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Determination of phenolic profile of Gemlik olive cultivar

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The effect of variation of harvest time and location on the total phenolic content, antiradical scavenging activity and phenolic compounds composition of leaves of Gemlik olive cultivar obtained from two different location (Gemlik and Mudanya in Marmara Region of Turkey) were investigated. It was determined that the highest total phenolic content (373.36 and 336.70) were observed in April for both region. Among the olive leave samples the highest (93.69%) antioxidant activities were determined in olive leaves grown in Mudanya and harvested in December and (93.86%) in either olive leaves grown in Gemlik and harvested in April. Determination of phenolic compounds in olive leaves was carried out by LC-DAD-ESI-MS/MS. The results of analysis phenolic compounds showed that oleuropein content ranged from 94.21 mg/kg to 860.21 mg/kg in olive leaves. Differences were observed in the concentration of phenolic compounds depending on the harvest time and location in all olive leaf samples.

Keywords: Antioxidant activity, LC-DAD-ESI-MS/MS, Oleuropein, Phenolic compounds

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Olive tree (*Olea europaea* L.), is the most characteristic and drought-tolerant plant species in Mediterranean area. Olive leaves obtained from both harvesting and cleaning of olives and pruning of olive trees[1]. Olive leaves represent about 10% of the weight of olives collected for olive oil extraction and 25% of the total weight of by-products after olive tree pruning[2,3]. Olive leaves have widespread phenolic compounds and also contain phenolic compounds belonging to the secoiridoids family such as oleuropein[4]. Bioactive phenolic compounds present in olive leaves waken interest of researchers around the world[5]. Olive leaves were reported to have biological properties such as antioxidant, hepatoprotective[6], cardioprotective[7], antihypertensive[8], antiproliferative[9], antiplatelet[10], antimicrobial[11], antidiabetic[12] and antiinflammatory[13]. The leaves of the olive plant (*Olea europaea*) are rich in antioxidants and the bioactive compounds present in olive leaves are used as natural additives in the food industry[14]. For instance, olive leaves extracts are used as replacement of synthetic additives and the shelf life extension of crackers[15], salmon burgers[16], baked snacks[17], fresh pasteurized milk[18] and ready-to-use vegetable pate[19].

The phenolic compounds of olive leaves is affected depending on several conditions such as, climatic conditions, origin and different (biotic and biotic stress factors)[20], Several studies have been performed for determination of the effect of harvest time on phenolic composition of olive leaf obtained from Italian cultivars[21], Portuguese cultivars[22], Tunisian cultivars[23], Syrian cultivars[24], Greek cultivars[25], Spanish cultivars[26]. Moreover, fewer data reports focused on the impact of harvest time and location on the phenolic composition of olive leaves of Gemlik cultivar. The objective of this study was to investigate the effect of the harvest time and location on the phenolic composition of olive leaves of Gemlik cultivar harvested from fruit setting until the end of harvest period (April-December). Also this study aims to exhibit the convenient period of the year to harvest olive leaves of Gemlik cultivar.

Materials and Methods

Plant material

Olive leaves from Gemlik cultivar were harvested from the olive garden located in Mudanya and Gemlik provinces in Bursa. The selected cultivar was the most widespread cultivar in Marmara region. Olive leaves (approximately 100 g) were harvested by hand. The

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harvested leaf materials were kept in a freezer (-18°C). The leaves were collected during April (1), May (2), June (3), July (4), August (5), September (6), October (7), November (8) and December (9).

Chemicals and reagents

HPLC grade methanol and acetic acid were obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent and DPPH were purchased from Sigma-Aldrich. The following reagents were used as reference compounds for the HPLC: caffeic acid, p-coumaric acid were obtained from Sigma Aldrich hydroxytyrosol, tyrosol, oleuropein obtained from Extrasynthese Co. (Genay France). All reagents used had purity of analytical grade. Distilled water was used throughout the study.

Extraction of phenolic compounds

Olive leaf extraction was carried out as explained by *Morsy et al.*[27] after slight modification. 1.5 g leaf samples was vortexed with 3 x 5 mL of methanol/water (80/20) for 1 min and followed by 15 min centrifugation. After that supertanant filtered through a Whatman No.1 filter paper and evaporated under nitrogen gas until methanolic phase was completely removed. Then, the residues were dissolved in 4 mL methanol and kept at 4°C until used for analysis.

Determination of total phenolic content

Total phenolic content was determined according to the Folin-Ciocalteu colorimetric method[28]. The method is based on the colour reaction of the Folin-Ciocalteu reagent with the hydroxyl groups. In 1 mL of the diluted extract (1:10 v/v), 0.5 mL Folin-Ciocalteu reagent was added. The mixture was vortexed and allowed to stand for 3 min. 1 mL of 35% (w/v) sodium carbonate was added and the volume was completed to 10 mL. The mixture was vortexed and incubation for 90 min at 25°C for colour development. After time their absorbance was measured at 725 nm (Varian Cary 500 UV-Visible Spectrophotometer). Distillated water was used as a blank. Total phenolic content in the leaf samples expressed as mg/100 g gallic acid equivalent (GAE). The experiments were performed in triplicate.

Determination of antioxidant activity (DPPH radical scavenging assay)

DPPH radical scavenging assay is carried out according to the DPPH method described by Boskou *et al.*[29] with minör modifications (0.1 mL) diluted extract was added to 3.9 mL of 0,06 mM DPPH (2,2-difenil-1-pikrilhidrazil). methanol was used as control. The samples were vortexed and placed in the dark for 30 min. The absorbance (A) was measured at 517 nm using spectrophotometer. The inhibition percentage of DPPH was calculated using the formula was calculated using the formula $(A_0 - A_1)/A_0 \times 100$, where A_0 was the control absorbance and A_1 was the sample absorbance mixed with DPPH solution.

Separation, identification and quantification of phenolic compounds

An Agilent 1100 High-Performance Liquid Chromatography (HPLC) system was used for the chromatographic determination. The isolation of phenolic compounds was carried out on 150 mm × 4,6 mm, particle size 5 µm Agilent Zorbax Eclipse XDB-C18 reversed phase column maintained at 25°C DAD signals for every analyte were selected according to their spectrums obtained from Agilent ChemStation Software. Appropriate wavelengths were selected as: 240 nm for oleuropein and 280 nm for hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid. The identification each compound was performed by comparing retention times to authentic standards. The mobile phase consisted of 0.2% acetic acid (solvent A) and methanol with (solvent B). The flow rate was 0.4 mL min-1. The solvent gradient changed according to the following conditions: 0 min, 5% B, 1 min, 5% B, 8 min, 90% B, 10 min, 90% B, 12 min, 5% B, 15 min 5% B. Samples were filtered through a 0.45 µm membrane filter before injection. Samples were injected into the system as 10 µL. Peaks were identified by retention times compared with standards.

Mass spectrometric (MS/MS) analyses were performed on an Agilent Technologies 1100 series LC-MSD Trap with a electrospray ionization (ESI) source and operated in negative ion mode. Nitrogen was used as collision gas. Full scan mass spectra were recorded 50-1000 m/z. The nebulizer gas pressure (N_2):45 psi, the drying gas flow (N_2):12 L/min at 325°C, capillary voltage: 3500 V and MRM mode. Data analysis is performed using MassHunter software (Agilent). Data were expressed as mg of phenols per kg of fresh weight (FW).

The validation characteristics evaluated were linearity, accuracy, limits of detection (LOD) and quantification (LOQ) and precision**.** The pure standards of hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, oleuropein were prepared in methanol at a final concentration of 1000 µg/mL. Standard

solutions of for the calibration were prepared at concentrations of 1, 2, 5, 10, 25, 50 and 100 µg/mL. The calibration working solutions for each compound were prepared triplicate and evaluated by injecting 5 µL. Calibration curves were constructed by plotting peak areas against corresponding concentrations. The accuracy was determined through the percent recovery with addition of the standard solution to the olive leaves extract. Recovery was estimated using the following formulae: Recovery (%) = ([recovered amount - original amount/spiked amount]) × 100.

Statistical analyses

All data presented as mean values of three analysis and standard deviation. MINITAB 16 (Minitab Inc., State College, PA, USA) were used for statistical analysis of data. Tukey post hoc test was applied for the groups turning out significant, and 5% (*i.e.,* $p<0.05$) was accepted statistically significant.

Results and Discussion

Total phenolic content

Figure 1 shows the total phenolic content of all samples. The mean values of total phenolic content in olive leaf extracts ranged between 154.11 mg GAE/g (August) and 336.70 mg GAE/100 g (April) for Mudanya; 180.22 mg GAE/100 g (August) and 373.36 mg GAE/g (April) for Gemlik. Total phenolic content decreased from April to August ($p<0.05$) and then increased from August to December ($p<0.05$). The highest total phenolic content of olive leaves were determined in Mudanya (336.70 mg GAE/100 g) and Gemlik (373.36 mg GAE/100 g) harvested in April, while the lowest total phenolic content of olive leaves were determined in Mudanya (154.11 mg GAE/100 g) and Gemlik (180.22 mg GAE/100 g) harvested in August.

Total phenolic content of olive leaves showed high variability. In April, the total phenolic content of olive leaf samples was higher ($p<0.05$) with respect to other samples which have harvested different time. Hagidimitriou *et al.*[25] determined the total phenolic content of olive leaves collected in Greece at different harvest times. They exhibited that olive leaves harvested in April had higher total phenolic content compared to those collected in other harvest times. Dogancay & Cetınkaya[30] reported that Gemlik olive leaves harvested in August had lower total phenolic content compared to those collected in other months. In other study, the total phenolic content in the leaves of Turkish olive tree cultivar "Kilis Yaglik" was assessed monthly from January to December Cetinkaya & Kulak[31]. The authors reported that the content of phenols reached the lowest level in summer (August) and the highest level in April; these results are in agreement with ours. It was reported by Abdeljelil *et al.*[2] that the decrease of the total phenolic content in summer was associated with an increase of the activity of polyphenol oxidases, enzymes that catalyze the oxidation of phenols to quinones and are involved in the resistance against biotic and abiotic threats in plants. Probably, the decrease of the contents of total phenolics may have been occurred in the present study in ''Gemlik" olive leaves in August due to the increment of the polyphenol oxidase activity, which is consistent with the finding described by Abdeljelil *et al.*[2].

Antioxidant activity

Antioxidant activity of olive leaf extracts was analyzed by DPPH method. The results are shown in Figure 2 shows the antioxidant activity of all samples. The mean antioxidant activity values of olive leaf extracts ranged from 91.45-93.86%. The highest antioxidant activities of olive leaves were determined

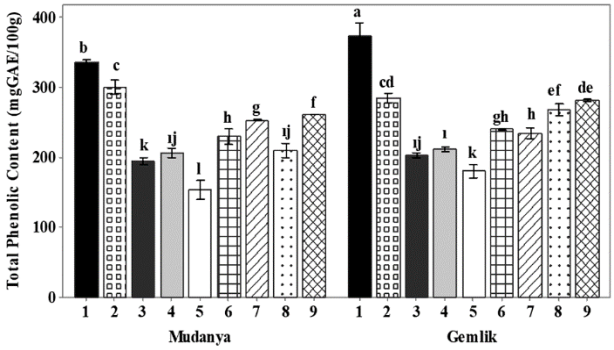

Fig. 1 — Total phenolic content (mg GAE/100 g, mean values) of olive leaves

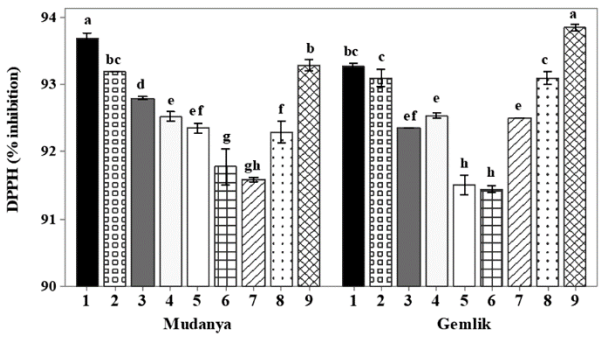

Fig. 2 — Antioxidant activity (% inhibition, mean values) of olive leaves

in Mudanya (93.69%) harvested in April and in Gemlik (93.89%) harvested in December, while the lowest antioxidant activities of olive leaves were determined in Mudanya (91.59%) harvested in October and in either Gemlik (91.45%) harvested in September.

Olive leaf extracts generally exhibited high antioxidant activity and influenced by several factors, among which harvesting period and cultivar[32]. Blasi *et al.*[21] who determined that the antioxidant activity of olive leaves obtained from Italian cultivars was the highest in March. Figure 2 shows the same trend for Mudanya location, antioxidant activity was the highest in March, while it decreased ($p<0.05$) until September, which is consistent with the finding described by Blasi *et al.*[21]. Olive leaves of Gemlik cultivar which analyzed in our study had quite high antioxidant activity values (Fig. 2). These observations were consistent with the results described by Khalıq *et al.*[33] who determined the antioxidant activity of leaves of eight cultivars in Pakistan and identified that Gemlik cultivar showed the highest antioxidant activity compared to other cultivars.

HPLC-DAD-ESI-MS/MS Analysis

Identification of phenolic compounds in olive leaves

The qualitative and quantitative determination of phenolic compounds was performed in olive leaves by HPLC-DAD-ESI-MS/MS. The phenolic compounds were identified by comparing the retention time with authentic standard in HPLC-DAD analysis. HPLC-DAD separation of the phenolic compounds of standard mixture is shown in Figure 3. For the isolation of phenolic compounds accurate molecular mass and formula, acquired by LC–ESI/MS/MS analysis and fragment ions were compared with those of authentic standards. Table 1 lists the phenolic compounds. Main and fragment ions of the determined compounds show similarity with other studies[34,35].

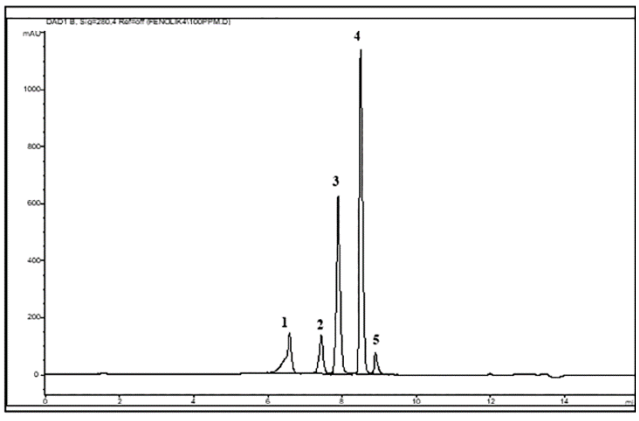

Fig. 3 — Obtained chromatogram of the 100 ppm standard mixture at 280 nm wavelength using the HPLC-DAD method

Quantification of phenolic compounds in olive leaves and method validation

Quantification was performed using HPLC-DAD data. Five standard calibration curves for the quantification of the phenolic compounds of olive leaves were prepared using five chemical standards. Chromatograms were acquired at two different wavelengths (240, 280 nm) according to absorption maxima of analyzed compound. Hydroxytyrosol, tyrosol, caffeic acid and p-coumaric acid were acquired at λ=280 nm; and oleuropein was acquired at λ=240 nm. The calibration plots indicated good correlations and regression values were higher than 0.990 in all cases and recovery values of five phenolic compounds were higher than 90%. The different analytical parameters of the HPLC-DAD method are summarized in Table 2.

The concentration of phenolic compounds of olive leaves of Gemlik cultivar expressed in mg/kg are given in Table 3. Among the five compounds detected by LC-MS/MS, the main phenolic compound in olive leaves and they were detected in olive leaves from all of the months for each location. The oleuropein content varied between 114.26 mg/kg (June) and

Table 1 — Phenolic compounds identified in olive leaf extract obtained by HPLC-DAD-ESI- MS/MS including: Retention time (RT), Molecular Formula (MF), Molecular Weight (MW), Molecular ion [M-H]- and Main fragments via MS/MS.

Phenolic compound	RT	MF	MW (m/z)	$[M-H]^-$ (m/z)	Fragment ions
Hydroxytyrosol	6.0	$C_8H_{10}0_3$	154	153	123
Tyrosol	7.0	$C_8H_{10}0_2$	138	137	-
Caffeic acid	7.8	$C_9H_8O_4$	180	179	135
p-coumaric acid	8.4	C_9H_8O	164	163	119
Oleuropein	9.0	$C_{25}H_{32}O_{13}$	540	539	275, 307, 377

Table 2 — Analytical parameters of the HPLC-DAD method

Phenolic compound	Regression equation	r^2	LOD µg/mL	LOQ µg/mL	Recovery (%)
Hydroxy tyrosol	Y=17.39x-61.86	0.9980	0.10	0.34	96.1
Tyrosol	Y=10.88x-12.07	0.9997	0.31	1.04	94.3
Caffeic acid	Y=32.25x-32.28	0.9995	0.22	0.75	91.9
p-coumaric acid	Y=86.11x-66.88	0.9995	0.30	0.99	94.8
Oleuropein	Y=22.90x-27.27	0.9993	0.31	1.04	98.7

Table 3 — Phenolic composition of olive leaves of Gemlik cultivar (results are presented as mean values ± SD expressed as mg /kg)

Location	Months	Hydroxytyrosol	Tyrosol	Caffeic acid	p-coumaric acid	Oleuropein
Mudanya	April	147.21±0.02^{b}	6.97±0.32^{g}	4.79±0.21^{e}	5.04±0.11^{d}	837.63±6.98^{a}
	May	52.20±0.99^{e}	19.40±0.80^{b}	nd	8.34±0.06^{b}	194.03±4.10^{fg}
	June	30.13±0.80^{j}	17.27±1.00^{c}	nd	3.92±0.06^{f}	114.26±4.27^{h}
	July	20.01±0.64^{k}	7.61±0.43^{g}	10.29±0.22^{d}	nd	208.32±0.39^{efg}
	August	36.35±1.00^{hı}	nd	nd	nd	213.16±2.19^{ef}
	September	19.38±1.42^{k}	nd	nd	3.78±0.04^{fg}	193.58±1.93^{fg}
	October	33.11±0.61^{j}	nd	14.20±0.63^{a}	4.80±0.29^{de}	188.00±1.06^{fg}
	November	30.06±0.40^{j}	nd	14.94±0.80^{a}	3.21±0.15^{ı}	341.61±17.01^{cd}
	December	35.13±1.79^{hı}	12.47±0.86^{e}	14.59±0.29^{ab}	8.47±0.40^{b}	338.20±5.67^{cd}
Gemlik	April	178.37±0.05^{a}	14.30±0.19^{d}	11.30±0.99^{cd}	5.38±0.33^{c}	860.21±29.01^{a}
	May	56.80±0.92^{d}	9.43±0.35^{f}	10.53±0.10^{d}	4.59±0.07^{e}	229,47±4,39^{e}
	June	43.00±0.20^{g}	21.18±0.17^{a}	nd	3.33±0.12^{hı}	94.21±5.41^{h}
	July	33.58±0.53^{ı}	22.27±0.22^{a}	13.00±0.12^{bc}	nd	208.32±0.39^{ef}
	August	46.84±0.75^{f}	nd	nd	nd	315.69±13.80^{d}
	September	42.92±0.64^{g}	nd	nd	3.56±0.08^{gh}	198.19±13.03^{fg}
	October	13.65±0.30^{ı}	nd	11.86±0.46^{cd}	3.97±0.08^{f}	174.03±6.38^{g}
	November	37.25±0.87^{h}	nd	12.35±0.63^{bc}	3.66±0.51^{fgh}	375.98±4.52^{b}
	December	66.84±3.07^{c}	11.60± 0.32^{e}	14.07±0.40^{ab}	11.98±0.21^{a}	358.15±9.38^{bc}

*The different letters in the same column show statistically significant differences according to the Tukey test ($p<0.05$)

837.63 mg/kg (April) for Mudanya and 94.21 mg/kg (June) and 860.21 mg/kg (April) for Gemlik). The highest oleuropein content in olive leaves were determined in Gemlik (860.21 mg/kg) harvested in April while the lowest oleuropein content of olive leaves was determined in Gemlik (94.21 mg/kg) harvested in June. A previous report conducted on olive leaves of seven Syrian cultivar, showed that the concentration of oleuropein in spring olive leaves samples was higher than that determined in fall samples[24]. Abdeljelil *et al*.[2] observed that the oleuropein content reached a maximum peak in April. In other study, the oleuropein content in the leaves of Australian olive tree cultivar "Hardy's Mammoth" was investigated during physiological development of the olive (1999-2000 season). The authors report that oleuropein content reached a maximum in April in old and young olive leaves[35] these results are in agreement with ours. Nevertheless, other studies report different trends of the oleuropein content in olive leaves. It was reported that oleuropein content in olive leaves belonging to the Spanish cultivars "Picual" and "Arbequina" reached a maximum peak in November and a minimum level in April[26]. The main finding from all these reports is that oleuropein is affected not only by harvest time, but also other abiotic and biotic stress factors. In this study, the highest oleuropein content and antioxidant activity was detected in April in both locations.

Hydroxytyrosol has been widely described as one of the main components of simple phenols in olive leaves[5,36]. The second most abundant phenolic compound observed after oleuropein was hydroxytyrosol (Table 3). The hyroxytyrosol contents varied between 19.38 mg/kg (September) and 147.21 mg/kg (April) for Mudanya and 13.65 mg/kg (October) and 178.37 mg/kg (April). Brahmi *et al*. (2013) reported lower values of hydroxytyrosol content for "Chemlali" "Neb Jmel" olive cultivar. Blasi *et al.*[21] studied hydroxytyrosol content of olive leaves collected from different four Italıan cultivar variety and they reported that the values ranged from 1.5 mg/g to 7.0 mg/g of. In other study, the phenolic profile in the leaves of Tunisian olive tree cultivar "Chemlali" was investigated Abaza *et al*.[36] The authors report that the hyrdoxytrosol content varied between 0.18 mg/g dry matter for aged leaves and 0.39 for young leaves. İn this study Hydroxytyrosol content for the olive leaves from Mudanya and Gemlik location are lower than those determined by Blasi *et al*.[21] and Abaza *et al*.[36]

Conclusions

In the present study, it has been shown that there are significant differences in antioxidant activity and total phenolic content of olive leaves extracts of Gemlik cultivar which has started to dominate most of the olive growing regions of Turkey, depending on harvest time and location. The highest antioxidant activity of olive leaves extract was obtained from Mudanya in April and from Gemlik in December. Moreover the highest contents of oleuropein and total

phenolics have been detected in harvested olive leaves in April both location. April and Deceember seem to be a good time to collect leaves as a source of phenolic compounds.

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Conflict of Interest

The authors declare that they have no conflict of interest in the study undertaken.

Author's Contributions

VU is the coordinating researcher. VU, EY contributed in designing and conduction of research. EY performed the experiments. EY calculated, analysed and interpreted data. EY wrote and revised the manuscript. All authors read and approved the final manuscript.

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