ORIGINAL ARTICLE

Determination of theoretical calculations by DFT method and investigation of antioxidant, antimicrobial properties of olive leaf extracts from different regions

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Abstract In this study, we studied samples of mature olive leaves from the districts of Incirli Ova within the province of Aydın and the district of Fethiye in Mugla/Turkey. Several processes were carried out on the olive leaves to use them in this study, including drying under different conditions, determination of moisture, extract output, overall determination of phenols, antioxidant activity determination and anti-microbial assays. The chemicals that were used in the study were Folin's reagent and gallic acid for total phenolic assays, DPPH (1,1-diphenyl-2-picrylhydrazyl) and Trolox for antioxidant activity assays and nutrient broth and nutrient agar for antimicrobial testing. In the theoretical part of the study, the structures of oleuropein and Trolox molecules were examined, and their oxidation properties were aimed to be determined and compared to experimental results. According to the results of total phenolic assays, the phenol contents in the olive leaves from Aydın and Mugla were observed to be very close to each other. In the anti-microbial assay, it was observed that the samples of olive leaves from Mugla were more antioxidant than those from Aydın.

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Introduction

Turkey is one of the leading countries in the world in olive culture and cultivation and comes fourth after Spain, Italy and Greece in terms of the quantity of olive trees and table olive production. Widely spreading throughout Turkey, olive trees are indigenous to the Aegean Region, Marmara Region, Mediterranean Region, Southern Anatolia Region and Black Sea Region, in particular. Studies conducted on the ingredients of olive fruits and leaves primarily reported that phenolic compounds in olive leaves are abundant. The most concentrated substances in an olive leaf are phenolic substances such as oleuropein and hydroxytyrosol which is the product of degradation (Bouaziz et al. 2008; Barbaro et al. 2014).

Thanks to the structure of these two phenolic compounds and particularly their higher antioxidant capacity, we considered that olive leaves have therapeutic effects against heart attack, rhythm disturbance, vascular occlusion, cancer, hypertension, diabetes mellitus and hypoglycemia, and because of their high anti-microbial properties, researchers started to investigate use of olive leaves against many viral diseases, particularly HIV (Samuelsson 1951; Figueiredo-Gonzalez et al. 2018a, b).

Phenolic fraction is partially responsible for the healthy and sensory properties, together with the higher oxidative stability of the olive oil, in comparison with the rest of edible vegetable oils (Reboredo-Rodrıguez et al. 2016; Mora-Ruiz et al. 2017). They are also used to cure neurological disorders including Parkinson's and Alzheimer's diseases. Oleuropein is one of main polyphenolic antioxidants and the most active phenolic compound of olive leaves. Besides, evidence indicates that phenolic compounds from olive oils can exert chemopreventive effects against different cancers (Reboredo-Rodriguez et al. 2018; Figueiredo-Gonzalez et al. 2018a, b). The phenolic compounds are found in the virgin olive oil (EVOO) the major source of energy in the Mediterranean diet. Recent epidemiological evidence and clinical trials connect the Mediterranean diet (Med Diet) with protective effects against the health (Figueiredo-Gonzalez et al. 2018a, b). It is the component which is available in every part of an olive tree and provides bitterness to olives. Elenolic acid, an ingredient in oleuropein, and calcium elenolate, its derivative, have an ability to keep out a wide variety of microorganisms. Oleuropein, which may provide a long lifespan to olives, is used in Europe in a wide range from rejuvenating creams to pills to milk quality improvement (Barbaro et al. 2014).

Galano et al. (2016) reported that the most important reaction mechanisms involving flavonoids and their derivatives are: (1) hydrogen atom transfer (HAT), (2) single electron transfer (SET), (3) sequential electron proton transfer (SEPT) and (4) sequential proton loss electron transfer (SPLET). In this study, two types of olive leaves were used, one was from Aydın, and the other was from Mugla. Different drying techniques were applied to perform moisture determination assays. Extracts of different types of olive leaves were obtained using an evaporator, and analyses for antioxidants, antibiotics and total phenolic compounds were completed. According to the results, different drying techniques had significant effects on the amounts of antioxidant, antimicrobial and phenolic compounds in olive leaves.

Materials and methods

Materials

Chemicals Folin's reagent, sodium carbonate, DPPH (1,1-diphenyl-2-picrylhydrazyl), methanol, gallic acid, ethanol, Trolox, nutrient agar, nutrient broth and Tris Hydrochloric acid (HCl) buffer were provided by Sigma Aldrich. Bacteria strains including *Escherichia coli* (*E. coli),* *Salmonella,* *Listeria monocytogenes* (*L. monocytogenes*), and *Staphylococcus aureus* (*S. auresus*) were procured by Aziz

Sancar Scientific Research Center at Istanbul Aydin University.

Methods

Four different drying methods were applied on two types of olive leave, one the province of Aydin (A) and the other from the province of Mugla (M) in Turkey. These were the processes of drying in a microwave at 360 W (I), in a microwave at 540 W (II), in an oven at 70 °C for 24 h (III) and in atmospheric conditions for 20 days (IV). Thereby, eight samples (AI, AII, AIII and AIV, and MI, MII, MIII and MIV) were obtained. For the drying processes, samples of 50 g were weighed for both types of olive leaves. Drying was completed in 5 min in microwaves (360 and 540 W), 24 h in an oven and 20 days in atmospheric conditions. The dried samples were grinded, preserved at 4 °C and prepared for extraction.

Moisture analysis

Moisture assays were carried out in parallel 3 times for A and for M. Firstly, the tare weights of the petri plates 2 and 3 were measured, and 5 g was weighed from olive leaves to reduce the fixed weight through drying at 105 °C for 2 h. The same procedure was also applied for two different samples, and their moisture contents (%) were calculated using the following Eq. 1.

$$\%\mathrm{Moisture} = \frac{(\mathrm{final\ weighing} - \mathrm{tare})}{\mathrm{Sample\ quantity\ (g)}} \times 100 \qquad (1)$$

Extraction of olive leaves

We weighed 0.5 g of the grinded olive leaves and added methanol (2 mL) and distilled water (8 mL), and a homogeneous mixture were produced through a 3-min vortex process. Then, the sample was centrifuged for 20 min a centrifuge device at 6000 rpm. Afterwards, the extract was transferred into a flask, and this process was repeated three times by adding the same methanol–water mix for each time. Consequently, the extracts were run through the evaporator and put into a 25-mL volumetric flask and the process was completed by using methanol.

Determination of total phenolic compounds

For total phenolic assays, a Folin's solution of 1:10, a gallic acid solution as a reference to the Folin's solution and a blank solution were prepared. 20 mL of distilled water was added into the Folin's solution with a volume of 10 mL to prepare a stock Folin's solution. 1:3 from the preparate was filled up to 25 mL with distilled water in a volumetric

flask. For a 2% sodium carbonate (Na_2CO_3) solution, 0.5 g was weighed and completed to 25 mL with distilled water in a volumetric flask. A calibration equation was obtained through dilution by 1/3 from a gallic acid stock solution of 3%. 0.1 mL of Folin's solution was added after diluting by 1/3 from the prepared stock Folin's solution, and 0.1 mL and 4.5 mL of distilled water and 0.3 mL of Na_2CO_3 were added after diluting by 1:3 from the gallic acid solution of 3%. (Reboredo-Rodrıguez et al. 2016; Reboredo-Rodriguez et al. 2018). To plot a calibration curve, the solutions in 8 test tubes were blended for 3 min through vortex for a homogeneous mixture. For each extract, the aforementioned experimental process was applied by the same measures, replacing gallic acid with extract sample, it was kept in darkness for 2 h, and then, its absorbance values were analyzed in UV spectrophotometer at 760 nm (Ryan et al. 2002; Visioli et al. 2002). The calibration equation obtained as a result of the experiments is y = 14084x + 0, 0154. R^2 value is 0.9995.

Antioxidant activity analysis

The DPPH method was used for the antioxidant assays. The assays were made by studying two parallel tests. In this method, the solutions of DPPH and Trolox were prepared. For the DPPH solution, 0.0023 g of DPPH was weighed and completed with methanol in a volumetric flask (100 mL). For the Trolox solution, 10 μM Trolox at a weight of 0.0625 g was weighed and completed with methanol in a volumetric flask (25 mL). Besides these, a control solution was also prepared. For this, stock DPPH solution at a volume of 3.9 mL and 0.1 mL of methanol were added into a test tube. To prepare for the plot of the calibration curve, the stock Trolox solution was diluted at the concentrations of 0.2 μM, 0.25 μM, 0.4 μM, 0.5 μM and 0.7 μM. To plot a calibration curve, 100 μL of the solution was transferred from each of six test tubes in total with Trolox solutions including the stock Trolox solution into six separate test tubes (Reboredo-Rodriguez et al. 2018). 3.9 mL of the stock DPPH solution was added into each of these new tubes, and a homogeneous mixture was obtained using Vortex. The control solution and these preparates were kept in darkness for 60 m. The absorbance values were read at 517 nm in the UV spectrophotometer, and the calibration curve was plotted. For the extracts, 1.5 mL of each extract and 3.9 mL of stock DPPH solution were added into 8 test tubes. The preparates were kept in darkness, and their absorbance values were read at 517 nm in the UV spectrophotometer. Antioxidant values (%) were computed by applying these absorbance values to the following Eq. 2 (Czerwinska et al. 2012; DeLeonardis et al. 2007; Figueiredo-Gonzalez et al. 2018a, b).

$$\% \text{ Antioxidant activity} = 100 \frac{(\text{abs}_{\text{sample}} - \text{abs}_{\text{blind}})}{\text{abs}_{\text{control}}} \times 100 \quad (2)$$

The calibration equation obtained as a result of the experiments is y = 0.0326x + 0.0438. R^2 value is 0.9994. % Antioxidant activity values were calculated using this equation.

Antimicrobial analysis

For these assays, to prepare the medium, 19.65 g of nutrient agar was weighed, distilled water at a volume of 700 mL was added onto it, and then, it was kept in a sterilizer for 2 h to be reduced to sterilized conditions. The preparate of the agar solution was poured onto 32 petri dishes and completely spread over the bases. For freezing, these were preserved in a refrigerator. To prepare the nutrient broth solution, nutrient broth of 2.63 g was weighed, and 200 mL of distilled water was added into it. Eight test tubes were filled in half with this solution and kept in a freezer for 2 h to be reduced to sterilized conditions. We waited for it to freeze at room temperature. Four bacteria strains (*E. coli*, *Salmonella*, *L. monocytogenes* and *S. auresus*) were taken out of the freezer and thawed. 4 out of 8 broth solution preparates were labelled for each microorganism. Out of the strains, sampling was made through extraction and added into these test tubes. The prepared tubes were put into an oven at 37 °C for 24 h, and the bacteria were left to refresh. 1000 μL of the broths taken out of the oven (i.e. refreshed microorganisms) was added into the other labelled broths kept in another refrigerator. Cultivation was achieved using swabs in the agars prepared with the latter (i.e. slightly blurry broths). Sterilized filter papers were added onto these agars following dripping individual extracts, and they were incubated in an oven at 37 °C for 24 h. Then, the areas of inhibition zones were computed in mm^2 (Baysal and Çelik 2018).

Computational detail

The calculations of quantum mechanics were carried out in Gaussian 09. In the first step, oleuropein and Trolox were optimized using the DFT method (Stephens et al. 1994). DFT calculations were performed with the Lee–Yang–Parr correlation function and the Becke change terms hybrid B3LYP, (Becke 1993) functional. A 6-31-g (d), (Ditchfield et al. 1971) base set was used. Secondly, methanol was used as the solvent to be consistent with the experimental results. Therefore, solvent effect was applied by the IEFPCM method (Scalmani and Frisch 2010). The third

step was to calculate band dissociation energy (BDE) and ionization energy (IE) according to Eq. 3–4.

$$BDE = H(X - O\cdot) + H(H\cdot) - H(X - OH)(8) \tag{3}$$

$$IE = H(X - OH\cdot +) + H(e-) - H(X - OH) \tag{4}$$

Results and discussions

Moisture analysis

3 parallel % moisture assays were completed for two different types of olive leaf from the provinces of Aydin and Mugla. The results show that the moisture content was higher in olive leaves from Mugla. The reason why may be that the precipitation rate is greater in the province of Mugla than in that of Aydın, and the former has more humid climate conditions. % Moisture analysis values of olive leaves of Aydın Province at 105 °C are 12.8, 47.4 and 29.6 for samples 1, 2 and 3, respectively. The average % moisture analysis value is 29.93. % Moisture analysis values of % of olive leaves belonging to Muğla Province at 105 °C are 68.42 and 36.48 for samples 1 and 2, respectively, and the average % moisture analysis value is 52.45.

Total phenolic compound analysis

The phenolic compound contents of the olive leaf extracts are shown in Table 1. The total phenolic substance contents in different drying methods applied on olive leaves may be ranked in size as: AIII > AII > AIV > MII > AI > MIV > MIII > MI. The olive leaf samples (1.36×10^{-5} mg/100) from the province of Aydin which were dried in an oven had the highest total phenolic content. The one with the lowest content was the sample (3.97×10^{-7} mg/100) from the province of Mugla whose drying process was carried out in a microwave at 360 W.

The highest mean value of total phenolic content in both groups of samples of olive leaves from the provinces of Mugla and Aydin was in those which were dried in an oven (2.067×10^{-5} mg/100). The drying methods that followed were respectively 540 W microwave drying (9.31×10^{-6} mg/100), drying in atmospheric conditions (6.61×10^{-6} mg/100) and oven drying (2.067×10^{-5} mg/100) for both the A and M samples.

Computational Detail Analysis

In Fig. 1, the molecular structures of trolox and oleuropein were characterized by the DFT method. Table 3 shows B3LYP/6-31 g (d,p) BDE and IE of trolox and oleuropein.

While the reactions in the gas phase are endothermic, they are exothermic by the action of solvent in methanol. A series of calculations were performed to find antioxidants characterization of oleuropein. The same calculations were made for Trolox. The two OH groups are indicated in Trolox given in Fig. 1a. All calculations were performed in gas phase and in methanol. The 6 OH groups on oleuropein were numbered as given in Fig. 1b.

The BDE was calculated according to the equation given in Eq. 3 and IE was calculated according to the equation given in Eq. 4 for the two structures (Table 2). Calculations show that the IE value in methanol is lower than gas phase for two structure. This result shows that solvent effect has positive attraction. Oleuropein is lower IE than Trolox. BDE was calculated for Trolox 2 OH groups and oleuropein 6 OH groups. Calculations show that number 1 OH group is more active than number 2 for Trolox. While the reactions are endothermic in the gas phase, they are exothermic in methanol. The most active OH group for oleuropein is number 2 and 3. Figure 1c shows molecular structure of *oleuropein radical.*

Oleuropein cation radical structure was optimized to calculate IE. For third OH group, O–H bond length was measured as 1.02 Å. Other OH groups were measured as 0.97 Å. Bond distances result, BDE and molecular orbital shows that the most active OH group for oleuropein is number 3. According to the calculations, it was confirmed that oleuropein has higher negative BDE than trolox and thus has more antioxidant properties. Figure 1d shows molecular orbital of Oleuropein HOMO and LUMO.

Antioxidant activity analysis

Trolox solution was used as a reference solution for antioxidant assays. The absorbance value for the control sample was read as 0.499. The antioxidant activity values (%) of the specimens are presented in Fig. 2.

Based on the results, oven drying in Aydın samples was found to provide the highest antioxidant activity value (10.22%), while the lowest value (70.34%) was in the microwave drying process at 360 W. The antioxidant

Table 1 The amount of phenol compounds

	360 W (mg/100) (1)	540 W (mg/100) (2)	Oven (mg/100) (3)	Atmosphere (mg/100) (4)
Aydın (A)	3.09×10^{-6}	5.58×10^{-6}	13.6×10^{-6}	4.58×10^{-6}
Mugla (M)	0.397×10^{-7}	3.73×10^{-6}	7.07×10^{-6}	2.03×10^{-6}

Fig. 1 Molecular structure of trolox (**a**), detailed molecular structure of oleuropein (**b**) and molecular structure of Oleuropein Radical (**c**) and molecular orbital of Oleuropein (1) HOMO (2) LUMO
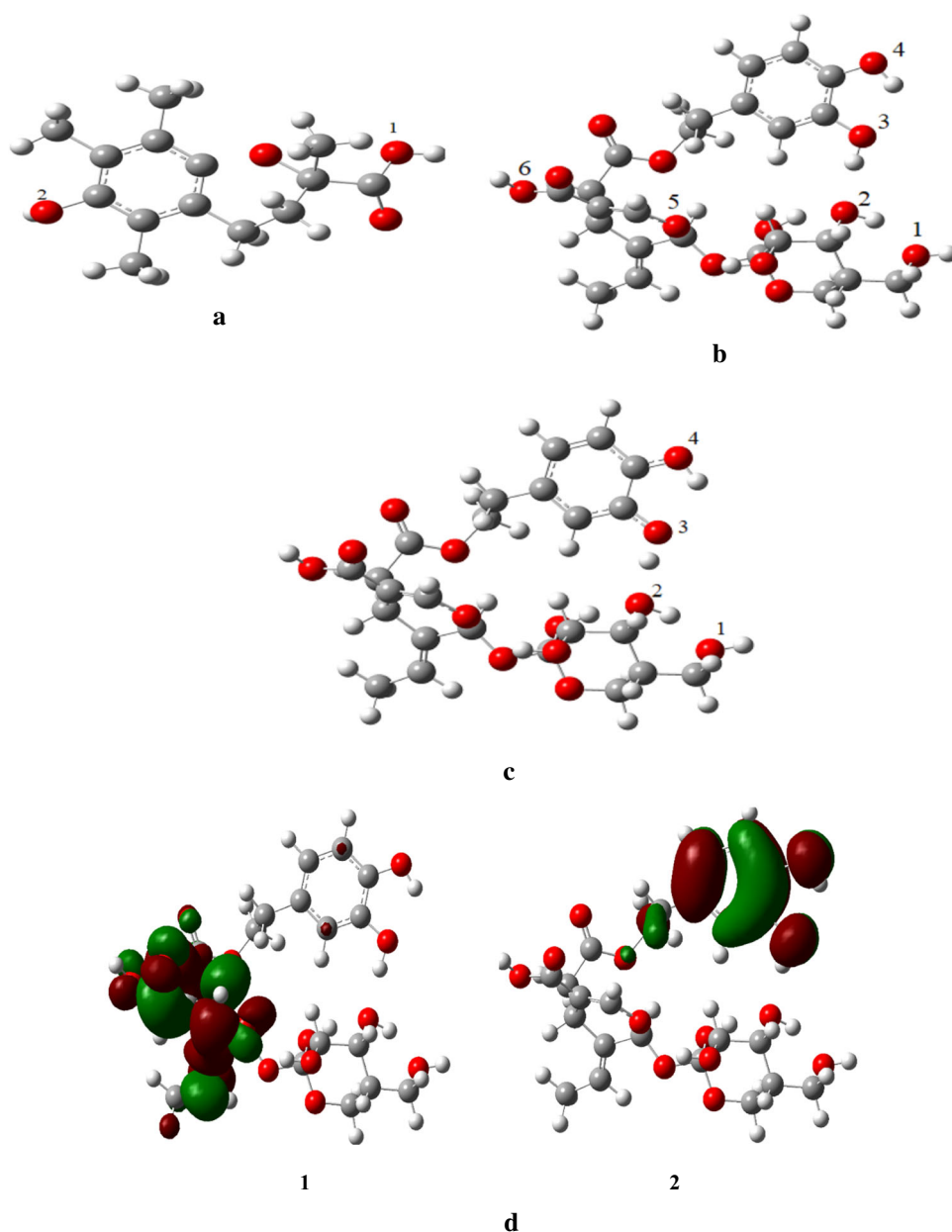

Table 2 B3LYP/6-31 g (d,p) Bond Dissociation Enthalpy (BDE) and ionization energy (IE) of oleuropein and trolox in gas phase and methanol

|	Gas phase		Methanol	
	IE (kcal/mol)	BDE (kcal/mol)	IE (kcal/mol)	BDE (kcal/mol)
Trolox	−119.74		−172.90	
TROLOX1		113.449559		−157.3761591
TROLOX2		119.8581287		−142.3464862
Oleuropein	−133.11		−165.38	
Oleuropein 1		−16.19387367		−352.2082263
Oleuropein 2		83.36547085		−358.3379914
Oleuropein 3		82.78588675		−333.3050335
Oleuropein 4		82.78588675		−331.1429697
Oleuropein 5		113.3641563		−324.886801
Oleuropein 6		113.4019255		−342.6274496
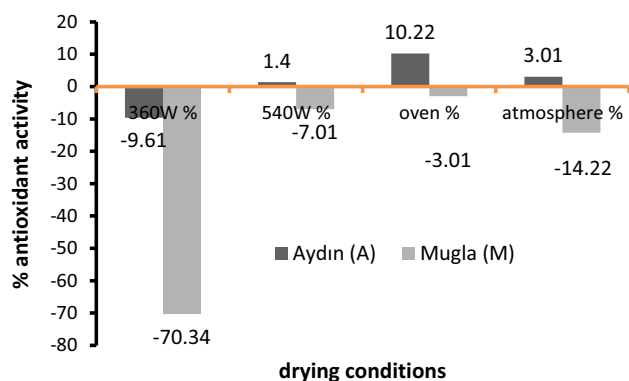
Fig. 2 Antioxidant activity values of samples

activity values (%) of the olive leaf samples from the province of Aydin may be ranked in size as: AIII > AIV > AII. The negative values account for no antioxidant activity of the olive leaf. According to the results, antioxidant effect was observed only in the olive leaves from the province of Aydin, while those from the province of Mugla had no antioxidant effect.

Antimicrobial analysis

The antimicrobial effects of the olive leave samples in different drying methods against *S. aureus*, *L. monocytogenes*, *Salmonella* and *E. coli* are presented in Table 3, Fig. 3. For the bacterium strain of *Staphylococcus aureus*, the antimicrobial effects in the drying methods that were applied were ranked from the highest to lowest as: microwave drying of A at 540 W > microwave drying of M at 540 W > microwave drying of A at 360 W > oven drying of M > microwave drying of M at 360 W > atmospheric drying of M > oven drying of A > atmospheric drying of A. In the general comparison of the Samples A and M, the olive leaves from Mugla had higher antimicrobial effects against the bacterium *S. aureus*. For the

Table 3 Antimicrobial resistance of the samples in inhibition zone (mm^2)

	S. aureus	*L. monocytogenes*	*Salmonella*	*E. coli*
AI	452	415	615	283
AII	1017	227	415	346
AIII	254	201	452	254
AIV	227	346	572	254
MI	380	227	1194	531
MII	716	415	531	177
MIII	445	283	855	254
MIV	373	346	615	452

bacterium strain of *L. monocytogenes,* the antimicrobial effects were ranked from the highest to the lowest as: AI = MII > MIV = IV > MIII > AII = MI > AIII. In the general comparison of the Samples A and M, the olive leaves from Mugla had higher antimicrobial effects against the bacterium *L. monocytogenes*.

For the bacterium strain of *Salmonella,* the antimicrobial effects were ranked from the highest to lowest as: MI > MIII > MIV = AI > AIV > MII > AIII > AII. In the general comparison of the Samples A and M, the olive leaves from Mugla had higher antimicrobial effects against the bacterium *Salmonella*. For the bacterium strain of *E. coli,* the antimicrobial effects were ranked from the highest to lowest as: MI > MIV > AII > AI > AIII = MIII = AIV > MII. In the general comparison of the Samples A and M, the olive leaves from Mugla had higher antimicrobial effects against the bacterium *E. coli*. Consequently, in the comparison of the Samples M and A, the highest antimicrobial resistance was found in the Samples MI and MIII from Mugla against the bacterium *Salmonella,* and the Sample AII from Aydin was strongly resistant against the bacterium *S. aureus,* followed by sample MII.

As a result, the growth of intestinal pathogens in the intestinal lumen is prevented and the effect of the disease is minimized. It is suggested that the anaerobic bacteria of the intestine provide colonization resistance against pathogenic organisms such as Salmonella. Acetate and other short chain fatty acids, mandatory anaerobic metabolism products inhibit the growth of Salmonella. The phenolic compounds, which are basically hydrolyzed from the glucoside form, are essentially polar and only slightly dissolved during the hemalamation process. Besides, most of the phenolics are now found in by-product olive pomace, so this is another and inexpensive source of bioactive compounds. In some cases, the same phenolic compounds found in virgin olive oil (VOO) are extracted and then used to enrich their own VOO to allow the preparation of oil samples with larger and well-known amounts and types of phenolic compounds (Reboredo-Rodriguez et al. 2018; Figueiredo-Gonzalez et al. 2018a, b). It is also possible to enrich the VOO using phenolic extracts, using appropriate technological parameters during processing, mainly grinding density and temperature–time conditions of malaxation (Reboredo-Rodriguez et al. 2017).

Conclusion

In this study, the antioxidant effects, antimicrobial effects and total phenolic compound contents of olive leaves from two different provinces were examined under different drying conditions. According to the antimicrobial resistance measurements, the olive leaves, in consistency with

Fig. 3 Inhibition zones of MI (**a**), MII (**b**), MII (**c**) and MIV (**d**) samples of Mugla province and inhibition zones of AI (**e**), AII (**f**), AIII (**g**) and AIV (**h**) samples of Aydın province

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(a)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(b)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(c)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(d)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(e)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

(f)

Salmonella   *S.aureus*   *L. monocytogenes*   *E.coli*

Fig. 3 continued

| Salmonella | S.Aureus | L. Monocytogenes | E.coli |

(g)

| Salmonella | S.aureus | L. monocytogenes | E.coli |

(h)
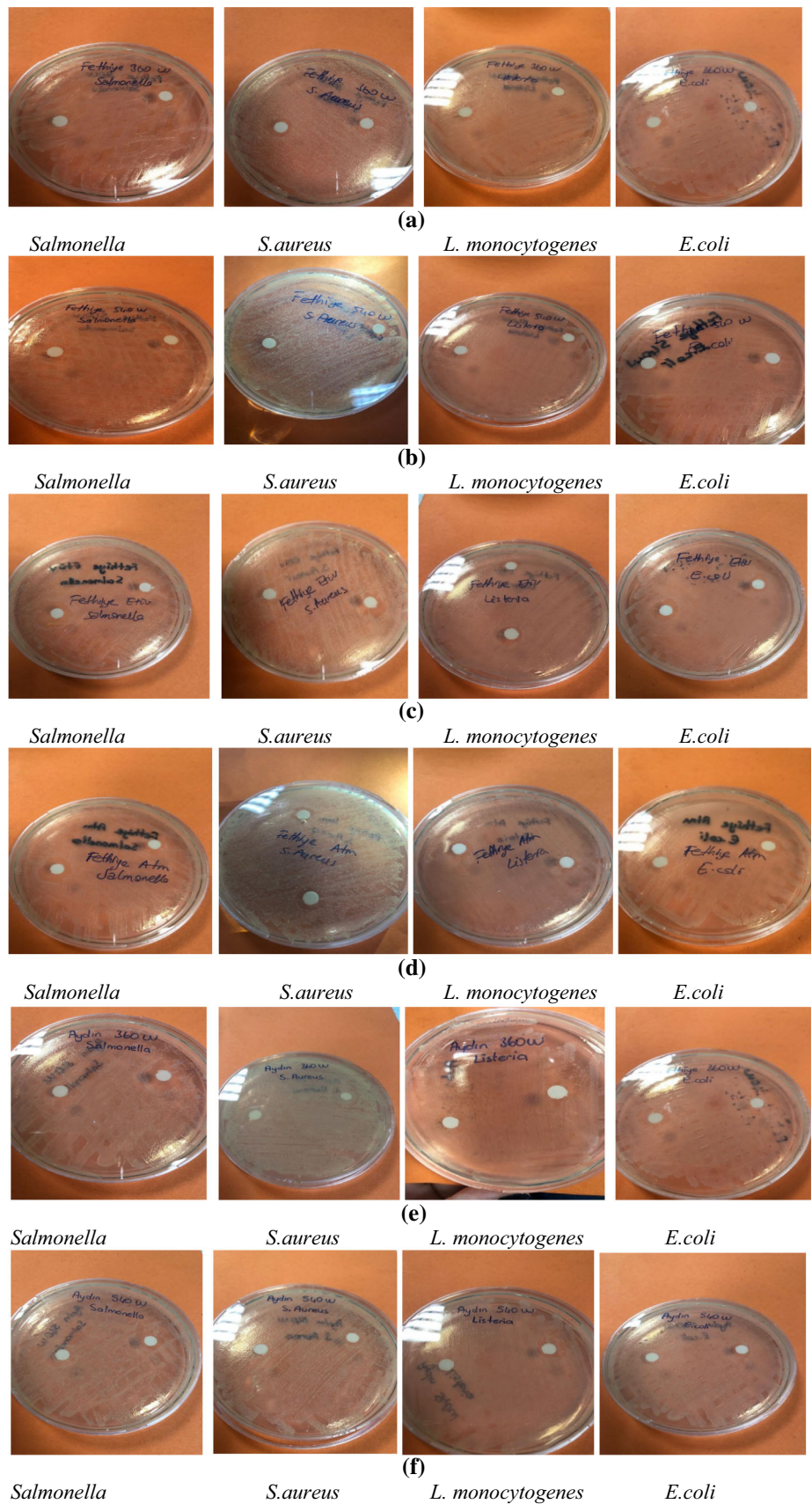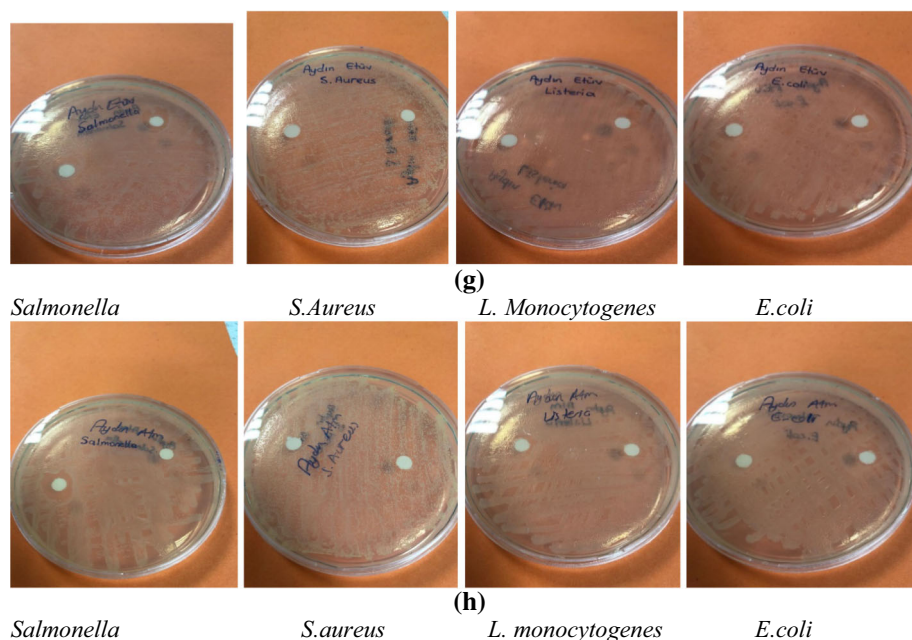
the literature, had strong effects against the bacteria *S. aureus*, *L. monocytogenes*, *Salmonella* and *E. coli*. Microwave drying processes had a key part in increased antimicrobial resistances. The results of the antioxidant assays showed that the olive leaves from the province of Aydin had antioxidant properties. In comparing the total phenolic compound contents of the olive leaves, especially the specimens from the provinces of Aydin and Mugla had higher phenolic compounds when they were put in an oven to dry at 70 °C for 24 h. According to these results, types of olive leave and different drying conditions created strong effects on their characteristics of total phenolic compound content, antioxidant effect and antimicrobial resistance.

Compliance with standards

Conflict of interest The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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