



A comparative study on chemical composition, antibiofilm and biological activities of leaves extracts of four Tunisian olive cultivars



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ABSTRACT

Olea europaea L. is one of the most important fruit trees in Tunisia because of its content of many potentially bioactive compounds. The aim of this study was to evaluate the chemical composition, antibiofilm, antiradical and acetylcholinesterase inhibitory activities from four Tunisian cultivars of *Olea europaea* L., i.e. 'Chetoui', 'Meski', 'Oueslati' and 'Jarboui'. By means of standardized methods, total phenols were determined and some of them characterized by HPLC. The total phenols and flavonoids contents were found to be the highest in the leaves of Chetoui cultivar. The Chetoui cultivar exhibited an important antioxidant and anticholinesterasic activity and an important anti-biofilm activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans*, *Enterococcus faecalis* and *Escherichia coli*, with percentages of inhibition comprised between 83 and 93% at 2xMIC values. Olive leaves extracts could be used in the control of bacterial biofilms in food and food-related environments.

1. Introduction

The study of biologically active compounds from natural sources has always been of great interest to scientists looking for new sources of drugs to treat infectious diseases. Indeed, disorders caused by microorganisms remain a major threat to public health, despite the considerable progress of medicine (Morse, 2004; Edziri et al., 2010).

Olea europaea L. is a species of the Oleaceae family and it is an important source of nutrients and biologically active principles throughout the history of civilization. It is an evergreen tree or shrub native to the Mediterranean region. Typically, it is a short tree, that rarely exceeds 10–15 meters in height. In Tunisia, *O. europaea* is one of the most important cultivated fruit crop, with approximately 70 million trees, covering 1600 thousand hectares. Olive leaves extracts have reported to have health benefits, such as increasing energy levels, lowering blood

pressure, and supporting the cardiovascular and immune systems (Khayyal et al., 2002; Covas, 2007; El and Karakaya, 2009).

Olives contain many potentially bioactive compounds having antioxidant, anti-inflammatory, antiviral, and hypocholesterolemic properties (Covas, 2007). *O. europaea* is widely studied in nutrition research, whereas the leaves are important for their content in secondary metabolites, in particular the secoiridoid derivatives oleacein and oleuropein, the former being responsible for the hypotensive and hypoglycemic activities (Gonzalez et al., 1992).

It has been shown that the qualitative and quantitative phenolic composition of the olive tree strongly differs among cultivars, plant parts and environmental conditions (Techathuvanan et al., 2014).

The cultivar Chetoui, together with three other minor ones, Meski, Oueslati and Jarboui, are cultivated in Tunisia. The olive cultivar, Chemlali, accounts for about 80% of the national olive oil production and

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is grown in central and southern Tunisia. The second most cultivated one, Chetoui is mainly widespread in the north of the country, and provides about 20% of the national production. Jarbouï is mainly grown in the North-West of Tunisia (Teboursouk), while Oueslati in central part of the country (Kairouan). No scientific report about the chemical composition, antibiofilm and acetylcholinesterase inhibitory activities concerning the leaves extracts of these four Tunisian four cultivars is present in the literature.

So the present study aims to compare the chemical composition, antibiofilm, antioxidant and anticholinesterase activities of the methanol leaves extracts obtained from these four Tunisian cultivars grown in the north and center of Tunisia.

2. Materials and methods

2.1. Plant and sample preparation

Olea europaea L. leaves of the four Tunisian cultivars Chetoui, Meski, Oueslati and Jarbouï were respectively collected from the North (Beja and Tunis), the center (Kairouan) and the Sahel region (Sousse) of Tunisia. Collection was performed in February, when the leaves completed their growth cycle. The leaves were manually isolated to obtain a weight of 100 g. Leaves were washed under running tap water to remove dust particles, insects and plankton, and then dried under the shade at room temperature. The dried plant materials were milled into fine powder using mechanical grinder and stored at 4 °C. Voucher specimens (Rus.1212) have been deposited in the Faculty of Pharmacy of Monastir University.

2.2. Chemicals

Methanol, Folin–Ciocalteu reagent, Gallic acid, Catechin, Butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, USA). AlCl₃ and NaOH was supplied by Applichem (Darmstadt, Germany), Phosphate-buffered saline (Aldrich, St. Louis, USA)

2.3. Reagents and standards

2-(4-Hydroxyphenyl)ethanol (tyrosol), cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside and apigenin were purchased from Fluka (Buchs, Switzerland); verbascoside, Hydroxytyrosol, luteolin-7-O-glucoside and oleuropein were obtained from Applichem (Darmstadt, Germany); p-coumaric acid, rutin and luteolin were supplied from Sigma (St. Louis, USA); methanol, hexane, acetic acid and formic acid were from Merck (Darmstadt, Germany) while trans cinnamic acid was obtained from Aldrich (St. Louis, USA).

2.4. Preparation of methanol extracts

Extracts were prepared according to the methodology proposed by Casas-Sanchez et al. (2007), with minor modifications. Briefly, 100 g of dried leaves at 70 °C temperature for 24 hrs, were soaked in 500 mL of methanol for 24 h at room temperature (25 ± 2 °C). Extraction was repeated three times, and the obtained extracts were combined and filtered through number 1 Whatman filter paper n°1 (Sigma Aldrich, France). The solutions were concentrated to dryness under reduced pressure using a rotary evaporator at 45 °C. Extracts were stored at 4 °C until further experiments.

2.5. Determination of pigments content

The procedure was carried out in the dark at 4 °C. A leaf sample (0.25 g) was mashed using a mortar and pestle with 80% acetone (v/v). The extract was filtered and centrifuged in sealed tubes at 15000 g for 5 min. The supernatant was collected and the absorbance was read at 663 and 647 nm for determination chlorophyll a and chlorophyll b, respectively,

and at 470 nm for the carotenoid content using an UV–VIS spectrophotometer (Spectro Double Beam PC UVD-2950, Labomed). The concentrations of chlorophyll a, chlorophyll b, and the sum of leaf carotenoids (xanthophylls and carotenes) were given in µg ml⁻¹ of extract solution, according to the equations of Lichtenthaler and Buschmann (2001):

$$\text{Chlorophyll}_a = 12.25 A_{663} - 2.79 A_{647}$$

$$\text{Chlorophyll}_b = 21.50 A_{647} - 5.10 A_{663}$$

$$\text{Carotenoids} = (1000 A_{470} - 1.82 \text{ Chlorophyll } a - 95.15 \text{ Chlorophyll } b) / 225$$

2.6. Determination of total phenols

Total phenols were determined using a slightly modified Folin–Ciocalteu procedure as described by Montedoro et al. (1992). Briefly, 0.125 ml of extract solution were mixed with 0.5 ml distilled water and 0.125 ml of Folin–Ciocalteu reagent. After 3 min, 1.25 ml of 7% Na₂CO₃ solution was added to the mixture. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. The absorbance of the resulting blue complex was then measured at 760 nm after incubation for 90 min at 23 °C in dark (Spectro UV–VIS, Double Beam PC UVD-2950, Labomed). The total phenols content was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW). The calibration curve range for gallic acid was 0–400 µg/ml. Triplicate measurements were taken for all the samples.

2.7. Total flavonoids

Total flavonoids contents were measured by the colorimetric assay developed by Zhishen et al. (1999). An aliquot of suitable diluted samples or standard solution of catechin was mixed with 0.075 ml of (5% w/v) NaNO₂ solution and kept at rest for 6 min. Afterwards, 0.15 ml of a fresh (10%) AlCl₃ solution was added to the mixture, followed by 0.5 ml of (1 M) NaOH solution after 5 min. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. The absorbance of the mixture was determined at 510 nm (Spectro UV–VIS, Double Beam PC UVD-2950, Labomed) against the same mixture without the sample (blank). Total flavonoids of plant organs were expressed as mg catechin equivalents/g dry weight (mg CE/g DW).

2.8. Identification of phenolic compounds using HPLC

The presence and amount of phenolic compounds in the extracts were studied by reversed-phase HPLC analysis by means of a binary gradient elution using an Agilent Technologies 1100 series liquid chromatography (HPLC, Palo Alto, CA, USA) coupled with an UV–VIS multi-wavelength detector. The separation was carried out at room temperature on a Eurospher-100 C₁₈ reversed-phase column. The mobile phase consisted of acetonitrile (solvent A) and water containing 0.2 % sulfuric acid (solvent B). The flow rate was 0.5 ml min⁻¹. The gradient program was as follows: 15% A/85% B, 0–12 min; 40% A/60% B, 12–14 min; 60% A/40% B, 14–18 min; 80% A/20% B, 18–20 min; 90% A/10% B, 20–24 min; 100% A, 24–28 min. The injection volume was 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Peaks were identified by congruent retention times compared with standards. Quantification of phenolic compounds was achieved by High-performance liquid chromatography (HPLC) comparing peak areas with those of resorcinol used as internal standard. Data were expressed as mg of phenols/100 g of dry weight (DW).

2.9. Antimicrobial activity

2.9.1. Micro-well determination of MIC, MBC and MFC

The MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) were determined on the plant extracts that showed antimicrobial activity, by a broth microdilution method proposed by Novy et al. (2015) with minor modifications. Briefly, 100 μ L of Mueller-Hinton Broth (Difco) plus different concentrations of plant extracts were prepared and transferred to each microplate well to obtain serial dilutions of the active extract, ranging from 4 to 512 mg/mL. Then, 10 μ L of a fresh culture (final concentration of 1×10^6 CFU/mL) of test organisms was added. Microplates were incubated at 37 °C for 24 h. Wells with no added plant extract were used as a positive growth control. Wells without added bacteria were used as a negative growth control. MIC was defined as the lowest concentration of the extract that restricted the visible growth of microorganism tested.

To determine MBC and MFC (Minimum Fungicidal Concentration), 100 μ L from each well that showed no visible growth were re-inoculated on Müller-Hinton agar (MH) agar plates incubated at 37 °C for 24 h. MBC and MFC was defined as the lowest extract concentration showing no bacterial and fungi growth.

2.10. Inhibition of biofilm formation

The inhibition of biofilm formation was tested only against seven type strains including *B. subtilis*, *S. aureus MR*, *S. aureus MTR*, *E. coli*, *P. aeruginosa*, *C. albicans*, *E. faecalis*, was examined by using the modified micro dilution method (Changwei et al., 2008). Prevention of biofilm formation was examined by microdilution, similar to the MIC assay for planktonic cells (Montedoro et al., 1992). Overnight cultures grown in Brain Heart Infusion (BHI) were diluted to 10^6 CFU/mL in BHI supplemented with 2% glucose (w/v). A 200 μ L aliquot was transferred to a 96-well microtiter plate, and 100 μ L of extracts with final concentration corresponding to MIC, 2xMIC, 4xMIC was added. The medium without extract was used as a control. Each assay was repeated three times. After incubation at 37 °C for 24 h, the culture supernatant was discarded, and the wells were washed twice with phosphate-buffered saline (PBS) to remove non adherent cells. The plates were air-dried, and the surface-attached cells were stained with 200 μ L of 0.1% crystal violet for 30 min. then, the crystal violet was removed and the plate was washed with water. Crystal violet stained biofilm cells were determined at 570 nm with with a Multiskan reader (BioRad, Tokyo, Japan). In order to access the ability of the extract to prevent biofilm formation, the percentage of biofilm inhibition was calculated using the equation:

$$\left[\frac{\text{OD}(\text{growth control}) - \text{OD}(\text{sample})}{\text{OD}(\text{growth control})} \right] \times 100$$

where OD (growth control) refers to the absorbance of the bacteria growth without extract, and OD(sample) refers to the absorbance of the extract with bacteria.

2.11. Antiradical activities

2.11.1. DPPH radical-scavenging assay

The DPPH method (Jacob and Shenbagaraman, 2011) was used to determine the antioxidant activity of the olive extracts. A 20 μ L sample from the stock solution was dissolved in absolute ethanol to a final volume of 1 ml and then added to a 1 ml of 0.1 mM DPPH (in absolute ethanol). The reaction mixture was kept at room temperature. The optical density (OD) of the solution was measured after 20 min at 517 nm using a Spectro UV-VIS, Double Beam PC UVD-2950 (Labomed) spectrophotometer. The optical densities of the samples in the absence of DPPH were subtracted from the corresponding OD in presence of DPPH. The percent reduction values were determined and compared with appropriate standards. The percent inhibition of the free radical DPPH (I %) was calculated using the equation:

$$I\% = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100$$

where A_{blank} is the absorbance of the control reaction containing all reagents except the tested compound, and A_{sample} is the absorbance of the tested compound. Butylated hydroxytoluene (BHT) was used as positive control.

2.11.2. ABTS radical-scavenging activity

For the determination of the antiradical activity, a protocol based on the ABTS + free radical decolorization assay was used, according to the method of Lavelli (2002). In brief, 5.0 ml of a 7.0 mM ABTS solution were treated overnight in the dark with 88.0 μ L of a 140 mM potassium persulfate solution to yield the ABTS radical cation. Prior to use in the assay, the ABTS radical cation was diluted with ethanol to an initial absorbance of about 0.700 (ratio of 1:88) at 734 nm and 30 °C. Free radical-scavenging activity was assessed by mixing 1.0 ml of diluted ABTS + radical cation with 10 μ L of sample. Absorbance was measured after 20 minutes after addition of the antioxidant at 520 nm using a Spectro UV-VIS, Double Beam PC UVD-2950 (Labomed) spectrophotometer. Results were expressed as percent inhibition. All tests were carried out in triplicate. Trolox was used as positive control.

2.12. Acetylcholinesterase (AChE) inhibition

The enzymatic activity was measured using a slightly modified version of the method described by Bahloul et al. (2016). Briefly, 50 μ L of buffer 0.1 M (pH 8), 25 μ L of the methanol extract dissolved in ethanol at different concentrations, then 25 μ L of 0.22 U/mL of AChE enzyme were mixed. After 20 min incubation at 37 °C, 25 μ L of 15 mM acetylthiocholine iodide (AChI) and 125 μ L of 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) were added, and the resulting mixture incubated for 30 min at room temperature. The absorbance of the mixture was measured at 405 nm using an UV-VIS SpectraMax 190 Microplate Reader. The inhibitory effect of the test compound was determined by comparison to the negative control: inhibition percentage (I%) = $\frac{(A_0 - A_1)}{A_0} \times 100$, where A_0 refers to the absorbance of the blank sample, and A_1 to the absorbance of the sample. The test was repeated three times. The inhibition of the enzyme activity was expressed as IC_{50} (the concentration of the sample required to inhibit 50% of enzyme), which was calculated by a linear regression analysis.

2.13. Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Statistical analysis involved a one-way analysis of variance (ANOVA). A value of p less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results and discussion

3.1. Total phenols, flavonoids and chlorophyll contents

The results of the total phenols, flavonoids and chlorophyll contents are listed in Table 1. The methanol extracts of the leaves of the Chetoui cultivar is characterized by the highest total phenols (47.47 mg GAE/g), flavonoids (7.29 mg CE/g) and chlorophyll (38.5 μ g/ml) amounts, followed by the Meski, Oueslati and Jarboui cultivars. Statistical analysis of the total phenols and flavonoids contents showed significant differences between the four cultivars, whilst the total chlorophyll amounts were not statistically different between cultivars. It can be hypothesized that the significant differences observed for total phenols and flavonoids may be related both to genetics and to the geographical origin (Kallithraka et al., 2004; Felhi et al., 2016).

The results on total phenols and flavonoids contents support the observed activity of the extracts against free radicals (Felhi et al., 2016). In other scientific report Blasi et al. (2016) demonstrated that Phenolic contents from four Italian cultivars increased from December to March (P

Table 1

Total polyphenols, flavonoid contents and chlorophyll contents of leaves methanolic extracts of four olive cultivars (*Olea europaea* L.).

	Methanol leaves extracts			
	Chetoui	Meski	Oueslati	Jarboui
Total polyphenols (mg GAE/g extract)	47.47 ± 0.45a	34.55 ± 0.6b	28.97 ± 0.9c	18.96 ± 0.85d
Total flavonoids (mg CE/g) extract)	7.29 ± 0.50a	5.34 ± 0.20b	4.86 ± 0.2c	3.08 ± 0.16c
Total chlorophyll content ch(a+b) µg/ml	38.5 ± 1.7a	37.5 ± 0.2a	31.6 ± 1.5a	29.8 ± 1.2a
Acetylcholinesterase inhibitory activity IC ₅₀ (µg/ml)	500 ± 15.21d	500 ± 12.14c	750 ± 10.24b	750 ± 17.35a

Means with different letters were significantly different at the level of $p < 0.05$. GAE: Gallic acid equivalent, CE: Catechin equivalent, Galanthamine: positive control with IC₅₀ of 0.4 µg/ml).

≤ 0.01) and from March to June ($P \leq 0.01$), and they reported that the highest total phenols values ($P \leq 0.05$) were found in June for all cultivars. so we can conclude that phenol content showed marked variations with plant growth, in fact probably the phenol storage in the leaves is a time dependent regulated process, according to the life cycle of olive leaves. In addition Özcan et al. (2019) demonstrated that fatty acid composition and phenols contents of the olive oils showed differences depending on the olive variety.

Akbaş et al. (2017) reported that The highest total phenol contents were found in olive leaves heated in microwave 540 W and atmospheric air.

3.2. Identification and quantification of phenolic compounds by HPLC

The HPLC analysis of the olive leaves extracts of the four cultivars allowed the identification of nine phenolic compounds (Table 2). Hydroxytyrosol, tyrosol, 4-hydroxybenzoic acid, rutin, luteolin-7-O-glucoside, apigenin-7-O-glucoside, oleuropein, apigenin and catechin hydrate. All these compounds were previously characterized in olive leaves (Meirinhos et al., 2005; Sahin and Bilgin, 2012). The major phenolic components common to all the four cultivars were oleuropein and apigenin-7-O-glucoside, with oleuropein as the main one. This component was present in highest amounts in the Meski cultivar. As shown in Table 2, Chetoui and Meski cultivars had the highest phenolic content compared to Oueslati and Jarboui ones. Apigenin was the least represented derivative.

These differences are not surprising because of the influence of both genetic and geographic factors (North, Sahel and Center Tunisia). These results are in agreement with Brahmi et al. (2013), who found that the olive organs from the northern cultivars (Chetoui) had the highest level of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids than the southern ones (Chemlali).

Additionally, these results are also in good agreement with those reported by Benavente-Garcia et al. (2000), which showed that the polyphenols in olive leaves consists of oleuropeosides (oleuropein and

Table 2

Contents of phenolic compounds (µg g⁻¹ DW) determined by HPLC analysis in the olive leaves extracts obtained from Chetoui, Meski and Jarboui cultivars.

Compounds	Chetoui	Meski	Jarboui	Ouslati
Hydroxytyrosol	91.31	89.61	89.30	75.7
Tyrosol	141.33	113.86	86.17	83.5
4-Hydroxybenzoic acid	83.76	66.33	81.10	54.8
Rutin	156.26	210.39	248.64	145.6
Luteolin 7-O-Glucoside	176.35	116.02	217.26	112.98
Apigenin 7-O-Glucoside	380.92	327.96	419.08	312.56
Oleuropein	427.962	520.11	258.93	245.5
Apigenin	34.2713	29.23	43.27	21.65
Catechin hydrate	-	-	86.17	-

verbascoside), flavones (luteolin, diosmetin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, and diosmetin-7-O-glucoside), flavonols (rutin), flavan-3-ols (catechin), and substituted phenol (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid).

Rocchetti et al. (2019) reported that homogenizer-assisted extraction using methanol 100% produced an extract of *M. oleifera* leaves with the highest amounts of phenolic compounds. These findings demonstrate that each extraction method promoted the recovery of specific phenolic subclasses with different efficiencies. So we can conclude that extraction methods and conditions (solvent, time, temperature, ...) influence the type of phenolic compounds of leaves extracts.

3.3. Antibacterial activity

Table 3 shows the MIC values for the four olive cultivars obtained using the microdilution method. The values varied between 32 and 128 µg/ml, without large difference between the four olive cultivars extracts. The Chetoui methanol extract had the best antibacterial activity against all gram negative bacteria and against all *S. aureus* strains, with a MIC and MBC values of 32 µg/ml. In addition, this extract showed a good antifungal activity against *Candida* strains, with a MIC between 32 and 64 µg/ml Techathuvanan et al. (2014) found that *Olea europaea* leaf extracts have antimicrobial activities against foodborne pathogens, such as *S. aureus*, *E. coli*, *Salmonella* spp., and *L. monocytogenes*, with MIC values ranging between 1.4 and 5.2 mg/ml. This good antibacterial activity of Tunisian olive varieties could be attributed to oleuropein, which is the major compound identified in the present study or this is may be due to the climatic difference between tunisian varieties and turkich variety.

Liu et al. (2017) demonstrated that at 62.5 mg/ml, ethanolic extracts of olive leaves almost completely inhibited the growth of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enteritidis*. In addition, they observed that ethanol extracts were able to destroy the flagella of *L. monocytogenes* and to reduce the motility of the pathogens. Furthermore they found that the extracts inhibited biofilm formation in *L. monocytogenes* and *S. enteritidis*. According to Masoko and Makgapeetja (2015), the methanol extract of *Olea africana* have a good antibacterial activity against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*, with MIC values comprised between 0.24 and 0.63 mg/ml. The differences observed between the present study results and these findings are probably due to differences between the two olive species.

Owen et al. (2003) also reported that olive leaves have antimicrobial activity against *E. coli*, *S. aureus*, *B. cereus* and *S. typhi*. The results reported in the present study show similarity with previous ones (Markin et al., 2003; Pereira et al., 2007; Sudjana et al., 2009; Lee and Lee, 2010; Gökmen et al., 2014) about the antimicrobial activity of olive leaves extracts. According to Pereira et al. (2007), the antimicrobial mechanism of the extract consists in the denaturation of the proteins and increasing cell membrane permeability. Similarly, Lee and Lee (2010) reported that the combined phenols mixture prepared from an olive leaves extract showed inhibition effects against *B. cereus* and *S. enteritidis*. In addition, Gökmen et al. (2014) showed that the MICs of olive leaves extract against *L. monocytogenes*, *E. coli* O157, *E. sakazakii* and *P. aeruginosa* was ≥32 µg mL⁻¹, while the MIC against *B. cereus*, *S. aureus*, *E. faecalis*, *P. vulgaris*, *E. coli*, *Salmonella typhimurium* was ≥16 mg mL⁻¹.

3.4. Antibiofilm activity

The four olive cultivars showed variable effects on the development of a preformed biofilm (Fig. 1). Chetoui and Meski extracts showed the best antibiofilm activity against *P. aeruginosa*, *E. coli*, *B. cereus*, *E. faecalis*, *S. aureus*, *S. aureus MTR* and *C. albicans*, with inhibition values of >50% at MIC doses. In detail, they all inhibit the biofilm of all the tested strains between 72 and 89.8% at doses of 2MIC, and the difference between these two concentrations was statistically significant. The Chetoui cultivar showed important anti-biofilm activities against *P. aeruginosa*, *B. cereus*, *S. aureus*, *C. albicans*, *E. faecalis* and *E. coli*, with percentage of

Table 3
Determination of MIC (µg/mL) and MBC (µg/mL) of methanol extracts of olive leaves of four different cultivars.

Strains	MCh		MM		MO ^b		MJ		Ampicillin
	MIC ^a	MBC ^b	MIC	MBC	MIC	MBC	MIC	MBC	MIC ^a
Gram positive bacteria									
<i>Pseudomonas aeruginosa</i> CI122	64	64	64	128	128	128	128	128	0.012
<i>Pseudomonas aeruginosa</i> CI311	64	64	64	128	128	128	128	128	0.012
<i>Staphylococcus aureus</i> ATCC25923	64	32	32	64	64	128	128	128	0.08
<i>Staphylococcus aureus</i> MRSA ¹¹²	64	32	32	128	64	128	128	128	0.09
<i>Staphylococcus aureus</i> MRSA ¹²⁶	- ^c	32	32	128	64	128	128	128	50
<i>Staphylococcus aureus</i> MRSA ²³⁴	64	32	32	128	64	128	128	128	100
<i>Staphylococcus aureus</i> MRSA ⁶⁷⁵	64	32	32	128	64	128	128	128	50
<i>Bacillus cereus</i> ATCC 11778	64	64	32	128	64	128	64	128	0.078
<i>Bacillus subtilis</i> ATCC 14579	64	64	32	128	64	128	64	128	32
Gram negative bacteria									
<i>Escherichia coli</i> ATCC 25922	64	32	64	64	64	128	64	128	0.025
<i>Klebsiella pneumoniae</i> CI29	32	32	64	64	64	128	128	128	0.25
<i>Escherichia coli</i> CI423	64	32	64	64	64	128	128	128	0.025
<i>Enterococcus faecalis</i> ATCC29212	32	32	64	64	64	128	64	128	0.2
<i>Enterococcus faecium</i> CI234	32	32	64	64	64	128	64	128	0.025
Yeast									
<i>Candida glabrata</i> ATCC 90030	MIC	MFC	MIC	MIC	MFC	MIC	MIC	MFC	Amphotericin B
<i>Candida albicans</i> ATCC 90028	32	64	64	64	64	64	64	64	0.5
<i>Candida parapsilosis</i> ATCC 22019	32	64	64	64	64	64	125	64	0.5
<i>Candida kreusei</i> ATCC 6258	32	64	32	64	64	64	64	64	0.5

^a Minimal inhibitory concentration in µg/ml.

^b Minimal bactericidal concentration in µg/ml.

inhibition comprised between 83 and 93% at 2MIC values (Fig. 1). Also the methanol extracts from Jarboui and Oueslati showed a good antibiofilm activity against all the tested strains, with percentages of inhibition ranging from 54.5 to 83.8% at a concentration of 2 MIC (Fig. 1 C D). Only the Jarboui extract presented lesser level of biofilm inhibition activity (29.3%) against *P. aeruginosa* at MIC dose. For Jarboui and Oueslati cultivars, a significant difference was observed between the two concentrations (MIC and 2MIC). To the best of our knowledge, the present study is the first one about the antibiofilm activity of methanol extract of olive leaves obtained from these four Tunisian olive cultivars on the selected microorganisms.

In a previous study, it was shown that the olive mill waste was able to reduce biofilm formation in *E. coli* (Carraro et al., 2014). On the contrary, according to Liu et al. (2017), olive leaves extracts slightly inhibited the biofilm formation in *L. monocytogenes* at 7.8 mg/ml. In the same study, the biofilm formation in *S. enteritidis*, was more inhibited (74% at 15.6 mg/ml).

The observed antibiofilm activity is generally attributable to the high concentrations of phenolic compounds, such as oleuropein, which

antibiofilm activity was previously tested (Carraro et al., 2014) or may be due to a synergistic effect of some phenols contained in the olive extracts.

The mechanisms of antibacterial and antibiofilm activities of oleuropein are not completely understood. However, phenolic compounds have the ability to increase the permeability of cell membranes, thus facilitating their rupture (Taweekhaisupapong et al., 2012). Casas-Sanchez et al. (2007) reported the interaction of oleuropein with phosphatidylglycerol at the surface of the bacterial cell membrane, causing changes that lead to the disruption of the cell envelope. The observed antimicrobial activities could be due, at least in part, to these properties of the phenol derivatives contained in the four olive leaves extracts.

3.5. Antioxidant and acetylcholinesterase (AChE) inhibition activity

DPPH and ABTS are free radicals that provide information about the potential of the extract components to inhibit oxidative cell damages by preventing reactive radical species from attacking key biomolecules in biological and food systems. They are also frequently used by the food industry and agricultural researchers to measure the antioxidant

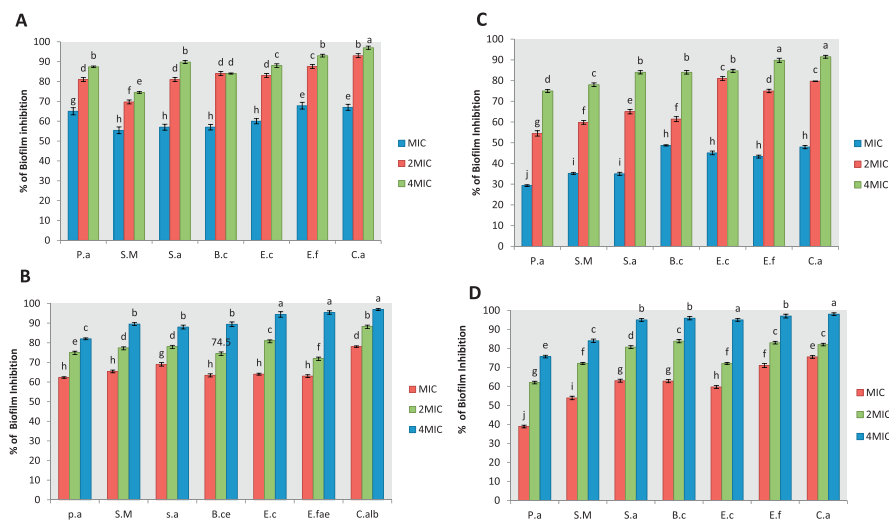


Fig. 1. The effect of different concentrations of Chetoui (A), Meski(B), Jarboui(C) and Oueslati (D) methanolic extracts on biofilm formation expressed as percentage of inhibition. *P. a:* *Pseudomonas aeruginosa* ATCC 9023, *S.M:* *Methicillin resistant S. aureus*, *S. a:* *Staphylococcus aureus* ATCC25923, *B. c:* *Bacillus subtilis* ATCC6633, *E. c:* *Escherichia coli* ATCC 25922, *E. f:* *Enterococcus faecalis* ATCC29212, *C. a:* *Candida albicans* ATC 90028. Results are means of three different experiments. Means with different letters were significantly different at the level of $p < 0.05$.

capacities of foods. The results on the antioxidant activity using the DPPH assay of the four olive cultivars are shown in Fig. 2. It is apparent that the Chetoui and Oueslati methanol extracts have the highest antioxidant activity (87.05 and 70.47%, respectively). Noteworthy, the Chetoui extract exhibited a better DPPH scavenging activity than the reference antioxidant BHT (81.5%). Instead, a lesser activity was observed for the methanol extracts of Meski and Jarbouli cultivars (47.3 and 61.6%, respectively). These differences were statistically significant and in good agreement with Brahmi et al. (2013) that reported a greater activity for the methanol extracts of various olive organs cultivated in the North of Tunisia than those obtained from the southern cultivars (see Fig. 3).

The antioxidant activity was evaluated using also the ABTS methods, obtaining results that substantially confirmed the trend observed with the DPPH assay. Fig. 2 shows that Chetoui and Meski extracts had the best antioxidant activity by this method compared to other cultivars and the reference antioxidant Trolox. These high antioxidant activities could be attributed to their high levels of Total phenolic content (TPC) and flavonoids, especially can be due to oleuropein, which was known by its antioxidant activity (Özcan and Matthaus, 2017). As demonstrated by Cheung et al. (2003), free radical scavenging activity is strongly related

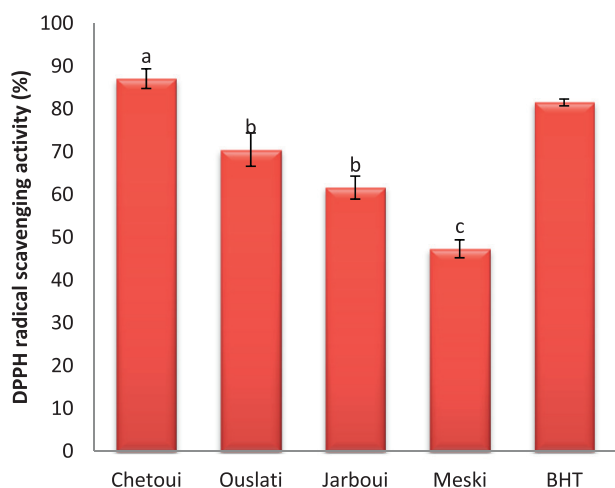


Fig. 2. Free radical scavenging capacities of methanolic extracts from the leaves of Chetoui, Meski, Ouslati and Jarbouli cultivars by the ABTS assay. Values are expressed as means \pm standard deviation ($n = 3$). Means with different letters were significantly different at the level of $p < 0.05$.

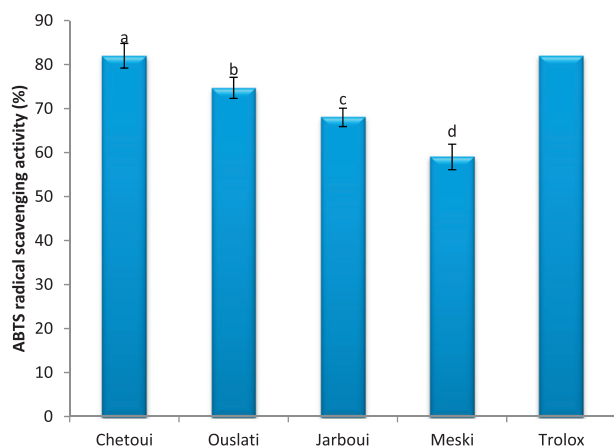


Fig. 3. Free radical scavenging capacities of methanolic extracts from leaves of Chetoui, Meski, Ouslati and Jarbouli cultivars by the ABTS assay. Values are expressed as means \pm standard deviation ($n = 3$). Means with different letters were significantly different at the level of $p < 0.05$.

to the presence of polyphenol compounds. Indeed, the results of the HPLC analysis demonstrated the abundance of oleuropein, a wellknown antioxidant derivative (Fukumoto and Mazza, 2000; Stamatopoulos et al., 2014). The antioxidant activity was correlated with the presence of phenolic compounds also in other studies (Fukumoto and Mazza, 2000; Brahmi et al., 2013).

Generally, it can be observed that all olive leaf extracts exhibited high radical scavenging activity even if it is well known that antioxidant capacity is influenced by several factors, among which harvesting period and cultivar (Yorulmaz et al., 2012; Brahmi et al., 2015). The results reported by Blasi et al. (2016) showed that the antioxidant activity of four Italian cultivars was the highest in March, when the leaves had completed their growth, while it decreased slightly until September, when started the ripening of the fruit. They demonstrated that The lowest values had been found for all cultivar samples harvested in December (from 40.9 of Frantoio to 67.1% of Dolce Agogia), while olive leaves from Leccino cultivar harvested in March exhibited the highest activity (86.1%). Further more the antioxidant activity determined by ABTS showed significant differences also between Moraiolo cultivar and Frantoio and between Moraiolo and Leccino ($P = 0.05$) with regard to September harvest.

Furthermore, the results reported by Abaza et al. (2011) showed that the DPPH radical scavenging activity increased with the extract concentration (at 0.5 mg/mL the activity was 59.74%, using 70% EtOH). But Urbani et al. (2015) reported that all methanolic saffron samples (Italy) showed very low radical scavenging activity with DPPH method while higher values were observed with hydrolysed sample but saffron methanolic extracts exhibited higher antioxidant activity with ABTS test than the respective hydrolyzed, they also demonstrated a strong correlation between antioxidant activity and secondary metabolit content (Urbani et al., 2015).

Akbaş et al. (2017) tested the effect of drying on antioxidant activity of Turkish olive leaves they demonstrated that the antioxidant activity of olive leaves heated in microwave 180 W change between 76.99% (Akdeniz Yerli) and 82.26% (Sarı Ulak) and they reported that antioxidant activity values of leave samples heated in microwave 360 W varied between 81.20% (Yaglık) and 82.24% (Gemlik).

We can conclude that antioxidant activity depend on cultivars, extraction and drying methods and influenced by chemical composition in the tested plants.

The methanol extracts of Chetoui and Meski also showed the highest rates of acetylcholinesterase inhibition capacity ($IC_{50} = 500 \mu\text{g/ml}$) (Table 1). Results were higher than galanthamine (positive control; $IC_{50} = 0.4 \mu\text{g/ml}$), a drug approved for the treatment of the Alzheimer's disease. Crowch and Okello (2009) found that galanthamine was 500 times more potent than the aqueous extracts of *Rhamnus prinoides* ($IC_{50} = 0.2 \text{ mg/ml}$). Ferreira et al. (2006) reported the extract obtained from *Hypericum undulatum* as a powerful inhibitor, showing 81.7% inhibition at 5 mg/ml.

To the best of our knowledge, the present study is the first to report the inhibition of acetylcholinesterase of extracts obtained from these olive cultivars. A positive correlation was observed between the IC_{50} values and the total phenols and flavonoids contents.

Acetylcholinesterase is the principal enzyme involved in the hydrolysis of acetylcholine. The great reduction of this neurotransmitter in the cerebral cortex is a significant factor in Alzheimer's disease. Therefore, the research of new compounds with the capacity of inhibiting that enzyme has been considered as a promising strategy for the treatment of neurological disorders in which an insufficient cholinergic neurotransmission is involved.

4. Conclusions

In summary, the present study evaluated the differences in total phenols and flavonoids contents of four different leaves extracts obtained from Tunisian olive cultivars. Moreover, by means of HPLC analyses, the

differences in type and amounts of each compound were assessed for every olive cultivar. The Chetoui cultivar was the richest in flavonoids and polyphenols. Furthermore, in this cultivar oleuropein was the main phenol. Chetoui and Meski showed the highest antioxidant and acetylcholinesterase inhibition activities. Significant differences were observed for antioxidant and acetylcholinesterase inhibition activities for the extracts of the four olive cultivars. It has been shown that Chetoui and Meski methanol extracts exhibit a good antibacterial activity against Gram positive and negative strains and were able to inhibit the biofilm formation on polystyrene surface, with inhibition values of >50% with at MIC doses.

In addition, this study provides data for supporting the use of Tunisian olive leaves extracts as natural antioxidant agents, and confirms that these extracts represent a significant source of phenolic compounds. Therefore, it is suggested that further works should be performed on the isolation and identification of the antimicrobial, antioxidant, antibiofilm and anticholinesterase active constituents and their possible synergistic/antagonistic interactions.

Declarations

Author contribution statement

Hayet Edziri: Conceived and designed the experiments; Wrote the paper.

Raouf Jaziri, Luc Verschaevé, Dalenda Boujnah, Mahjoub Aouni: Contributed reagents, materials, analysis tools or data.

Hechmi Chehab, Mohamed Hammami: Analyzed and interpreted the data.

Guido Flamini: Performed the experiments.

Maha Mastouri: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- Abaza, L., Youssef, N.B., Manai, H., Haddada, F.M., Methenni, K., Zarrouk, M., 2011. Chétoui olive leaf extracts: influence of the solvent type on phenolics and antioxidant activities. *Grasas Aceites* 62, 96–104.
- Akbaş, U.G., Nurhan, U., Juhaimi, F., Özcan, M.M., Ghafoor, K., Babiker, E.E., Gbemisola, F., 2017. The effect of drying on phenolic compound, antioxidant activity, and mineral contents of leaves of different olive varieties. *Int. J. Food Mic* 251, 48–66.
- Bahloul, N., Bellili, S., Aazza, S., Chérif, A., Faleiro, M.L., Antunes, M.D., Miguel, M.G., Mnif, W., 2016. Aqueous extracts from Tunisian *Diplotaxis*: phenol content, antioxidant and anti-acetylcholinesterase activities, and impact of exposure to simulated gastrointestinal fluids. *Antioxidants* 5, 12–15.
- Blasi, F., Urbani, E., Simonetti, M.C., Chiesi, Cl., Cossignani, L., 2016. Seasonal variations in antioxidant compounds of *Olea europaea* leaves collected from different Italian cultivars. *J. Appl. Bot. Food Qual.* 89, 202–207.
- Benavente-Garcia, O., Castillo, J., Lorente, J., Ortuno, A., Del Rio, J.A., 2000. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem.* 68, 457–462.
- Brahmi, F., Mechri, B., Dhibi, M., Hammami, M., 2013. Variation in antioxidant activity and phenolic content in different organs of two Tunisian cultivars of *Olea europaea* L. *Acta Physiol. Plant.* 36, 169–178.
- Brahmi, F., Mechri, B., Dhibi, M., Hammami, M., 2015. Effect of growth stage and solvent extract on the antioxidant potential of olive leaves. *J. Plant Sci.* 3, 1–7.
- Carraro, L., Fasolato, L., Montemurro, F., Martino, M.E., Balzan, S., Servili, M., Novelli, E., Cardazzo, B., 2014. Polyphenols from olive mill waste affect biofilm formation and motility in *Escherichia coli* K-12. *Mic. Biotech.* 7, 265–275.
- Casas-Sanchez, J., Asuncion Alsina, M., Herrlein, M.K., Mestres, C., 2007. Interaction between the antibacterial compound, oleuropein, and model membranes. *Colloid Polym. Sci.* 285, 1351–1360.
- Changwei, A., Anping, L., Abdelnaser, A.E., Tran, D.X., Shinkichi, T., 2008. Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. *Ext. Food Cont.* 19, 940–948.
- Cheung, L.M., Cheung, P.C.K., Oi, V.E.C., 2003. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* 81, 249–255.
- Covas, M.I., 2007. Olive oil and the cardiovascular system. *Pharmacol. Res.* 55, 175–186.
- Crowch, C.M., Okello, E.J., 2009. Kinetics of acetylcholinesterase inhibitory activities by aqueous extracts of *Acacia nilotica* (L.) and *Rhamnus prinoides* (L'Hér.). *Afr. J. Pharm. Pharmacol.* 3, 469–475.
- Edziri, H., Mastouri, M., Mahjoub, M.A., Patrich, G., Matieu, M., Ammar, S., Ali, S.M., Laurent, G., Zine, M., Aounia, M., 2010. Antibacterial, antiviral and antioxidant activities of aerial part extracts of *Peganum harmala* L. grown in Tunisia. *Toxicol. Environ. Chem.* 7, 1283–1292.
- El, S.N., Karakaya, S., 2009. Olive tree (*Olea europaea*) leaves: potential beneficial effects on human health. *Nutr. Rev.* 67, 632–638.
- Felhi, S., Hajlaoui, H., Ncir, M., Bakari, S., Ktari, N., Saoudi, M., Gharsallah, N., Kadri, A., 2016. Nutritional, phytochemical and antioxidant evaluation and FT-IR analysis of freeze dried extracts of *Ecballium elaterium* fruit juice from three localities. *Food Sci. Tech.* 36, 646–655.
- Ferreira, A., Proenca, C., Serralheiro, M.L.M., Araujo, M.E.M., 2006. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. *J. Ethnopharmacol.* 10, 831–837.
- Fukumoto, L.R., Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* 48, 3597–3604.
- Gökmen, U., Kara, R., Akkaya, L., Torlak, E., Önen, A., 2014. Evaluation of antimicrobial activity in olive (*Olea europaea*) leaf extract. *Am. J. Microbiol.* 5, 37–40.
- Gonzalez, M., Zarzuelo, A., Gamez, M.J., Utrilla, M.P., Jimenez, J., Osuna, I., 1992. Hypoglycemic activity of olive leaf. *Planta Med.* 58, 513–515.
- Jacob, S.J.P., Shenbagaraman, S., 2011. Evaluation of antioxidant and antimicrobial activities of the selected green leafy vegetables. *Int. J. Pharm. Tech. Res.* 3, 148–152.
- Kallithraka, S., Mohdaly, A.A.A., Makris, D.P., Kefalas, P., 2004. Determination of major anthocyanin pigments in Hellenic native grape varieties (*Vitis vinifera* sp.). *J. Food Compos. Anal.* 18, 375–386.
- Khayyal, M.T., El-Ghazaly, M.A., Abdallah, D.M., Nassar, N.N., Okpanyi, S.N., Kreuter, M.H., 2002. Blood pressure lowering effect of an olive leaf extract (*Olea europaea*) in L-NAME induced hypertension in rats. *Antihyp* 11, 797–802.
- Lavelli, V., 2002. Comparison of the antioxidant activities of extra virgin olive oils. *J. Agric. Food Chem.* 50, 7704–7708.
- Lee, O.H., Lee, B.Y., 2010. Antioxidant and antimicrobial activities of individual and combined Phenolics in *Olea europea* leaf extract. *Bioresour. Technol.* 101, 3751–3754.
- Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids—Measurement and characterization by UV-VIS. In: Lichtenthaler, H.K. (Ed.), *Current Protocols in Food Analytical Chemistry (CPFA)*, pp. 726–728 (Supplement 1), New York.
- Liu, Y., McKeever, L., Malik, N.S., 2017. Assessment of the antimicrobial activity of olive leaf extract against foodborne bacterial pathogens. *Front. Microbiol.* 8, 113.
- Markin, D., Duek, L., Berdicevsky, I., 2003. *In vitro* antimicrobial activity of olive leaves. *Mycoses* 46, 132–136.
- Masoko, P., Makgapeetja, D., 2015. Antibacterial, antifungal and antioxidant activity of *Olea africana* against pathogenic yeast and nosocomial pathogens. *BMC Complement Altern. Med.* 15, 409–411.
- Meirinhos, J.B.M., Silva, P., Valentao, R.M., Seabra, J.A., Pereira, A., Dias, P.B., Andrade, F., Ferreres, R., 2005. Analysis and quantification of flavonoid compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. *Nat. Prod. Res.* 19, 189–195.
- Montedoro, G.F., Servili, M., Baldioli, M., Miniati, E., 1992. Simple and hydrolysable compounds in virgin olive oil. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* 40, 1571–1576.
- Morse, S.S., 2004. Factors and determinants of disease emergence. *Rev. Sci. Tech.* 23, 443–451.
- Novy, P., Davidova, H., Serrano-Rojero, C.S., Rondevaldova, J., Pulkrabek, J., Kokoska, L., 2015. Composition and antimicrobial activity of *Euphrasia rostkoviana* Hayne essential oil. *Ev. Based Compl. Alt. Med.* 7, 34–41.
- Owen, R.W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B., Bartsch, H., 2003. Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food Chem. Toxicol.* 41, 703–717.
- Özcan, M.M., Matthauss, B., 2017. A review: benefit and bioactive of olive (*Olea europaea* L.) leaves. *Eur. Food Res. Technol.* 243, 89–99.
- Özcan, M.M., Al Juhaimi, F., Uslu, N., Ghafoor, K., Ahmed, I.A.M., Babiker, E.E., 2019. The Effect of olive varieties on fatty acid composition and tocopherol contents of cold pressed virgin olive oils. *J. Oleo Sci.* 68 (4), 307–310.
- Pereira, A.P., Ferreira, I.C.F.R., Marcelino, F., Valentao, P., Andrade, P.B., Seabra, R., Estevinho, L., Bento, A., Pereira, J.A., 2007. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrancosa) leaves. *Molecules* 12, 1153–1163.
- Rocchetti, G., Blasi, F., Montesano, D., Ghisoni, S., Marcotullio, M., 2019. Impact of conventional/non-conventional extraction methods on the untargeted phenolic profile of *Moringa oleifera* leaves. *Food Res. Int.* 115, 319–327.
- Sahin, S., Bilgin, M., 2012. Study on oleuropein extraction from olive tree (*Olea europaea*) leaves by means of SFE: comparison of water and ethanol as co-solvent. *Separ. Sci. Technol.* 47, 2391–2398.
- Stamatopoulos, K., Katsoyannos, E., Chatzilazarou, A., 2014. Antioxidant activity and thermal stability of oleuropein and related phenolic compounds of olive leaf extract

- after separation and concentration by salting-out-assisted cloud point extraction. *Antioxidants* 3, 229–344.
- Sudjana, A.N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N., Riley, T.V., Hammer, K.A., 2009. Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. *Int. J. Antimicrob. Agents* 33, 461–463.
- Taweechaisupapong, S., Ngaonee, P., Patsuk, P., Pitiphat, W., Khunkitti, W., 2012. Antibiofilm activity and post antifungal effect of lemongrass oil on clinical *Candida dubliniensis* isolate. *S. Af. J. Bot.* 78, 37–43.
- Techathuvanan, C., Reyes, F., David, J.R., Davidson, P.M., 2014. Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. *J. Food Prot.* 77, 269–275.
- Urbani, E., Blasi, F., Chiesi, C., Maurizi, A., Cossignani, L., 2015. Characterization of volatile fraction of saffron from central Italy (Cascia, Umbria). *Int. J. Food Prop.* 18, 2223–2230.
- Yorulmaz, A., Poyrazoğlu, E.S., Özcan, M.M., Tekin, A., 2012. Phenolic profiles of Turkish olives and olive oils. *Eur. J. Lipid Sci. Tech.* 14, 1083–1093.
- Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64, 555–559.