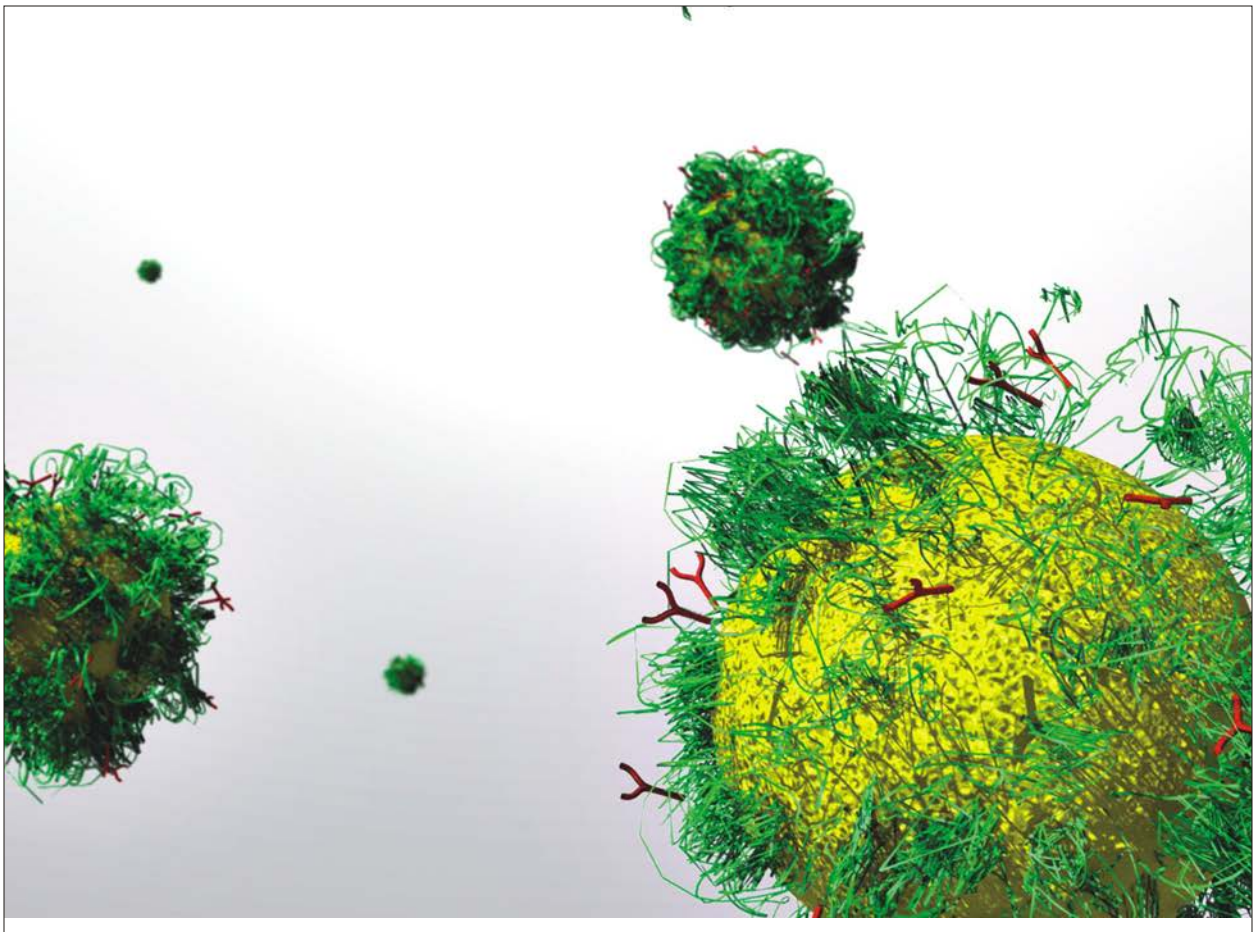

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Composition and Antioxidant and Antimicrobial Activity of the Volatile Oils from *Olea europaea* L. Fruit and Stem

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ABSTRACT

The aims of this study was to analyze the chemical composition of fruits and stem volatile oils of *Olea europaea* L. cv. Chemchali and to test the efficacy of volatile oils against four pathogenic bacteria and four phytopathogen strains and their antioxidant activity. The GC-MS analysis revealed 38 compounds representing 88.5 and 73.6% of the total oils containing 3-ethenylpyridine (12.5%), (E)-2-decenal (11.4%) and 2-ethylbenzaldehyde (7.7%) in fruit and nonanal (9.9%), (E)-2-decenal (9.6%) and benzyl alcohol (9.00%) in stem as major components, respectively. The antibacterial and antifungal activities of stem volatile oils were relatively good. Therefore, the stem of *Olea europaea* L. demonstrated higher activities against tested fungi and bacteria than the fruit. The DPPH and ABTS-radical-scavenging activities of the oils showed that the lowest EC50 value was detected in volatile oil from fruit.

Key words: *Olea europaea* L., organ, volatile oils, volatile constituents, antioxidant activity, antibacterial activity, antifungal activities

INTRODUCTION

Essential oils of plants are of growing interest both in the industry and scientific research because of their antibacterial, antifungal and antioxidant properties and make them useful as natural additives in foods (Pattnaik *et al*., 1997). Free radical oxidation of the lipid components in food due to the chain reaction of lipid peroxidation is a major strategic problem for food manufacturers. Due to undesirable influences of oxidized lipids on the human organisms, it is essential to decrease lipid peroxidation products in food (Karpinska *et al*., 2001). Reactive oxygen species are reported to be a causative agent of various diseases such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson's disease (Perry *et al*., 2000). Plant essential oils and their extracts have had a great usage in folk medicine, food flavoring, fragrance and pharmaceutical industries (Kusmenoglu *et al*., 1995). *Olea europaea* is an emblematic species and one of the most widespread and economic important fruit trees in the Mediterranean basin. Olive and its different products are basic ingredients of the Mediterranean diet which provide numerous

health benefits. In fact, olive (*Olea europaea* L.) is one of the most important fruit crops throughout the Mediterranean Basin. The species include thousands of cultivars, most derived from empirical selections over many centuries, now well-adapted to different local conditions. Aroma compound composition in table olives depends on several factors, such as genetics, ripening degree of fruits and processing conditions (Garrido Fernandez *et al.*, 1997; Ruiz *et al.*, 2005). A pleasant fragrance derives from the equilibrium of a number of volatile substances, such as hydrocarbons, alcohols, aldehydes, ketones, esters and other compounds.

To the best of our knowledge, there has been little information regarding antioxidant activities of different parts of olive (mostly reported on fruit), especially stem. Therefore, the aim of the present work was carried out to study *in vitro* antioxidant, antibacterial and antifungal activities of the volatile oils in fruits and stems of *Olea europaea* L. cv. *Chemchali* in addition to evaluate the component of volatile oils by GC-MS.

MATERIALS AND METHODS

Plant materials: Fruits and stems of the cultivar *Chemchali* of *Olea europaea* L. were collected from the south (Gafsa) of Tunisia. Olive fruits were isolated manually from the aerial parts in our laboratory to obtain a weight of 500-700 g of each part. The stems were separated from the other parts. Voucher specimens have been deposited in the Herbarium of the Laboratory of Biochemistry, Faculty of Medicine of Monastir, Tunisia.

Extraction method: Each sample (250 g) of the fresh fruits and stems was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The volatiles obtained after trapping in diethyl ether were dried over anhydrous sodium sulphate, evaporated and concentrated under a gentle stream of N_2 and stored at 4°C until tested and chemically analyzed.

Identification of the volatile constituents: GC analyses were accomplished with an HP-5890 series II instrument equipped with a HP-5 capillary column (30 m×0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and detector temperatures, 250°C; carrier gas, nitrogen (2 mL min^{-1}); detector, dual FID; split ratio, 1:30; injection, 0.5 μL. The identification of the components was performed, by comparison of their retention times with those of pure authentic samples and by means of their Linear Retention Indices (LRI) relative to the series of n-hydrocarbons.

Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses were performed with a Varian CP 3800 gas chromatograph (Varain, Inc. Palo Alto, CA) equipped with a DB-5 capillary column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany; 30 m = 0.25 mm; coating thickness×0.25 mm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature at 250 and 240°C, respectively; oven temperature was programmed from 60-240°C at 3°C/min; carrier gas, helium at 1 mL min^{-1}; splitless injector. Identification of the constituents was based on comparison of the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons and on computer matching against commercial (NIST 98 [U.S. National Institute of Standards and Technology] and ADAMS (Adams, 1995) and homemade library mass spectra

built from pure substances and components of known samples and MS literature data (Stenhagen *et al.*, 1974; Massada, 1976; Jennings and Shibamoto, 1980; Swigar and Silvestein, 1981; Davies, 1990; Adams, 1995). Moreover, the molecular weights of all the identified substances were confirmed by gas chromatography-chemical ionization mass spectrometry (GC-CIMS), using methanol as chemical ionization gas.

Antioxidant activity

DPPH radical scavenging assay: Radical scavenging activity was determined by a spectrophotometric method based on the reduction of a methanol solution of DPPH as reported by Blois (1958). One millititre of various concentrations of the volatiles in methanol was added to 1 mL of a 0.004% methanol solution of DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. Then the absorbance was measured with a UV-VIS a spectrophotometer (Secommam, U-1789, France), at 517 nm against a blank. Percent inhibition of the free radical DPPH, was calculated according to Equation:

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

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. Concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against sample concentration. Tests were carried out in triplicate. Butylated hydroxytoluene (BHT) was used as a positive control.

$ABTS^+$radical cation scavenging: The $ABTS^+$ radical cation scavenging activity of each volatile oil and ascorbic acid (control) was determined according to the literature (Yvonne *et al.*, 2005). In brief, 5.0 mL of a 7.0 mM ABTS solution was treated overnight in the dark with 88.0 μL 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, with 30°C. Free radical scavenging activity was assessed by mixing 1.0 mL of diluted $ABTS^+$ radical cation with 10 μL of sample and monitoring the change in absorbance after 0, 0.5 and 1 min and than after 5 min intervals, until a steady state was achieved. The antioxidant capacity of the volatile fractions was expressed as IC_{50}, the concentration necessary for 50% reduction of $ABTS^+$.

Antibacterial and antifungal activities

Microbial strains: The employed bacterial strains were *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 27950, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27950. In the case of yeasts, *Candida glabrata* ATCC 90030, *C. kreusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028.

Micro-well dilution assay: Minimum Inhibitory Concentration (MIC) values were determined by micro-titre plate dilution method (Sahin *et al.*, 2004). The inocula of the bacteria and yeasts were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard

turbidity. The volatile oils were first dissolved in 10% DMSO and then diluted to the highest concentration (10 mg mL^{-1}) to be tested and then serial twofold dilutions were made in 10 mL sterile test tubes containing nutrient broth.

In brief, the 96-well plates were prepared by dispensing into each well 95 µL of nutrient broth and 5 µL of the inocula. An aliquot (100 µL) from the stock solutions of volatiles initially prepared at the concentration of 10 mg mL^{-1}, was added into the first wells. Then 100 µL from their serial dilutions were transferred into each consecutive well. The last well containing 195 µL of nutrient broth without compound and 5 µL of the inocula on each strip was used as negative control. The final volume in each well was 200 µL. The plate was covered with a sterile plate sealer and then incubated for18 h at 37°C. The MIC was defined as the lowest concentration of the compounds able to inhibit the growth of microorganisms, after incubation. The results were expressed in microgram per milliliter (Smania *et al.*, 2006; Zacchino, 2001).

Statistical analysis: All experiments were performed in triplicate. Data were recorded as means±standard deviations and were analyzed with SPSS (2003). Differences were considered significant at $p<0.05$.

RESULTS

Chemical composition of volatile oils: The volatile oil yields of *Olea europaea* L. var. *chemchali* varied between different parts with 0.90% MS in fruits and 0.92% MS in stems. GC/MS analysis of the volatiles of olive fruits and stems led to the identification of their components which are listed in Table 1 among their percentages and retention indices. The fruits volatile oil composition was characterized by high levels of 3-ethenylpyridine, (E)-2-decenal and 2-ethylbenzaldehyde with, respectively 12.5, 11.4 and 7.7%. Nonanal, (E)-2-decenal and benzyl alcohol were the main components in the stem volatile oil with, respectively 9.9, 9.6 and 9.0%. Other, representative compounds were found as nonanal (6.6%), (E,E)-2,4-decadienal (5.2%) and (Z)-2-heptenal (4.9%) in the fruit and phenol (8.0%), linalool (5.7%) and methyl salicylate (4.9%) in the stem. The minor fraction in the fruits contains other compounds whose rates do not exceed 1%; globulol, heptanal, (Z)-4-decenal, tetradecane, hexadecane and (E)-β-damascenone.

A total of 38 volatile c ompounds were detected, namely 3 esters, 8 alcohols, 2 terpenoids, 17 aldehydes, 3 ketones, 3 saturated hydrocarbons, 1 pyridine and 1 carboxylic acid (Table 1). The identified compounds in fruits and stems represented 88.5 and 73.6% of the total volatile oil, respectively. Quantitatively, alcohols and aldehydes were found to be the most important groups of volatiles in the fruit and stem of *Olea europaea* L. var. *chemchali*. The percentage of alcohols represented 15.7 for fruits and 19.9% for stems (Table 1). This class was characterized by the presence of appreciable relative percentage of phenylethyl alcohol (3.1%) in fruit and benzyl alcohol in stems (9.0%). The fruits of var. *chemchali* were characterized by the highest percentage of aldehydes (52.7%) because their important amounts of (E)-2-decenal (11.4%) and 2-ethylbenzaldehyde (7.7%) (Table 1). In comparison to fruits, the stems of the variety *Chemchali* are characterized by their superiority in total C6 aldehyde and total C6 alcohol (Fig. 1). The main ester was represented by methyl salicylate, produced in the stems in percentage of 4.9% (Table 1). In fact, the ester (Z)-3-hexenyl acetate was found only in the fruits. The highest relative percentage of terpenoids was found in stems (8.5%), with linalool (5.7%) as the most abundant component. However, the carboxylic acids and pyridine were present only in the fruits, with an important percentage of pyridine (12.5%).

Table 1: Volatile compounds[a] (%) evaluated by GC-MS in the fruit and stem samples from the olive cultivar grown in the area studied

Constituents	LRI[b]	Fruit	Stems
Alcohols			
(E)-3-hexenol	855	2.9	-
1-hexanol	868	2.0	Tr[c]
Phenol	977	-	8.0
Benzyl alcohol	1036	2.9	9.0
1-octanol	1069	1.3	Tr
Phenylethyl alcohol	1111	3.1	2.9
4-vinylguaiacol	1316	2.9	-
Globulol	1585	0.6	Tr
Identified alcohols (%)		15.7	19.9
Esters			
(Z)-3-hexenyl acetate	1005	1.0	
1-hexyl acetate	1009	1.1	-
Methyl salicylate	1190	-	4.9
Identified esters (%)		2.0	4.9
Aldehydes			
(E,Z)-2,4-hexadienal	801	2.7	4.7
Heptanal	904	0.5	Tr
(Z)-2-heptenal	938	4.9	Tr
Benzaldehyde	957	3.4	3.8
Octanal	1002	-	1
(E,Z)-2,4-heptadienal	1016	0.5	Tr
Phenylacetaldehyde	1045	2.1	Tr
(E)-2-octenal	1064	2.1	-
Nonanal	1103	6.6	9.9
(E)-2-nonenal	1165	1.0	Tr
(Z)-4-decenal	1192	0.8	-
(E,E)-2,4-nonadienal	1218	1.6	-
2-ethylbenzaldehyde	1221	7.7	-
(E)-2-decenal	1266	11.4	9.6
(E,Z)-2,4-decadienal	1295	2.2	-
(E,E)-2,4-decadienal	1318	5.2	3.8
(E)-2-undecenal	1349	-	2.1
Identified aldehydes (%)		52.7	34.9
Terpenoids			
Linalool	1098	0.5	5.7
α-terpineol	1188	-	2.8
Identified terpenoids (%)		0.5	8.5
Ketones			
3-octen-2-one	1042	1.0	-
(E)-β-damascenone	1384	0.5	2.8
(E)-β-ionone	1387	-	Tr
Identified ketones (%)	1.5	1.1	3.8
Saturated hydrocarbons			
Dodecane	1200	0.6	-
Tetradecane	1400	0.9	-
Hexadecane	1600	0.8	1.6
Identified sat. hydroc (%)	2.3	1.6	-
Carboxylic acid			

Table 1: Continue

Constituents	LRI[b]	Fruit	Stems
Nonanoic acid	1278	1.3	-
Identified carboxylic acid (%)	1.3	-	-
Pyridine			
3-ethenylpyridine	970	12.5	Tr
Identified pyridine (%)	12.5	-	Tr
Total identified (%)	88.5	73.6	-

[a]Percentages obtained by flame ionization detector (FID) peak area normalization (HP-5 column). [b]Linear retention indices (DB-5 column). [c]Traces (tr)<0.1%

Table 2: Antioxidant activities of the volatile oils from fruit and stem of the *Olea europaea* L. cultivar *chemchali*

Samples	Fruit	Stem
DPPH IC_{50} (mg mL^{-1})	2.630	3.54*
$ABTS^{+}$ IC_{50} (mg mL^{-1})	10.900	>20**
BHT	0.034	
Trolox	0.012	

Values are the means of the three different samples (n = 3). *p<0.05; **p<0.01

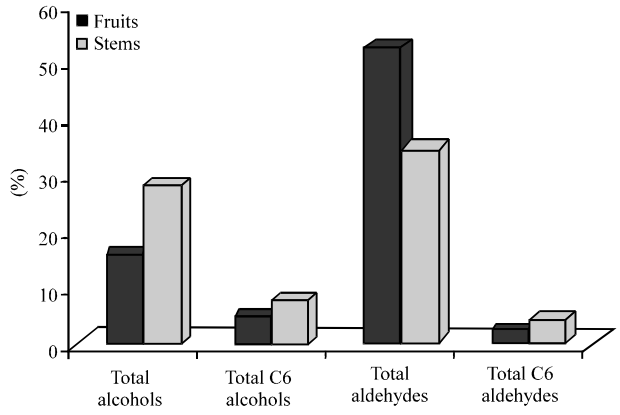

Fig. 1: Total alcohols, total C6 alcohols, total aldehydes and total C6 aldehydes in the volatile oils from fruit and stem of the *Olea europaea* L. cultivar *chemchali*

Antioxidant activity: The effects of antioxidants in the DPPH-radical-scavenging test reflect the hydrogen-donating capacity of a compound. When the radical form of DPPH is scavenged by an antioxidant through the donation of hydrogen to form a stable DPPH molecule, this leads to a colour change from purple to yellow and a decrease in absorbance. The DPPH-radical-scavenging activity of the volatile oils is shown in Table 2. Lower IC_{50} value indicates higher antioxidant activity. The fruit (IC_{50} = 2.63 mg mL^{-1}) showed higher scavenging ability on DPPH radicals than the stem (IC_{50} = 3.54 mg mL^{-1}). DPPH scavenging abilities of the volatile oils from fruit and stem were lower than that of synthetic antioxidant BHT (IC_{50} = 0.034 mg mL^{-1}). The same results were observed with the other assay, with the volatile oils from fruit and stem showed moderate activity (Table 2). The lowest EC50 value was detected in volatile oil from stem. These results were found to be statistically significant ($p<0.05$).

Table 3: Antibacterial and antivities of volatile oils from fruit and stem of *Olea europaea* L. cultivar *chemchali*

	MIC[a] values (mg mL^{-1})			
Variables	Fruits	Stems	Amphotericin[d]	Levofloxacin[e]
Gram-positive bacteria				
Enterococcus fæcalis ATCC 29212	n.a.[b]	n.a.	n.d.[c]	1.22
Staphylococcus aureus ATCC 27950	0.31	0.07	n.d.	0.3
Gram-negative bacteria				
Escherichia coli ATCC 25922	n.a.	0.62	n.d.	0.61
P. aeruginosa ATCC27950	n.a.	0.07	n.d.	0.3
Fungi *Candida parapsolosis* ATCC 22019	0.15	0.07	0.05	n.d.
Candida albicans ATCC90028	0.31	0.15	0.05	n.d.
Candida glabrata ATCC90030	0.31	0.07	0.05	n.d.
Candida kreusei ATCC6258	n.a.	0.31	0.05	n.d.

[a]MIC: Minimum inhibitory concentration. [b]:Not active. [c]:Not determined. [d]:Control antifungal. [e]:Control antibiotic

Antibacterial and antifungal activity: The antibacterial and antifungal activities of the volatile oils from *Olea europaea* L. fruit and stem were assayed *in vitro* by a broth micro-dilution method against four pathogenic bacteria and four phytopathogen strains. Table 3 summarizes the microbial growth inhibition by each volatile oil and their combination effects. Minimal Inhibitory Concentration (MIC) values of the volatiles oils toward the selected bacteria and fungi were determined in mg mL^{-1}. The comparison with the control antibiotic, led to the conclusion that the volatiles were able to inhibit the bacterial and fungal growths but with different effectiveness. The antibacterial activity of the fruits was seen only against Bacillus cereus with a MIC value of 0.31 mg mL^{-1}. On the other hand, *Staphylococcus aureus* and *P. aeruginosa* showed best susceptibility towards the volatile oil of stem with a MIC value of 0.07 mg mL^{-1} followed by *Escherichia coli* with a MIC value of MIC 0.62 mg mL^{-1}. As shown in Table 3, the antifungal activity against the phytopathogen strains of fruit and stem volatile oils were relatively good. For comparison, the stem of *Olea europaea* L. demonstrated higher activities against tested fungi.

DISCUSSION

In the present study, the fruit volatile oil of *Olea europaea* L. var. *chemchali* was rich in 3-ethenylpyridine, (E)-2-decenal and 2-ethylbenzaldehyde; therefore the stem volatile oil was rich in nonanal, (E)-2-decenal and benzyl alcohol. This result is not in accordance with Wannes *et al.* (2010), who showed that the volatile oils of the stems and fruits of two varieties of myrtle (*Baetica* and *italica*) are similar. The stems volatile oil had a difference qualitative and quantitative composition that of fruits. Data showed that the fruit of var. *chemchali* was characterized by the highest percentage of aldehydes because their important amounts of (E)-2-decenal; aldehyde typical from olive fruit flavorings; this bioactive compound is known for its antibacterial activity (Kubo *et al.*, 1995). On the other hand, the stems of the variety *chemchali* are characterized by their superiority in total C6 aldehyde and total C6 alcohol. This result is confirmed by Angerosa *et al.* (2000) who showed that the proportions of the C6 aldehydes, C6 alcohols and their acetate (LOX products) are mainly dependent on different enzymatic reactions present in different tissues. These compounds are formed by the lipoxygenase pathway that is present in various tissues in plants and involves different reactions enzyme (Gargouri *et al.*, 2008). Moreover, the ester (Z)-3-hexenyl acetate was detected only in the fruits. This ester is synthesized also by means of lipoxygenase (LOX) where the enzymatic reduction of aldehydes catalyzed by ADH, gives rise to the corresponding alcohols. These alcohols serve as substrates for Alcohol Acyltransferase (AAT)

for producing esters (Bartley *et al.*, 1985). Thus, it is important to note that the enzymatic activity is strictly dependent of organ of olive. Result showed the heterogeneous distribution of volatile compounds between the different olive parts.

Antioxidant activities of the volatiles oils from olive fruit and stem of the variety *chemchali* were tested by the DPPH and ABTS-radical-scavenging test. The volatile oils from fruit and stem showed moderate activity. The results showed that the volatile oil from fruit showed higher scavenging ability on DPPH and ABTS radicals when compared to those reported for stems. Volatiles oils are, from the chemical point of view, quite complex mixtures constituted by several tens of components and this complexity makes it often difficult to explain the activity pattern. Even many reports on the antioxidant potentials of the essential oils often refer to concepts such as synergism, antagonism and additivity. Both minor and major compounds should make a significant contribution to the oil's activity (Wang *et al.*, 2008). Moreover, trying to correlate the observed activity with the chemical composition of the oils, it is noteworthy to cite that considering the molecular characteristics of aldehydes present in a good amount in the volatile oil from fruit, these compounds may contribute to the observed antioxidant activity of the tested volatile oils as far as they were described by Ricci *et al.* (2005) to be implied in such activities.

The antibacterial and antifungal activities of the volatile oils from *Olea europaea* L. fruit and stem toward the selected bacteria and fungi were determined. For comparison, the volatile oils from fruit were less active against gram-negative bacteria than g-positive bacteria. This might be due to the protection by a hydrophilic outer membrane of the g-negative bacteria which suppressed the passage of the lipophilic volatile oil (Mann *et al.*, 2000). The highest antibacterial activity in the volatile oil stem could, in part, be associated with the superiority of percentages of terpenoids in stem with comparison from fruit. As described previously by other authors, the essential oils containing terpenoids are active against bacteria (Djoukeng *et al.*, 2005). Thus, the antifungal activity against the phytopathogen strains of fruit and stem volatile oils were relatively good. Compared with fruits, the stem of *Olea europaea* L. demonstrated higher activities against tested fungi and this is attributed to the high content of monoterpene alcohol, linalool (Pattnaik *et al.*, 1997).

CONCLUSION

The present study provides data about the stems olive volatiles and reveals the interesting character of the Tunisian olive in its volatiles content. As far as it concerns the antioxidant and antimicrobial activities, it should be noted that the volatile oils from fruit and stem showed moderate activity; therefore the stem sample showed higher activities against tested fungi and bacteria than those of the fruit. This also underlines the importance of the ethnobotanical approach for the selection of organ olive in the discovery of new bioactive substances and places these species among the most promising of indigenous drugs.

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