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Chemical compounds, antioxidant properties, and antimicrobial activity of olive leaves derived volatile oil in West Anatolia

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Abstract: In this article, the chemical compounds, antimicrobial and antioxidant activity of the volatile oil from leaves of *Olea europaea* L. cultivar from Turkey (Ayvalık) has been studied. The essential oil was provided with a Clevenger apparatus and analyzed by GC-MS/FID. This analysis leads to the detection of 42 compounds representing 99.59±1.15% of the total oil. The major constituents were a-pinene (9.82±0.33%), benzylalcohol (8.83±0.27%), phenethylalcohol (8.52±0.25%), 2-monopalmitin (8.13±0.28%), palmitic acid (5.53±0.41%), octadecanoic acid 2,3-dihydroxypropylester (5.84±0.42%), phytol (4.22±0.17%), and benzaldehyde (4.21±0.38%). The antimicrobial activities of the dried leaves essential oils were assessed against seven bacterial and four fungal strains. Significantly, the essential oil has an efficient antibacterial activity toward to the bacterial strains such as *Bacillus cereus* ATCC 14579, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212 and *Klebsiella pneumonia*e ATCC 13883. The olive a leaf essential oils showed significant antimicrobial and antioxidant effects. This study gives more knowledge for the development of this crucial therapeutic plant.

Keywords: *Olea europaea L. Leaves volatile oils, GC-MS/FID, Chemical composition, Antimicrobial activity, Antioxidant activity*

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INTRODUCTION

Olea europaea L. is one of the most important fruit trees in Mediterranean countries, especially in Spain, Italy, France, Greece, Turkey, Tunisia, and Morocco (1). In Turkey, Ayvalık region of the Balıkesir province has the most plentiful olive variety representing more than 19% of in Turkey's whole olive trees.

Olive leaf is the origin of many phytochemicals such as polyphenolics and flavonoids, which accomplish many antioxidant, antibacterial, antifungal, and anti-inflammatory activity (2-10). In folk medicine, it is reported that the decoction prepared from the leaves is used for hypertension, arrhythmia, intestinal muscle spasms, and cancer treatment (11-14). In Turkey, congenital coronary artery anomalies are commonly seen at the rate of 0.2-1.2%. Coronary artery anomalies such as angina, significant hemodynamic abnormalities and myocardial infarction are essential because of the occurrence and sudden death (15).

Essential oils possess specific volatile odors or flavors obtained from various plant parts like

flowers, seeds, and leaves. Essential oils with various chemical components such as phenols, flavones, flavonoids, and terpenes, etc. show antibacterial, anti-cancer, antifungal, and anti-oxidant activities (16).

There are very few studies on the extract obtained by hydrodistillation of *Olea europaea* L. leaves and its essential oil content, antioxidant and antimicrobial effect in the literature. This study's primary purpose was to identify the constituents of *Olea europaea* L. essential oil by GC/MS spectrometric method and investigate the chemical characterization, antioxidant, and antimicrobial activities of the volatile oil from Ayvalık flora of Turkey.

EXPERIMENTAL SECTION

Plant material

Fresh leaves of *Olea europaea* L. cv. were picked up in October 2017 from Ayvalık (Turkey), a region in Balıkesir Province on the Aegean Sea coast located at 39°16′40.55N and 26°42′47.77E. The Mediterranean climate is dominant in this region, characterized by hot and dry summers and mild and rainy winters. The mean temperature is between 24-34 °C. West winds from the weather cool the region. The average annual rainfall is about 700 mm. The altitude of the region is 270 m.

The voucher specimen was described and stocked at Herbarium Turcicum, Ankara Herbarium Voucher No: 60542 (Department of Biology, Ankara University, Ankara, Turkey). Part of the leaves was washed, cleaned, and shade-dried at room temperature without an airflow (25 °C) for 15 days. After drying, olive leaf samples were milled and became ready for hydrodistillation.

Isolation of the volatile oils

The volatile oils were obtained by hydrodistillation in a Clevenger-type apparatus from *Olea europaea* L. leaves. To get a colorful oil with a yield of 0.06% (w/w), each dried sample (200 g) consisted of the leaves of upper branch parts of the plants were exposed to water (500 mL) distillation in a Clevenger apparatus for 3.5 h. The gained volatile oil was dried over anhydrous Na_2SO_4, then filtered, evaporated, and concentrated under a gentle stream of N_2 (nitrogen gas) and stored at 4 °C until analyzed.

Volatile oil analysis

GC analyses were made with a Shimadzu (Kyoto, Japan) GC17B instrument equipped with TC-5 capillary column (50 m×0.25 mm, film thickness 0.25 µm). The working conditions: oven temperature program consisted of a 10-min hold at 60 °C, followed by a 5 °C/min rise to 220 °C. The injector and FID detector temperature were maintained at 250 °C. The detector carrier gas was nitrogen (2 mL/min), FID split ratio was 1:25, and injection volume was 1 µL. The identification of the components was made by comparing their retention times with those of pure authentic samples. Simultaneously, linear retention indices (LRI) according to n-alkane series were also evaluated for component identification (1,17). Relative amounts of the individual components were calculated based on GC peak areas with FID response factor correction. The oil samples were analyzed by direct, splitless injection.

A Shimadzu (Kyoto, Japan) GC/MS QP2010 apparatus via the capillary column (TC-5/MS; 50 m x 0.25 mm i.d. film thickness 0.25 µm) equipped with electron ionization quadrupole detector (m/z 35-650) was used to determine the chemical composition of the samples. 1.2 mL/min flow rate for the carrier helium gas, 240 °C for injection heat, and 290 °C temperatures for the MS transfer line was selected. The column temperature was initially set at 50 °C and held for 3 min., then increased to 280 °C at the rate of 3 °C/min. and fit for 5 min at that temperature. Diluted samples (1:15 [v/v], in acetone) of 1.0 µL amounts were injected in a splitless manner.

The constituents were identified based on comparing their mass spectra with those of NBS75K, Wiley 7, NIST MS 2.0 library search data of the GC-MS system, standards of the main components, and literature data.

Total antioxidant activity (TAA)

DPPH (diphenylpicrylhydrazyl) assay

The TAA of the volatile fraction was measured by the reduction of alcoholic DPPH solutions in the existence of an electron-donating antioxidant (EDA) by modifying the method described by Gil et al. (18). Briefly, 100 µL aliquots of various concentrations of the volatile olive leaf extracts were added to 2.9 mL of a 2,2-diphenyl-1-picrylhydrazyl (DPPH; 6.10^{-5} M DPPH; 2.4 mg/100 mL of methanol). After a 40 min. incubation at 30 °C temperature in the dark, the absorbance was read at 520 nm. Percentage inhibition of free radical DPPH was calculated in the following manner:

$$\text{Inhibition \%} = [(A_{control}-A_{sample})/ A_{control}] \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample.

The volatile oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting of inhibition free radical DPPH in percentage (%) against essential oil concentration. As a positive control, butylated hydroxytoluene (BHT), a synthetic

antioxidant reagent, was used. All tests were carried out in triplicate.

Antimicrobial activity (AmA)

Source of Microorganisms

The bacterial strains tested were *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Lactobacillus plantarum* ATCC 8014, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureu*s ATCC 27950. In the case of yeasts, *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 90030, *Candida kreusei* ATCC 34077, and *Candida parapsilosis* ATCC 22019. All these cultures were purchased from the culture collections of the National Type Culture disperse Collection Unit, Microbiology Reference Laboratory and Biological Products Department, Public Health General Directorate, Ministry of Health, (Ankara, Turkey).

Disc Diffusion Method

All method steps, including preparation conditions of test microorganisms, inoculation instructions, bacteria and yeast incubation times, were studied according to Vardar-Ünlü et al., (19). Briefly, a suspension of the test micro-organism (0.1 mL, 108 cells mL^{-1}) was spread on solid medium plates. Filter paper discs (6 mm in diameter) (Schleicher and Schüll, No.2668, Germany) were soaked with 30 µL of the oil and placed on the inoculated plates. After keeping the plates at 4 °C for 2 h, they were incubated at 37 °C for 24 h (bacteria) or at 30 °C for 48 h (yeasts). Following the incubation period, the diameters (mm) of the inhibition zones were measured. The diameters (mm) of the inhibition zones were quantified, and the results were classified into three categories according to the diameters obtained in the test: resistant (> 7 mm), medium (> 12 mm) and sensitive (> 18 mm) (20).

Minimum inhibitory concentration (MIC) Method

A microdilution broth susceptibility assay for bacteria and yeast was used to determine the MIC (21). Preparation, incubation, and counting of bacterial strains, yeasts, and test strains were performed consistent with the method of Ünlü et al. (22). All tests were performed in Mueller Hinton broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5%, v/v) to enhance the oil solubility. Bacterial strains were cultured overnight at 37 °C in MHA and the yeasts were cultured overnight at 30 °C in Sabouraud dextrose broth (SDB). Test strains were suspended in MHB to give a final density of 5 x 10^5 colony-forming units (CFU)/mL and were confirmed by viable counts. The essential oil's geometric dilutions were prepared in a 96-well microtiter plate, including one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. British Society for Medical Mycology (BSMM) guidelines were used for broth microdilution testing for yeasts. The essential oil was resolved in Yeast Nitrogen Base Glucose (YNBG) supplemented with Tween 80 detergent (final concentration 0.5% v/v) and ultimate concentrations ranged from 100.00 mg/mL to 0.025 mg/mL (22).

Statistical Analysis

The data were statistically analyzed by ANOVA (n=3) and statistical significance was accepted at a level of $p< 0.05$ (23).

RESULTS AND DISCUSSION

Chemical Composition of volatile oil

The composition of *Olea europaea* L. volatile oil was analyzed by GC-MS. 42 compounds, representing 99.59±1.15% of the oil, were identified. The main components are α-pinene (9.82±0.33%), benzyl alcohol (8.83±0.27%), phenethyl alcohol (8.52±0.25%), 2-monopalmitin (8.13±0.28%), palmitic acid (5.53±0.41%), octadecanoic acid 2,3-dihydroxypropyl ester (5.84±0.42%), phytol (4.22±0.17%), benzaldehyde (4.21±0.38%) (Table 1). In our study, alcohols were characterized by the presence of eleven compounds (32.92±0.56 %), the most important of them were benzyl alcohol (8.83±0.27%), phenethyl alcohol (8.52±0.25%), followed by phytole (4.22±0.17%). The second priority group was aldehydes composed of five compounds (9.42%) mainly, benzaldehyde (4.21±0.38%) and, 2,4-heptadienal (2.91±0.23%). The ester group was characterized by 2-monopalmitin (8.13±0.28%).

Table 1. The main leaf volatiles (%) *of Olea europea L*. collected in the Ayvalık, Turkey.

	Constituents	LRI $_{lit.}$	LRI $_{cal.}$	%	Method of identification
Alcohols	1-penten-3-ol	683	678	1.81±0.09	MS
	2-ethoxyethanol	717	715	2.22±0.22	MS
	cis-3-hexene-1-ol	857	860	1.23±0.07	MS
	trans- 2-hexene-1-ol	868	870	2.62±0.24	CO, MS
	1-hexanol	884	881	1.21±0.09	CO, MS
	Benzyl alcohol	1007	1005	8.83±0.27	MS
	Phenethyl alcohol	1110	1112	8.52±0.25	MS
	p-cymen-8-ol	1183	1187	0.72±0.11	CO, MS
	Eugenol	1356	1350	0.33±0.02	CO, MS
	Isoeugenol	1384	1388	1.21±0.09	CO, MS
	Phytol	1840	1841	4.22±0.17	CO, MS
Aldehydes	n-Hexanal	784	785	0.73±0.12	CO, MS
	Benzaldehyde	996	998	4.21±0.38	MS
	2,4-heptadienal	1009	1008	2.91±0.23	MS
	n-octanal	1023	1022	0.23±0.02	MS
	Nonanal	1098	1100	1.34±0.08	CO, MS
Esters	2-Monopalmitin	2498	2493	8.13±0.28	CO, MS
Terpenes	α- pinene	941	968	9.82±0.33	CO, MS
	β- caryophyllene	1421	1420	2.01±0.18	MS
Carb. Acids	Hexanoic acid	1085	1092	0.42±0.03	MS
	Caprylic acid	1179	1182	0.64±0.05	MS
	Nonanoic acid	1280	1280	0.72±0.12	MS
	Myristic acid	1720	1718	0.71±0.11	MS
	Palmitic acid	1973	1968	5.53±0.41	MS
	Stearic acid	2124	2125	1.32±0.07	CO, MS
Hydrocarbons	2,2,6-trimethyloctane	1029	1030	2.44±0.22	CO, MS
	Undecane,5-methyl	1154	1157	0.23±0.02	CO, MS
	Dodecane,4,6-dimethyl	1285	1288	1.22±0.06	MS
	n-Tetradecane	1399	1400	1.53±0.06	MS
	n-Hexadecane	1600	1601	1.82±0.07	MS
	n-Heptadecane	1700	1700	0.82±0.11	MS
	1-chloro octadecane	2070	2068	1.47±0.09	MS
	Heneicosane	2100	2100	0.94±0.08	MS
	Tetracosane	2500	2501	0.88±0.05	MS
	Octadecanoic acid, 2,3-dihydroxypropyl ester	2689	2690	5.84±0.42	MS
Others	1-hydroxy-2-propanone	698	695	0.93±0.08	MS
	Trichloroethene	702	698	0.47±0.02	CO, MS
	4-Ethenylpyridine	1037	1040	2.25±0.18	MS
	Methyldiethanolamine	1053	1055	1.53±0.07	MS
	5-chloro-n- amylacetate	1129	1131	1.34±0.04	MS
	4-Ethenyl-2-methoxyphenol	1283	1284	1.32±0.05	MS
	Benzene, (2-propenyloxy) methyl	1405	1404	2.92±0.23	MS
	Total identified			**99.59±1.15**	

LRI: linear retention indices (HP5-MS column); MS, mass spectrometry; CO, co-injection with standards; LRI_{lit}, retention indices from the literature (10); LRI_{calc}, experimental retention indices calculated against a C_8–C_{32} n-alkanes mixture on the HP5-MS column
All values are mean ± standart deviation of triplicates.

We were able to find a limited number of articles associated with the essential oil compositions of *Olea europaea* L. (1, 24-28). In several previous studies, the chemical composition of the volatile fractions from *Olea europaea* L. cultivars was investigated. Most of these studies focused on the regions, showing similar climatic and geographic characteristics of the Mediterranean basin. In one of these researches, three Italian *Olea europaea* L. cultivars (Leccino, Frantoio, and Cipressino) were investigated in different years and months (July and November) by Campeol et al. (24, 25). In a Tunisian investigation, Chemlali cultivar was studied by Haloui et al. (26) and Nebjemel, Chemchali, Chemlali and Chetou cultivars were studied in October by Brahmi et al. (1, 27).

In the northern part of Algeria, olive leaves collected in September at an altitude of 800 m were investigated (28). The essential constituents in either plant were determined as (E)-2-hexenal, nonanal, kongol, benzene-acetaldehyde, (E)-β-

damascone, (E)-β-damascenone, (E,E)-a-farnesene and (E)-2-hexen-1-ol (24, 25). The main components were found as (E)-3-hexenol, 3-ethenylpyridine, (E)-β-damascenone, and phenylethyl alcohol (1).

Brahmi et al. (27) have identified the compounds forming 92.10% of the total volatile oil. Consistent with our study, Brahmi et al. (27) determined that there was the highest amount (6.10%) of phenylethyl alcohol in the alcoholic group, which was characterized by the presence of four compounds. Aldehydes were composed of nine compounds, primarily nonanal. Volatile compounds, characterizing at least 99.23% of the essential oils, were identified as a-pinene (52.70%), 2.6-dimethyl-octane (16.57%) being the most abundant components of the essential oil. The other chemical components were 2-methoxy-3-isopropylpyrazine (6.01%), tetracosane (4.38%) and docosane (3.58%). The following chemical components occurred in trace amounts: β-pinene (2.46%), z-3-hexanol (1.51%), (*E, Z*)- 2,6-nonadienal (1.46%), α-ionone (1.45%) and (*E*)-2-hexanol (1.26%) (26).

Boukhebti et al. (28) have analyzed and identified the volatile oil components of *Olea europaea* leaves which represent 94.10% of the total oil. The chemical composition of the essential oil is dominated by the compounds, palmitic acid (14.71%), Z-nerolidol (9.45%) and octacosane (6.32%).

Keskin et al. (29) reported that the chemical constitutions of aqueous extract (using a Soxhlet apparatus) from West Anatolia, Turkey were analyzed by GC/MS. GC/MS analysis of the extract resulted in the identification of fifteen constituents, representing 99.68% of the extracts; cyclotrisiloxane, hexamethyl (36.98%), cyclotetrasiloxane, octamethyl (15.18%) and cyclopentasiloxane, decamethyl (14.59%) being the main components.

In other articles focusing on the same cultivars or different cultivars growing in particular habitats, the role of environmental effects are studied and its importance is emphasized. Further investigations would probably explain and generalize the obtained data.

Total antioxidant activity (TAA) of volatile oil

The weakest TAA was exhibited by the volatile oil 70.68±2.4% and 3080±11.2 IC_{50} (µg/mL) (Table 2). The activities were compared with BHT. The volatiles of the dried leaves showed tolerable TAA and lower than the reference antioxidant, BHT (IC_{50}= 28.8 ±1.4 µg/mL; 85.12± 4.8%).

This observation is consistent with the other reports. In previous studies on the dried olive leaves, it is shown that DPPH IC_{50} (µg/mL) values vary in different cultivars. Brahmi et al. (1) reported that DPPH values were found 3430.70±51.36 (µg/mL), 3190.52±89.50 (µg/mL) and 3250.11±46.52 (µg/ml) respectively. Brahmi et al. (27) described that the essential oil exhibited the weakest TAA (49.92%), Haloui et al. (26) reported that the TAA was exhibited by the essential oil (74.44%±0.79).

Table 2. Antioxidant activity of the essential oil of *Olea europaea L.*

Sample	**DPPH radical scavenging activity, %**	**DPPH IC_{50}(µg/mL)**
Essential oil of *Olea europaea L* leaves	70.68 ± 2.40	3080.00 ±11.22
BHT	85.12 ± 4.80	28.20 ± 1.40

Results are means of three different experiments.

Antimicrobial activity (AmA) of volatile oil

The AmA of the volatile oil was examined for 7 bacteria and 4 Candida species using disk diffusion and MIC methods. (Table 3). Comparing the essential oils with the control antibiotic and control antifungal concluded that they could inhibit most bacterial growths with different effectiveness.

Table 3. Antimicrobial activity of essential oil from *Olea Europea* L. leaves.

	***Olea europaea L.* essential oil**			
Microorganisms	**Disc Diffusion[a]**	**MIC values (µg/mL)**	**Amphotericin** (Control Antifungal)	**Gentamicin** (Control Antibiotic)
Bacillus cereus ATCC 14579 (+)	29.00±0.50	1100	nd	1500
Enterococcus faecalis ATCC 29212	13.00±0.01	1750	nd	2100
Escherichia coli ATCC 25922	9.00±0.09	70	nd	1750
Klebsiella pneumoniae ATCC 13883	11.00±0.02	1500	nd	2250
Lactobacillus plantarum ATCC 8014 (+)	9.00±0.03	50	nd	750
Pseudomonas aeruginosa ATCC 15442	13.00±0.05	150	nd	1100
Staphylococcus aureus ATCC 27950 (+)	10.00±0.04	70	nd	2100
Candida albicans ATCC 10231	17.00±0.11	1250	600	nd
Candida glabrata ATCC 90030	9.00±0.15	150	550	nd
Candida kreusei ATCC 34077	7.00±0.06	250	500	nd
Candida parapsilosis ATCC 22019	7.00±0.21	-	450	nd

[a]DD, disc diffusion method; diameter of inhibition zone (mm) including disk diameter of 6 mm.
MIC, minimum inhibitory concentration; values given as µg mL^{-1} for the essential oils and antibiotics.
n.d. not determined
Disc diffusion values are expressed as mean±SD (n=3)
DMSO: Negative control

This study revealed that the volatile oil has efficient antibacterial activity toward bacterial strains, especially *Bacillus cereus* ATCC 14579, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 15442, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 27950, *Candida glabrata* ATCC 90030. On the flip side, there is an inference that the essential oil's antimicrobial activity (resistant >7 mm) has no significant effect on *Candida kreusei* ATCC 34077 and *Candida parapsilosis* ATCC 22019. Susceptibility of the oil, amphotericin (control antifungal) and, gentamicin (control antibiotic) was expressed as a minimum inhibitory concentration (MIC) and, DMSO was used as a negative control.

Brahmi et al. (1) also analyzed the AmA of dried olive leaves. They found that essential oil has very remarkable antibacterial activity towards bacterial strains *E. faecali*s ATCC 29212 70 *S. aureus* ATCC 27950 *E. coli* ATCC 25922 *P. aeruginosa* ATCC27950 *C. Kreusei* ATCC6258 *C. parapsilosis* ATCC *C. albicans* ATCC90028 *C. glabrata* ATCC90030. While Boukhebti et al. (21) established similar results with *Citrobacter freundii* ATCC 8090, *Pseudomonas aeruginosa* ATCC 27853 and, *Staphylococcus aureus* ATCC 25923, while not having great antibacterial activity against *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922.

CONCLUSION

Olive leaves are considered a by-product of the olive tree cultivation and oil industry. Interest in alternative uses of these agro-food by-products has increased significantly in recent years. Endowed with engaging biological activities, many studies focused on valorizing olive leaves in the food industry as a functional food or as a source of nutraceuticals.

In this study, to our knowledge, the antioxidant and antimicrobial activities of the essential oil obtained from the olive leaves in West Anatolia in Turkey were tested for the first time. According to the results obtained from this work, the volatile compounds of dried olive leaves have very high, antibacterial and antifungal properties that may benefit the pharmaceutical, food, and cosmetics industries. It has been determined that especially essential oils have a very impressive antibacterial activity against bacterial strains such as *Bacillus cereus* ATCC 14579, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212 and *Klebsiella pneumoniae* ATCC 13883. However, the leaves' essential oil showed a lower, tolerable antioxidant activity than the reference antioxidant BHT. On the other hand, it can also be concluded that the changes in the Olea europea L. volatile oil chemical compositions, from different geographical areas, might have been based on different variables, climatic, seasonal, geographical, and geological differences.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declare no conflict of interest.

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