total phenolic content of the olives and olive leaves by using Folin Ciocalteau assay. Table 2 shows TPC olive leaves from different cultivars during fruit ripening.

The total phenols content of olive leaves ranged between 0.28 and 0.74 g, while commercial OLE had 0.51 g caffeic acid/100 g dry matter of olive leaves. Later harvest time significantly reduced the TPC of olive leaves ($P<0.05$). This reduction was 44, 45 54 and 63% for Adana Topagi, Adana Yerli, Domat and Gemlik olive leaves, respectively. Due to this reduction early harvest even before september may be suggested for olive leaves to obtain even with higher TPC. Early harvest is also essential for olives to obtain them with high TPC with low oil yield but high oil quality. However, Domat olive leaves had the highest TPC during different harvest times ($P<0.05$) (Table 2). Therefore, cultivar is one factor that significantly affects the TPC of olive leaves. These results suggest that olive leaves, an agricultural waste, have great potential as a functional food ingredient, particularly as a source of phenolic compounds. Our results are in accordance with Sudjana *et al.* (2009) who reported that cultivar is one of the factor that can influence the composition of the olive leaves extract. Our results were also similar to Ortega-García and Peragón (2010) who found the TPC of olive leaves were decreased depending on fruit ripening. They found that TPC of olive leaves changed from 2.8 to 2.2, 3.7 to 2.8, 5 to 2.7 and 4 to 4.1 g caffeic acid per 100 g dry matter for Picual, Verdial, Arbequina and Frontio olive cultivars, respectively during fruit ripening from July to December. A wide range of total phenols were reported for olive leaves obtained from Magritiki, Kalamon, Koroneiki, Chemchali, Chemlali, Chetoi and Zarrazi olive cultivars ranging from 0.17 g caffeic acid/100 g fresh weight (Salta *et al.*, 2007), 0.28 to 14.4 g galic acid (Giao *et al.*, 2007), 0.88 to 2.6 g caffeic acid (Jimenez *et al.*, 2011) and 6.9 to 48.3 g (Kontogianni and Gerothanassis, 2012) per 100 g olive leaves. Our results were slightly lower than the values reported for olive leaves. However, Kiritsakis *et al.* (2009) found the phenol content of olive leaves as low as 0.047 and 0.088 g gallic acid per 100 g sample for Kalamon, Magritki and Koroneiki cultivars when they used dichloromethane as extraction solvent. Cultivar may have an important effect on TPC of olive leaves, and solvent used can also affect the concentration extracted.

Radical scavenging activity of olive leaves against DPPH

The RSA of OLEs was evaluated using a methanolic solution of the stable free radical, DPPH. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts over a short time scale (Ferreira *et al.*, 2007). Table 3 shows

Table 2. Total phenolic content (mean±SD) of extracts from olive leaves harvested at different times.

Cultivars	Total phenol contents (g caffeic acid/100 g dry matter)			
	HD1	HD2	HD3	HD4
Adana Topagi	0.63±0.00^{c}	0.53±0.01^{a}	0.35±0.00^{d}	0.28±0.01^{e}
Adana Yerli	0.66±0.00^{b}	0.53±0.01^{a}	0.34±0.01^{e}	0.30±0.01^{d}
Domat	0.74±0.01^{a}	0.48±0.01^{b}	0.48±0.00^{a}	0.40±0.01^{a}
Gemlik	0.51±0.00^{d}	0.36±0.00^{d}	0.35±0.00^{c}	0.32±0.00^{c}
Commercial extract	0.38±0.00^{e}	0.38±0.00^{c}	0.38±0.00^{b}	0.38±0.00^{b}

Significant differences in the same column are shown by different superscript letters ($P<0.05$).
HD = harvest date (Table 1).

Table 3. The EC_{50} values (mean±SD) of extracts from olive leaves harvested at different times.

Cultivars	EC_{50} (µg/ml)			
	HD1	HD2	HD3	HD4
Adana Topagi	0.25±0.00^{b}	0.23±0.00^{c}	0.25±0.00^{bc}	0.20±0.00^{c}
Adana Yerli	0.26±0.00^{b}	0.26±0.00^{bc}	0.24±0.00^{c}	0.19±0.00^{c}
Domat	0.20±0.00^{c}	0.22±0.00^{c}	0.26±0.00^{bc}	0.24±0.00^{b}
Gemlik	0.27±0.01^{b}	0.28±0.01^{b}	0.29±0.01^{b}	0.19±0.01^{c}
Commercial extract	0.11±0.00^{d}	0.11±0.00^{d}	0.11±0.00^{d}	0.11±0.00^{d}
Butylated hydroxyl toluene	0.69±0.03^{a}	0.69±0.03^{a}	0.69±0.03^{a}	0.69±0.03^{a}
Butylated hydroxyl aniline	0.28±0.00^{b}	0.28±0.00^{b}	0.28±0.00^{bc}	0.28±0.00^{b}

Significant differences in the same column are shown by different superscript letters (P<0.05).
HD = harvest date (Table 1).

radical scavenging capacity of olive leaves from different cultivars during fruit ripening.

The concentration of total phenols that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. EC_{50} is a parameter widely used to evaluate the antioxidant or radical-scavenging capacity. The lower EC_{50} shows that the antioxidant activity is higher (Achat *et al.*, 2012). According to EC_{50} values shown in Table 3 commercial extract had the highest radical scavenging activity than extracts from other olive leaves during fruit ripening, as well as BHT and BHA (P<0.05). The EC_{50} values of commercial extract, BHA and BHT were 0.11, 0.28 and 0.69 µg/ml, respectively. The OLEs from 4 different cultivars, regardless of the cultivar or harvest time in which the leaves had been picked, were able to interact with the stable free DPPH radicals efficiently and quickly EC 50 values ranged from 0.19 to 0.29 µg/ml during fruit ripening (Table 3). According to Table 3, OLEs from Adana Topagi, Adana Yerli, Domat and Gemlik were potently active and exhibited strong DPPH radical-scavenging ability similar to BHA (P≤0.05) and better than BHT (P<0.05). The higher DPPH radical scavenging activity; might probably due to the combined effect of the phenolic compounds and their high hydrogen atom donating abilities. Our results were in accordance with (Bahloul *et al.*, 2009; Bouaziz and Sayadi, 2005; Jimenez *et al.*, 2011; Kiritsakis *et al.*, 2009; Kontogianni and Gerothanassis, 2012). The EC_{50} values found in this research were lower (the higher antioxidant activity) than the OLEs reported by some other authors. Bouaziz *et al.* (2008) reported 1.25, 1.57 and 0.58 µg/ml and 0.87 the EC_{50} values for ethyl acetate, methanolic extract, hydroxytrsol and BHT. EC 50 values were found between 26 to 150 µg/ml for olive leaves from different olive cultivars including Chandorlia, Chandarolikis, Chemlali, Chemchlai, Chetoui and Zarrazi (Bahloul *et al.*, 2009; Jimenez *et al.*, 2011; Kontogianni and Gerothanassis, 2012).

The results of this study simply showed that olive leaf leave extracts from Adana Topağı, Adana Yerli, Domat and Gemlik showed higher radical scavenging activity than BHT (P≤0.05) and similar or lower activity than BHA (P≥0.05) during fruit ripening. Similarly, Benavente-Garcia *et al.* (2000) found that OLE showed similar or better 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) scavenging activity than hydroxtyrosol, caffeic acid and oleuropein, respectively (1.58 mM TEAC) as well as vitamin C and E. Lee and Lee (2010) found that a mixture prepared similar to OLE (58.1%) had similar DPPH scavenging activity to oleuropein (55.0%), rutin (57.0%) and better activity than vanillin (5.8%) at 50 mM (Ferreira *et al.*, 2007) found that methanolic extracts of olive leaves on DPPH radicals increased with the concentration increase and were comparable to the RSA values obtained for the standards BHA and α-tocopherol. Bouziz *et al.* (2010) found enzymatic hydrolysate of olive leaves had IC 50 values of 13.18 µg/ml where BHT had 7.94 µg/ml. Recently, Rafiee *et al.* (2012) found that methanol extract of Coranaiky olive leaf (86.81 µg/ml IC50 values) showed higher radical scavenging activity than BHA (89.05 µg/ml IC50) and can be used as a natural antioxidant replacement for BHA. Papoti and Tsimidou (2009a) suggested that olive leaf is a robust source of radical scavengers and flavonoids (Goulas *et al.*, 2010) regardless sampling parameters (olive cultivar, leaf age or sampling date).

Antioxidant activity of olive leaves in enriched bulk refined olive oil

Phenolic compounds increase the resistance of oil to storage and heating (Cerretani and Bendini, 2010) enrichment of refined olive oil with phenolics increased its oxidative stability (Artajo *et al.*, 2006; Bahloul *et al.*, 2009). Salta *et al.*(2007) found that oxidative stability of vegetable oils was increased when the oils were enriched with OLEs. Rafiee *et al.* (2012) found that methanolic extracts of olive leaf

obtained from Coronaiky variety can be used to increase oxidation stability in sunflower oil. The PV provides a measure of the degree of lipid oxidation and indicates the amount of oxidised hydroperoxides. Hydroperoxides are the primary products of auto-oxidation (De Abreu *et al.*, 2011). PV is used to measure the oxidation status of fats and oils (Rafiee *et al.*, 2012) (mainly as evidence of primary oxidation) after processing and storage (Rotondi and Magli, 2004). Table 4 shows the PV of oils either in the absence or presence of antioxidants stored at 60 °C for 21 days.

The initial PV of refined olive oil was 2.9 meq O_2/kg. After oxidation at 60 °C for 21 days the PV of control refined olive oils reached up to 374 meq O_2/kg compared to 20, 28 and 69 meqO_2/kg for BHT, BHA and commercial OLE. The results showed that enrichment of refined olive oil with 100 µg/g OLEs caused significant protection of refined olive oil and all olive leave extracts showed antioxidant activity as compared to control (P<0.05) regardless of harvest time and cultivar. However, Domat and Adana Topagi olive leaves showed better or similar antioxidant activity than 100 µg/g commercial OLE (P≥0.05). There was a slight decrease in the antioxidant activity of Domat OLE depending on harvest time (Table 4). The reason for this decrease in the antioxidant activity could be decrease in TPC of Domat OLEs depending on fruit ripening (Table 2). OLEs were less effective than BHT and similar or less effective than BHA during ripening (P≤0.05). The antioxidant activity of OLEs were also tested at 200 µg/g level as shown in Table 5.

The results showed that enrichment of refined olive oil with 200 µg/g OLEs caused significant protection of refined olive oil and all olive leave extracts were antioxidant and the activity was decreased depending on harvest time for Adana Yerli, Domat and Gemlik. The antioxidant activity of olive extracts from Adana Topağı, Domat and Gemlik were similar or better than commercial OLE (P≤0.05). OLEs were less effective than BHT (P<0.05) but the activity was similar or less than BHA during ripening for Adana Topagi OLE (P≤0.05). It was obvious from Table 4 and 5 that OLEs from Adana Topagi and Domat were more effective than other OLEs and commercial OLE depending on harvest time on reducing the formation of primary oxidation compounds during thermal oxidation of olive oil. This study also confirmed the strong relationship between TPC and oxidative stability measured by PV as stated before by (Morello *et al.*, 2005).

The oxidation of refined olive oils were also followed by measuring CD values during storage. The formation of CD, offers a good indication of the alterations that occur during the oxidative process, considering that OLEs, BHT and BHA present more stability, remaining in the heated oil. The CD of refined olive oil with OLEs, BHT and BHA during oxidation for 21 days at 60 °C is shown in Table 6 and Table 7.

The CD of the control increased from 0.20 to 0.69 during storage for 21 days. The CD of refined olive oil with commercial OLE, BHT, BHA during storage for 21 days increased to 0.57, 0.54 and 0.57, respectively. The CD of refined olive oils with OLEs from Adana Topagi and Adana Yerli at 2nd and 3rd harvest dates was lower or similar than those of the refined olive oils with BHT, BHA and Commercial extract during storage (P≤0.05). When different treatments are evaluated in each harvest time, it is possible to observe that only Domat OLE, commercial OLE the synthetic antioxidants BHT were effective in the protection against the formation of CD, differing from control (P≤0.05) at the end of the experiment after 21 days of heating at 60 °C. The antioxidant activity of Domat OLE at 100 and 200 µg/g was greater, differing significantly with

Table 4. Peroxide value (PV) (mean±SD) of refined olive oil samples enriched with 100 µg/g olive leaf extracts, commercial extract, butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) stored at 60 °C for 21 days.

Cultivars	PV (meq O_2/kg)			
	HD1	HD2	HD3	HD4
Control	373.5±2.8^{a}	373.5±2.8^{a}	373.5±2.8^{a}	373.5±2.8^{a}
Adana Topagi	40.6±1.3^{d}	36.4±1.1^{ef}	36.6±1.3^{d}	33.2±1.0^{e}
Adana Yerli	78.3±4.5^{b}	82.2±3.3^{c}	66.9±2.6^{b}	65.5±1.2^{c}
Domat	40.6±1.3^{d}	40.3±2.7^{e}	60.2±4.0^{bc}	53.7±3.3^{d}
Gemlik	47.3±0.9^{d}	102.8±5.6^{b}	57.2±1.1^{c}	75.1±0.9^{b}
Commercial extract	68.7±6.3^{c}	68.7±6.3^{d}	68.7±6.3^{b}	68.7±6.3^{bc}
BHT	19.9±1.1^{e}	19.9±1.1^{g}	19.9±1.1^{e}	19.9±1.1^{f}
BHA	28.4±1.0^{e}	28.4±1.0^{fg}	28.4±1.0^{de}	28.4±1.0^{e}

Significant differences in the same column are shown by different superscript letters (P<0.05).
HD = harvest date (Table 1).

Table 5. Peroxide value (PV) (mean±SD) of refined olive oil samples enriched with 200 µg/g olive leaf extracts, commercial extract, butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) stored at 60 °C for 21 days.

Cultivars	PV (meq O_2/kg)			
	HD1	HD2	HD3	HD4
Control	322.1±10.3^{a}	322.1±10.3^{a}	322.1±10.3^{a}	322.1±10.3^{a}
Adana Topagi	33.7±0.2^{d}	33.6±1.0^{d}	35.0±2.1^{c}	27.1±2.7^{e}
Adana Yerli	82.7±3.4^{b}	67.5±6.0^{b}	59.6±1.4^{b}	100.0±5.3^{b}
Domat	37.3±1.1^{d}	52.2±3.1^{c}	53.5±2.5^{b}	43.5±4.9^{d}
Gemlik	34.8±1.4^{d}	53.5±1.5^{bc}	54.7±0.8^{b}	47.0±0.7^{d}
Commercial extract	64.0±3.8^{c}	64.0±3.8^{c}	64.0±3.8^{c}	64.0±3.8^{c}
BHT	20.0±1.7^{e}	20.0±1.7^{d}	20.0±1.7^{d}	20.0±1.7^{e}
BHA	29.0±1.0^{e}	29.0±1.0^{d}	29.0±1.0^{cd}	29.0±1.0^{e}

Significant differences in the same column are shown by different superscript letters (P<0.05).
HD = harvest date (Table 1).

Table 6. Conjugated diene values (mean±SD) of refined olive oil samples enriched with 100 µg/g olive leaf extracts, commercial extract, butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) stored at 60 °C for 21 days.

Cultivars	Conjugated dienes (%)			
	HD1	HD2	HD3	HD4
Control	0.69±0.02^{b}	0.69±0.02^{c}	0.69±0.02^{b}	0.69±0.02^{c}
Adana Topagi	0.71±0.03^{b}	0.58±0.02^{d}	0.59±0.02^{c}	0.68±0.02^{c}
Adana Yerli	0.94±0.05^{a}	0.91±0.01^{a}	0.50±0.01^{d}	1.00±0.02^{a}
Domat	0.52±0.03^{c}	0.53±0.05^{d}	0.40±0.01^{e}	0.32±0.01^{e}
Gemlik	0.89±0.04^{a}	0.81±0.06^{b}	0.85±0.02^{a}	0.86±0.01^{b}
Commercial extract	0.58±0.04^{c}	0.58±0.04^{d}	0.58±0.04^{c}	0.58±0.04^{d}
BHT	0.54±0.04^{c}	0.54±0.04^{d}	0.54±0.04^{cd}	0.54±0.04^{d}
BHA	0.57±0.03^{c}	0.57±0.03^{d}	0.57±0.03^{c}	0.57±0.03^{d}

Significant differences in the same column are shown by different superscript letters (P<0.05).
HD = harvest date (Table 1).

later harvest date from the other OLEs (P≤0.05) and similar or better than BHT and BHA (P≤0.05) in refined olive oil.

At the end of 21 days of the heating process, the treatments that indicated the best protection of refined olive was obtained with the one containing Domat OLE added at 100 and 200 µg/g by reducing formation of CD 50 to 54% where 22, 17 and 16% reduction was obtained with BHT, BHA and commercial OLEs, respectively at 4th harvest date.

The results found in this study in accordance with previous research is quite important finding since there are some health concern about the potent antioxidants such as BHT and BHA and olive leaves can be very important source of phenolics showing comparable or lower activity than BHT and BHA. Our findings are in accordance with the results of studies which showed that the enrichment of oils with phenolics from different source increased oxidative stability of oils including olive, sunflower, corn and canola oils and butter (Orozco-Solano *et al.*, 2011; Rafiee *et al.*, 2012; Roussis *et al.*, 2008). Extracts were more effective than BHT and BHA (De Abreu *et al.*, 2011; Hayes *et al.*, 2010; Lambropoulos and Roussis, 2007; Soulti and Roussis, 2007) during storage and cooking.

Table 7. Conjugated diene values (mean±SD) of refined olive oil samples enriched with 200 µg/g OLEs, commercial extract, butylated hydroxyl toluene (BHT) and butylated hydroxyl aniline (BHA) stored at 60 °C for 21 days.

Cultivars	Conjugated dienes (%)			
	HD1	HD2	HD3	HD4
Control	0.69±0.02^{bc}	0.69±0.02^{b}	0.69±0.02^{a}	0.69±0.02^{c}
Adana Topagi	0.64±0.02^{c}	0.61±0.01^{c}	0.69±0.06^{a}	0.58±0.02^{d}
Adana Yerli	0.95±0.05^{a}	0.48±0.01^{de}	0.57±0.03^{bc}	0.82±0.01^{a}
Domat	0.47±0.03^{d}	0.35±0.02^{f}	0.46±0.02^{d}	0.34±0.02^{f}
Gemlik	0.73±0.02^{b}	0.90±0.01^{a}	0.72±0.03^{a}	0.75±0.04^{b}
Commercial extract	0.51±0.02^{d}	0.51±0.02^{d}	0.51±0.02c^{d}	0.51±0.02^{e}
BHT	0.47±0.01^{d}	0.47±0.01^{e}	0.47±0.01^{d}	0.47±0.01^{e}
BHA	0.63±0.01^{c}	0.63±0.01^{c}	0.63±0.01^{ab}	0.63±0.01^{cd}

Significant differences in the same column are shown by different superscript letters (P<0.05).
HD = harvest date (Table 1).

4. Conclusions

All OLEs had significant amount of phenols, although it decreased significantly, Domat had the highest TPC during different harvest time. OLEs from Adana Topagi, Adana Yerli, Domat and Gemlik were affective as radical scavenger regardless of harvest date and cultivar, their effect on protection of olive oil against oxidation was closely related to cultivar and harvest date. Their activity was similar or better than than BHA and BHT as DPPH radical scavenger, as oil protection was considered only Domat and Adana Topagi were effective on the formation of hydroperoxides and Domat was effective on the formation of CD and the activity of these olive extracts was lower or similar than BHT and BHA. This could be explained by the fact that their radical scavenging activity, the hydrogen-donating ability of antioxidants in a solvent model does not necessarily indicate their antioxidant activity in a lipid environment. It seems like the radical scavenging properties of Adana Topagi and Domat OLE exhibit a higher correlation with the inhibition of bulk lipid oxidation compared to Adana Yerli and Gemlik OLE which did not show any reduction on the formation of hydroperoxides or CD. Adana Topagi and Adana Yerli olive leaves were effective at some harvest time and they can be considered as a potential antioxidant source of natural origin. It was concluded extracts from Domat may have a food additive value due to its high phenolic content can serve as substitutes for synthetic antioxidants as safe, natural, effective and functional ingredients with health promoting properties may be used to enhance the stability and nutritional quality of oils. The antioxidant activity which renders Domat OLEs useful for the enhancement of the oxidative stability of edible oils and it may be useful for preventing radical-related food deterioration.

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