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Original Research

Assessment of Antioxidant and Antimicrobial Compounds of Volatiles from Leaves, Stems and Flowers of Olives

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Abstract

Protection of olive cultivars, *Olea europaea* L., from diseases and the development of more sophisticated control methods are indispensable for a renovated and competitive olive sector. In this context, the volatiles obtained by the main Tunisian oil cultivar Chemlali and both the introduced cultivars Arbequina and Koroneiki were tested for their antimicrobial activity against several dangerous pathogens by diffusion and dilution methods (in 2014). To evaluate the adaptation to biotic stress, the antioxidant potential was additionally evaluated. The volatiles extracted from leaves, stems and flowers of the tested cultivars exhibited interesting antimicrobial and antioxidant activities, reaching in many cases 100% of inhibition. To identify the bioactive compounds, GC-FID and GC-MS were performed, permitting to identify up to 97.8% of total compounds. Both non-terpene hydrocarbons and terpenes were present in important proportions among volatiles.

Keywords: olive cultivars, antimicrobial activity, antioxidant activity, non-terpene hydrocarbons, terpenes

Introduction

Tunisia is the main olive producing country in the southern Mediterranean. 34% of its cultivated land is devoted to olive growing, which extends from the north to the south of the country. This sector plays, economic, social and environmental roles, contributing to food security, job creation, equilibrium of the commercial balance, preservation of natural resources and limitation of the rural exodus. The olive forest is dominated by the oil cultivars, Chemlali in the center and south of the country, and Chetoui in the north. Chemlali alone occupies 56% of the olive-growing area and represents 69% of the total number of olive trees

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[1]. Other varieties have been introduced in the Tunisian olive system, such as Arbequina and Koroneiki, of Spanish and Greek origin respectively, to improve the productivity and to mitigate the fluctuation problem that characterizes the local varieties. To maintain satisfying productivity and defend the Tunisian position in the world, the olive tree must be well protected from microbes that may have adverse effects on final yield. Nevertheless in the Mediterranean region, olive production is affected by several diseases, Verticillium wilt, caused by *Verticillium dahlia* Kleb., is currently the most devastating disease correlated with low yield and high rates of olive tree loss [2]. *Fusarium solani* causes leaf drop, wilt, and mortality of the olive tree [3]. *Pseudomonas* is a very dangerous bacterium, *Pseudomonas savastanoi* and its pathovars *savastanoi*, *fraxini*, and *nerii* provoke a disease characterized by tumorous out growths [4]. The contamination of olive tree by *P. savastanoi* pv. *savastanoi* causes to hypertrophy of the stems and branches and, rarely, of the leaves and fruits [5]. Similarly, *Agrobacterium tumefaciens* leads crown gall disease on various plant species, especially olive cultivars, by introducing its T-DNA into the host genome, causing its proliferation and consequently plant tumors. The cited microbes, in the company of many others, have great economic consequences. Since no effective bactericides or fungicides exist, biological control using the naturally occurring antagonistic potential against pathogens is a potentially viable and environmentally friendly alternative [6].

Thus, the aim of the present study is to evaluate the behavior of the principal olive cultivar Chemlali and both the introduced cultivars Arbequina and Koroneiki against many dangerous pathogenic germs and to evaluate their antioxidant capacity to scavenge radicals that could be a consequence of such biotic stress.

Experimental

Plant Material

Chemlali, Koroneiki and Arbequina, 35 years old, were cultivated in intensive mode (6x6), in "Menzel El Mhiri", located in Kairouan governorate and Nasrallah delegation (35°21' North 9°49' East). From each cultivar, fresh leaves, flowers and stems were harvested during the flowering stage, in 2014.

Volatiles Extraction and Analyses

Volatiles were extracted from the aerial parts of the different cultivars. Fresh leaves, flowers and stems were weighted and crushed and submitted to steam distillation. Obtained samples were conserved at -16ºC until tests. The analyses of volatile compounds were performed with GC- FID and GC-MS systems, according to Saidana et al. [7].

Antimicrobial Activities

The bacterial strains investigated were: *Pseudomonas savastanoipvsavastanoi* EW2009*; Agrobacterium tumefaciens* C58; *Pseudomonas aureofaciens* NCPPB 3335*, Burkholderia glathei* LMG 14190T (U96935)*; Bacillus pumilus* QST2808.

While fungi strain were *Verticillium dahlia* MB196942, *Botrytis cinerea* TAX: 40559*, Fusarium solani* (Mar.) Sacc. 1881, *Penicilliu mitalicum* MB162660, *Fusarium oxysporum* f. sp. *Lycopersici* MB 416243. The inhibition zones, MIC, MBC and MFC were determined according to Saidana et al. [7, 8].

Antioxidant Activity

DPPH and $ABTS^{\bullet+}$ scavenging activities were performed according to Saidana et al. [9, 10].

Statistical Analysis

Statistical comparisons of the different parameters were performed with SPSS version 20. Analyses of one-way ANOVA, were followed by means comparisons ($P = 0.05$) and Tukey test.

Results

Volatiles Content

Volatiles yield in Chemlali, Koroneiki and Arbequina leaves, stems and flowers varied significantly from 0.01 to 0.024%.

The highest yield of essential oils was found in the flowers of all the cultivars. Compared to the other flowers, those of Chemlali showed the highest yield, reaching 0.024%.

Similarly, Chemlali appeared to be the richest in volatiles in all its organs, followed by Koroneiki then Arbequina. Indeed, the contents of volatiles were 0.019, 0.012 and 0.010% in the leaves, 0.024, 0.017 and 0.015% in the flowers and 0.012, 0.012 and 0.010% in the stems of Chemlali, Koroneiki and Arbequina respectively.

Antibacterial Activity

The antibacterial activities of the volatiles extracted from Chemlali, Arbequina, Koroneiki leaves, stems and flowers were tested against both pathogenic strains, *Pseudomonas savastanoi* and *Agrobacterium tumefaciens* and various soil bacteria, such as *Pseudomonas aureofaciens*, *Burkholderia glathei* and *Bacillus pumilus*.

Leaves of the tested cultivars exhibited an antibacterial activity against *P. savastanoi*, with inhibition diameters of 12, 13 and 13.5 mm for Chemlali, Arbequina and Koroneiki, respectively (Table 1). Flowers exhibited even more interesting

Table 1. Antibacterial activity of volatiles extracted from Chemlali, Arbequina and Koroneiki leaves, stems and flowers against pathogenic strains and soil bacteria.

		Pathogenic bacteria		Soil bacteria			Pathogenic fungi					
		P.s.	A.t.	B.p.	P.a.	B.g.	P. i.	V.d.	F.s.	F. o.	B. c.	A.n.
Ch.Le.	Ø	12±0.1^{d}	10±0.3^{b}	11±0.2^{b}	9±0.1^{c}	9±0.1^{d}	8±0.3^{b}	12±0.3^{b}	8±0.2^{c}	9±0.1^{b}	-	9±0.3^{b}
	MIC	125	125	-	-	-	125	125	125	125	250	125
	MBC/MFC	>1	>1	-	-	-	-	-	1	-	-	250
Ch.St.	Ø	20±0.1^{a}	6.5±0.1^{d}	7±0.3^{e}	8±0.2d	10±0.1^{c}	8±0.3^{b}	10±0.3^{b}	-	-	7.5±0.3	7.5±0.3^{c}
	MIC	125	125	-	-	-	125	125	-	125	250	250
	MBC/MFC	>1	>1	-	-	-	250		-	-	-	-
Ch.Fl.	Ø	14.5±0.3^{b}	10.5±0.2^{b}	-	5.5±0.3f	6±0.1^{f}	6±0.1^{c}	15±0.1^{b}	8±0.3^{c}	8±0.2^{c}	-	8±0.1^{c}
	MIC	225	225	-	-	-	-	-	-	125	250	125
	MBC/MFC	>1	>1	-	-	-	-	-	-	-	500	125
Ar.Le.	Ø	13±0.1^{c}	9±0.3^{c}	9±0.1^{c}	13±0.2^{b}	10.5±0.2^{b}	9±0.2^{a}	-	8±0.3^{c}	9±0.3^{b}	-	8±0.3^{c}
	MIC	-	-				125	125	125	250	250	125
	MBC / MFC	-	-	-	-	-	-	-	-	-	-	-
Ar.St.	Ø	10±0.3^{e}	-	-	-	-	-	12±0.1^{b}	10±0.2^{b}	8±0.3^{c}	-	7.5±0.3^{c}
	MIC	125	-	-	-	-	250	125	125	-	250	250
	MBC / MFC	>1	-	-	-	-	500	-	-	-	-	-
Ar.Fl.	Ø	13±0.3^{c}	-	11±0.3^{b}	9±0.1^{c}	8±0.2*^{e}	-	-	6±0.2^{d}	8±0.2^{c}	-	-
	MIC	-	-	-	-	-	125	125	-	-	250	125
	MBC / MFC	-	-	-	-	-	250		-	-	-	-
Ko.Le.	Ø	13.5±0.3^{c}	-	5.5±0.1^{f}	7±0.1^{e}	8±0.1^{e}	-	-	-	8±0.3^{c}	-	8±0.3^{c}
	MIC	-	-	-	-	-	-	-	-	-	250	250
	MBC / MFC	-	-	-	-	-	-	-	-	-	-	-
Ko.St.	Ø	10.5±0.3^{e}	-	8±0.1^{d}	5.5±0.1^{f}	-	-	10±0.3^{b}	-	-	-	9±0.3^{b}
	MIC	-	225	-	-	-	250	125	-	-	250	125
	MBC / MFC	-	>1	-	-	-	500	-	-	-	-	-
Ko.Fl.	Ø	13.5±0.3^{c}	-	12±0.1^{a}	9±0.2^{c}	10±0.1^{c}	-	11±0.3^{b}	-	8±0.3^{c}	-	8±0.3^{c}
	MIC	-	125	-	-	-	250	-	-	250	250	250

Table 1. Continued.

	MBC / MFC	-	>1	-	-	500	-	-	-	-
Positive controls	Ø	20.5±0.1a	23.5±0.3a	12±0.3a	30±0.1[a]	40±0.1[a]	-	48±0.3[a]	48±0.3[a]	57±0.1[a]	45±0.3	38±0.3[a]

Ch.Le.: Chemlali Leaves; Ch.St: Chemlali Stems; Ch.Fl: Chemlali Flowers; Ar.Le.: Arbequina Leaves; Ar.St.: Arbequina Stems; Ar.Fl.: Arbequina Flowers; Ko.Le.: Koroneiki Leaves; Ko.St.: Koroneiki Stems; Ko.Fl.: Koroneiki Flowers; Ø: inhibition diameter in mm; MIC: Minimal inhibitory concentration in µg/ml; MBC: minimal bactericidal concentration in µg/ml; MFC: minimal fungal concentration in µg/ml; –: not determined; *P.s.: Pseudomonas savastanoi; A.t.: Agrobacterium tumefaciens ; B.p.: Bacillus pumilus, P.a.: Pseudomonas aureofaciens, B.g : Burkholderia glathei; P.i.: Penicillium italicum; V.d.: Verticillium dahlia; F.s.: Fusarium solani; F.o.: Fusarium oxysporum; B.c.: Botrytis cinerea; A.n. Aspergillus niger*. Different letters indicate statistical significance at the p<0.05 level

activity, presenting inhibitions zones of 14.5, 13 and 13.5 mm, respectively.

However, stem volatiles of Chemlali showed the best antibacterial activity, which corresponded to the largest inhibition zone, reaching 20 mm. This activity was similar to that of Ampicillin, the antibacterial reference drug. On the contrary, volatiles extracted from stems of Arbequina and Koroneiki presented the least activity against *P. savastanoi,* with an inhibition zone diameter of 10.5 mm. The antibacterial activities of all tested volatiles against *A. tumefaciens* were feeble.

Only the volatiles extracted from all the organs of Chemlali and Arbequina leaves presented diameter inhibition zones varying from 7 to 11 mm (Table 1). *B. pumilus* seemed sensitive to flower volatiles of Arbequina and Koroneiki and leaf volatiles of Chemlali, with inhibition zone diameters of 11, 12 and 11 mm. These values were similar to that of Ampicillin, the antibacterial reference drug. *P. aureofaciens* was moderately sensitive to Arbequina leaf volatiles. While *B. glathei* appeared resistant to all the volatiles. The antibacterial activities of all the samples much smaller than that of Ampicillin against *P. aureofaciens* and *B. glathei,* which presented inhibition zones of 30 and 40 mm, respectively. Stem volatiles of the tested cultivars were inactive against all tested soil bacteria. All Chemlali volatiles extracted from leaves, flowers and stems exhibited an interesting antibacterial activity against *P. savastanoi* and *A. tumefaciens* at quite low concentrations (Table 1). Visible growth inhibitions of both cited bacteria were performed by leaf and stem volatiles of Chemlali at 125 µg/ml; whereas flower volatiles were active against these bacteria at 225 µg/ml. Bactericidal activities were not determined, being superior to 1.

Arbequina seemed to be active only through its stem volatiles against *P. savastanoi,* which visible growth was inhibited at 125 µg/ml.

While, Koroneiki was active against *A. tumefaciens* through its flower and stem volatiles, with visible growth inhibitions at 125 and 225 µg/ml.

Antifungal Activity

The antifungal activity of the three olive cultivars was tested against six phytopathogenic fungi, *Verticillium dahlia*, *Botrytis cinerea*, *Fusarium solani*, *Penicillium italicum*, *Fusarium oxysporum f. sp. Lycopersici* and *Aspergillus niger.* According to the results given in Table 1, *V. dahlia* appeared to be the most sensitive to volatiles of olive cultivars, especially leaf and flower volatiles of Chemlali, which presented inhibition zones of 12 and 15 mm, respectively. Stem volatiles of Arbequina and flower volatiles of Koroneiki caused inhibition zones of 12 and 11 mm. Leaf, flower and stem volatiles of Chemlali and leaf volatiles of Arbequina exhibited a moderate antifungal activity against *P. italicum,* but interestingly this activity exceeded that

Table 2. Antioxidant activities (%) of volatiles extracted from leaves (Le.), stems (St.) and flowers (Fl.) of Chemlali (Ch.), Koroneiki (Ko.) and Arbequina (Ar.) against the radical DPPH at different concentrations (mg/ml).

	Concentration mg/ml								
Samples	0.125	0.25	0.5	1	2	4	8	16	IC50
Ch.Le.	27.89 ±0.7^{d}	11.22 ±0.2^{d}	18.25 ±0.6^{c}	37.3 ±1.0^{e}	46.82 ±3.0^{g}	53.28 ±2.5^{g}	68.7 ±4.2^{g}	80.15 ±1.1^{e}	3 ±0.015
Ch.St.	30.95 ±2.1^{d}	30.04 ±0.5^{c}	37.3 ±1.2^{c}	57.02 ±2.1^{bc}	80.61 ±3.7^{c}	86.28 ±4.1^{bc}	89.9 ±1.2^{b}	89.06 ±3.6^{b}	0.9 ±0.013
Ch.Fl.	28.23 ±0.5^{d}	24.03 ±1.2^{c}	38.66 ±3.8^{b}	38.88 ±2.1^{de}	52.72 ±5.1^{e}f	64.85 ±0.1^{f}	74.71 ±0.2^{fg}	77.21 ±1.9^{f}	1.75 ±0.009
Ar.Le.	52.26 ±2.8^{b}	56.12 ±2.3^{b}	92.74 ±0.9^{a}	94.33 ±0.2^{a}	92.29 ±0.4^{b}	93.19 ±0.4^{ab}	98.54 ±4.4^{a}	98.16 ±1.2^{a}	0.1 ±0.008
Ar.St.	34.46 ±0.5^{cd}	29.5 ±0.1^{c}	43.19 ±0.2^{c}	68.48 ±0.3^{b}	79.02 ±1.1^{c}	84.35 ±0.7^{c}	84.7 ±1.0^{c}	85.51 ±2.7^{d}	0.75 ±0.003
Ar.Fl.	27.32 ±0.5^{d}	27.77 ±0.1^{c}	31.29 ±1.5^{bc}	36.05 ±2.3^{e}	49.09 ±4.3^{fg}	63.71 ±0.2^{f}	72.67 ±0.3^{g}	77.66 ±0.7^{f}	2.4 ±0.006
Ko.Le.	40.92 ±0.1^{c}	29.02 ±4.0^{c}	21.08 ±4.0^{c}	37.41 ±0.3^{e}	45.01 ±3.0^{g}	71.76 ±0.9^{ef}	83.21 ±0.6^{cde}	86.8 ±1.9^{cd}	2.45 ±0.014
Ko.St.	30.83 ±0.1^{d}	26.07 ±0.1^{c}	39.11 ±4.0^{b}	53.17 ±1.3^{bc}	73.24 ±2.8^{d}	82.32 ±1.5^{cd}	85.83 ±1.1^{bc}	87.77 ±1.5^{bc}	0.8 ±0.007
Ko.Fl.	8.27 ±0.2^{e}	25.96 ±1.9^{c}	41.49 ±1.7^{b}	49.77 ±1.4^{cde}	6.12 ±2.1^{e}	74.48 ±3.7^{de}	79.93 ±0.1^{de}	81.85 ±4.4^{e}	1.5 ±0.012
Trolox	95.39 ±0.1^{a}	95.32 ±1.2^{a}	94.98 ±1.5^{a}	94.45 ±2.2^{a}	100 ±0.1^{a}	100 ±0.0^{a}	100 ±0.1^{a}	100 ±0.1^{a}	

Different letters indicate statistical significance at the $p<0.05$ level for each concentration

of Carbendazime, the antifungal reference drug, which showed no inhibition zone.

Additionally, either *F. oxysporum* or *F. solani* were almost resistant to the tested volatiles, with inhibition zones varying between 6 and 10 mm. *B. cinerea* was the more resistant fungal: only stem volatiles showed an inhibition zone of 7.5 mm. According to the broth dilution method, *P. italicum* seemed to be the most sensitive against Arbequina and Koroneiki volatiles, where fungicidal activities were noted for the flower and stem volatiles at concentrations varying between 125 to 500 µg/ml (Table 1).

Visible growth inhibitions were reached at low concentrations (125-250 µg/ml). Chemlali seemed to be active against this fungus with its stem volatiles, showing a fungicidal activity at 125 µg/ml. Additionally, this cultivar exhibited a fungicidal activity against *B. cinerea* at a concentration of 500 µg/ml for its flower volatiles and a visible growth inhibition of this species by its leaf and stem volatiles at 250 µg/ml. Only Chemlali leaf volatiles exhibited a fungicidal activity against *F. solani* at a concentration of 1mg/ml.

Only Arbequina leaf and stem volatiles inhibited a visible growth at 125 µg/ml. Similarly, only Chemlali volatiles exhibited a fungicidal activity against *A. niger* at 250 µg/ml in the case of leaves and at 125 µg/ml in the case of flower volatiles. All the tested volatiles exhibited a visible growth inhibition against *A. niger*, at concentrations varying from 125 to 250 µg/ml. No fungicidal activity was registered for all the volatiles against *F. oxysporum f.* sp. *lycopersici,* which appeared to be the most resistant species.

However, its visible growth was inhibited by all the volatiles of Chemlali, those of leaves of Arbequina and flowers of Koroneiki.

Antioxidant Activity

DPPH Scavenging Activity

The scavenging activity of Chemlali, Koroneiki and Arbequina volatiles, tested at different concentrations and compared with Trolox, is summarized in Table 2.

All the volatiles extracted from the cultivar organs were active against the DPPH radical, and showed a very good antioxidant action. Indeed, almost over 80% of free radicals were scavenged by all the samples at 16 mg/ml. The inhibition of DPPH was legible even at lower concentrations.

Leaf volatiles of Arbequina showed an important radical scavenging activity (92.74%) even at 500 µg/ml, comparable to that of Trolox at the same concentration. Arbequina leaf volatiles maintained their action even at very low concentrations (125 µg/ml). Interestingly, 50% of DPPH radicals were inhibited at 100 µg/ml (Table 2). Thus, the leaves of Arbequina seemed to be the most active sample in this test.

$ABTS^{\bullet+}$ Scavenging Activity

The variation of the percentage inhibition of volatiles from leaves, stems and flowers of Chemlali, Arbequina and Koroneiki as a function of time and concentration is illustrated in Fig. 1. Volatiles extracted from all the olive tree parts exhibited an interesting scavenging capacity starting from the first five minutes of contact with the radical cation $ABTS^{\bullet+}$. This capacity increased gradually with contact time. Leaf volatiles of Chemlali, Arbequina, Koroneiki seemed to be very active against $ABTS^{\bullet+}$, with 87.5, 100 and 94.85% of radicals scavenged at a concentration of 16 mg/ml. Leaf volatiles of Arbequina were the most active ones, scavenging the totality of radicals at only 1.0 mg/ml and in a short time. Chemlali and Koroneiki exhibited a time depending activity, which became more important over time. The inhibitions of 50% of radicals by leaf volatiles were respectively reached after 30 min of contact at the concentrations of 1.81, 0.316 and 0.18 mg/ml for Chemlali, Arbequina and Koroneiki, respectively.

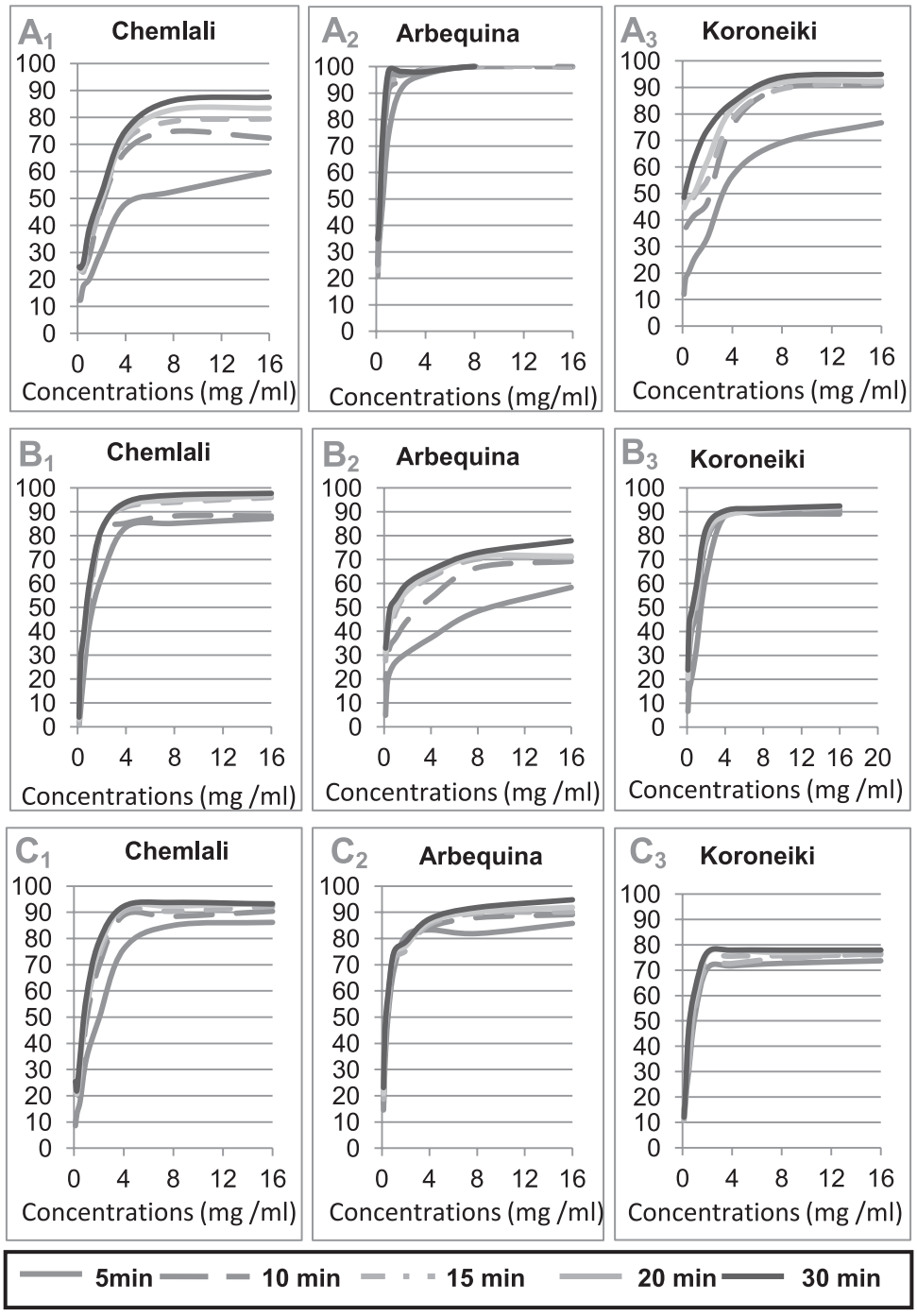

Fig. 1. Antioxidant activities (%) of volatiles extracted from leaves (A1, A2, A3), stems (B1, B2, B3) and flowers (C1, C2, C3) of Chemlali, Arbequina and Koroneiki against the radical cation $ABTS^{\bullet+}$.

Table 3. Radical cation scavenging activity of Chemlali (Ch.), Arbequina (Ar.) and Koroneiki (Ko.) volatiles extracted from leaves (Le.), stems (St.) and flowers (fl.), expressed as Trolox equivalent after 30 min of initial mixing and as 50% of inhibition.

	Concentration mg/ml								
Samples	0.125	0.25	0.5	1	2	4	8	16	IC50
Ch.Le.	0.60 ±0.1^{e}	0.59 ±0.1^{cd}	0.65 ±0.1^{e}	0.95 ±0.0^{d}	1.26 ±0.0^{d}	1.81 ±0.0^{bc}	2.08 ±0.0^{abc}	2.11 ±0.0^{bc}	1.810 ±0.007
Ch.St.	0.11 ±0.1^{e}	0.68 ±0.1^{bcd}	0.93 ±0.0^{e}	1.46 ±0.1^{cd}	1.99 ±0.0^{bc}	2.26 ±0.1^{a}	2.33 ±0.1^{a}	2.35 ±0.0^{ab}	0.722 ±0.002
Ch.Fl.	0.62 ±0.0^{e}	0.54 ±0.0^{d}	0.85 ±0.0^{e}	1.39 ±0.0^{cd}	1.89 ±0.0^{b}	2.22 ±0.0^{ab}	2.25 ±0.0^{ab}	2.24 ±0.0^{ab}	0.784 ±0.027
Ar.Le.	0.85 ±0.0^{b}	1.00 ±0.1^{ab}	1.75 ±0.0^{a}	2.36 ±0.0^{a}	2.36 ±0.0^{a}	2.36 ±0.0^{a}	2.40 ±0.0^{a}	2.40 ±0.0^{a}	0.316 ±0.028
Ar.St.	0.80 ±0.0^{b}	0.94 ±0.0^{abcd}	1.20 ±0.0^{bcd}	1.30 ±0.0^{de}	1.45 ±1.0^{cd}	1.58 ±0.0^{d}	1.76 ±0.0^{c}	1.88 ±0.7^{c}	0.580 ±0.003
Ar.Fl.	0.57 ±0.0^{c}	1.09 ±0.1^{ab}	1.41 ±0.0^{ab}	1.80 ±0.0b	1.90 ±0.0^{b}	2.11 ±0.0^{ab}	2.21 ±0.1^{ab}	2.28 ±0.0^{ab}	0.310 ±0.010
Ko.Le.	1.17 ±0.0^{a}	1.24 ±0.2^{a}	1.35 ±0.1^{abc}	1.53 ±0.0^{cd}	1.78 ±0.1^{bc}	2.03 ±0.1^{ab}	2.26 ±0.0^{ab}	2.28 ±0.1^{ab}	0.180 ±0.014
Ko.St.	0.58 ±0.2^{c}	1.05 ±0.1^{ab}	1.17 ±0.1^{bcd}	1.42 ±0.1^{cd}	1.99 ±0.0^{bc}	2.17 ±0.0^{ab}	2.20 ±0.1^{ab}	2.22 ±0.0^{ab}	0.513 ±0.007
Ko.Fl.	0.30 ±0.0^{d}	0.55 ±0.1^{d}	1.12 ±0.0^{bcd}	1.50 ±0.0^{cd}	1.85 ±0.1^{bc}	1.88 ±0.0^{bc}	1.88 ±0.0^{bc}	1.88 ±0.0^{c}	0.568 ±0.005

Different letters indicate statistical significance at the $p<0.05$ level for each concentration

Koroneiki presented the lowest IC_{50} after 30 min of contact (Table 3). Also stem volatiles of the studied cultivars were very active against $ABTS^{\bullet+}$, especially those of Chemlali, which inhibited almost the totality of radicals (IC_{50} = 0.722 mg/ml). Koroneiki and Arbequina seemed to be active even at lower concentrations, with IC_{50} of 0.513 and 0.580 mg/ml, respectively. Flower volatiles of Chemlali and Arbequina inhibited over 90% of radicals in almost all time intervals; whereas those of Koroneiki had a slightly weaker activity (80% after 30 min). IC_{50} of these cultivars were respectively 0.784, 0.310 and 0.568 mg/ml after 30 min of contact with radicals.

Trolox equivalent antioxidant capacity (TEAC), measured 30 min after the beginning of the reaction was summarized in Table 3. A TEAC value higher than 1 corresponds to a better antioxidant activity for the tested sample in comparison to Trolox. According to Table 3, Koroneiki leaf volatiles were more active against $ABTS^{\bullet+}$ than Trolox, even at the lowest tested concentration (125 µg/ml). Arbequina flowers were more active than Trolox at only 250 µg/ml, while Chemlali stems and flowers were more active at a concentration of 1 mg/ml. Starting from this concentration (1 mg/ml), all the olive tree volatiles always exhibited better antioxidant activity than Trolox.

Chemical Composition of Volatiles

The chemical investigation on the volatiles extracted from different organs of Chemlali, Arbequina and Koroneiki cultivars permitted to characterize 95.7, 94.9 and 91.7% of the total compounds in leaves; 80.6, 84.4 and 87.3% in stems and 95.1, 97.8 and 88.6% in flowers, respectively (Table 4).

Hydrocarbons appeared to be dominant in all the cultivars, especially in their leaves (49.0, 44.8 and 30.7% in Chemlali, Arbequina and Koroneiki, respectively). Similarly, terpenes seemed to be the main chemical class in stems and flowers.

Apocarotenes were particularly produced by stems of Chemlali (34.3%) and flowers of Arbequina (55.4%) and Koroneiki (44.9%). Aromatic derivatives were present in relevant amounts in all the cultivars, especially in the leaves (5.4, 16.0 and 14.1% for Chemlali, Arbequina and Koroneiki, respectively). Aldehydes were particularly detected in flowers (23.3, 18.6 and14.7%, respectively).

The major aliphatic compounds were 1-hexadecene (34.4%, Chemlali leaves), *n*-pentadecane (13.5%, Arbequina leaves) and *n*-dodecane (10.4%, Arbequina flowers). Nonanal was the major aldehyde in flowers of Chemlali (12.2%), Arbequina (10.7%) and Koroneiki (14.7%). Dihydroedulan IIA was the most represented apocarotene of Arbequina flowers (18.4%); while, dihydroedulan IA in Koroneiki flowers (17.8%) and *(E)*-β-damascenone in Chemlali leaves (16.8%).

(E)-nerolidol and liguloxide were the major oxygenated sesquiterpenes presents in all the cultivar volatiles, reaching the maximum in Koroneiki leaves (13.1%) and Arbequina stems (11%).

Table 4. Chemical composition of volatiles extracted from leaves (Le), stems (St) and flowers (Fl) of Chemlali, Koroneiki and Arbequina.

		Chemlali			Arbequina			Koroneiki		
Constituents (%)	**l.r.i.**	**Le**	**St**	**Fl**	**Le**	**St**	**Fl**	**Le**	**St**	**Fl**
2-methyloctane	864							0.6	1.4	
p-xylene	867				0.8			0.2	1.5	
n-nonane	900				4.1	6.5		3.6	9.0	
3-ethyl-1,5-octadiene	942				0.9	2.4		1.4	2.8	
1-ethyl-4-methylbenzene	965							0.9		
phenol	985				1.7					
2-methyldecane	1062		1.8							
linalool	1101		6.1	3.8			2.3	0.3		16.0
nonanal	1102		3.9	12.2	0.7		10.7	0.6		14.7
camphor	1145		1.5							
methyl nicotinate	1148			15.6						
Decane, 5,6-dimethyl-	1155									
Undecane, 2-methyl-	1167		1.8							
2-Decanol	1185									
(Z)-3-hexenyl butyrate	1188			2.4			2.1			
α-terpineol	1191		3.2	21.9						13.0
methyl salicylate	1192						1			
n-dodecane	1200			4.8	2.1		10.4	0.8	1.7	
decanal	1205				0.4					
trans-piperitol	1207					2.1				
β-cyclocitral	1222		1.4					0.2		
(E)-2-decenal	1263			11.1			5.3			
nonanoic acid	1275					2.8				
2,6,11-trimethyldodecane	1277		3.3							
dihydroedulan IIA	1285			8.8			18.4			12.7
p-cymen-7-ol	1290									
dihydroedulan IA	1292			2.3			7.4	0.6		17.8
theaspirane I	1298			4.9			13.6			4.5
4-vinylguaiacol	1313				1.3					
theaspirane II	1315		2.1	3.9			16.0	1.1	2.8	9.9
methyl 4-formylbenzoate	1370				7.1					
3-methyltridecane	1373				2.2					
(E)-β-damascenone	1382	16.8	3.2		6.1			4.0		
10-acethylmethyl-3-carene	1389	5.5	2.7		1.9			3.1	3.1	
1-tetradecene	1392		3.1		5.2	2.8		1.6	3.3	
dihydro-γ-ionone	1396		7.4			10.9			4.3	
n-tetradecane	1400	3.4	4.8		12.2	7.3		3.6	8.9	
(E)-β-damascone	1412	4.8			1.2			1.1		
trans-α-ambrinol	1414		2.4			3.2				

Table 4. Continued.

dihydro-α-ionone	1420				3.9			5.0		
nerylacetone	1436		8.0		1.3	9.3		3.9	10.3	
(E)-geranylacetone	1456		9.8		3.4	8.0		3.4	14.2	
(E)-β-ionone	1487				3.5			3.4	1.8	
1-pentadecene	1492				2.4			1.0		
n-pentadecane	1500		2.3		13.5					
(E,E)-α-farnesene	1507							1.6		
liguloxide	1532		9.5			11.0		2.0		
dihydroactinidiolide	1536				7.8			3.5		
epi-ligulyl oxide	1551		2.3			2.7			5.8	
4-methylpentadecane	1556				2.2					
2-methylpentadecane	1563							6.3		
(E)-nerolidol	1564	9.8			3.9			13.1	7.8	
(Z)-3-hexenyl benzoate	1570	5.4			5.1			11.3		
hexyl benzoate	1580							1.7		
caryophyllene oxide	1582					3.1				
1-hexadecene	1593	34.3				4.9		5.1	2.6	
n-hexadecane	1600	11.3				7.4		6.7	6.0	
Valerianol	1656	4.4								
Hydrocarbons		**49.0**	**17.1**	**8.2**	**44.8**	**31.3**	**18.4**	**30.7**	**35.7**	
Monoterpene hydrocarbons		5.5	2.7		1.9			3.1	3.1	
Oxygenated monoterpenes			10.8	25.7		2.1	2.3	0.3		29.0
Sesquiterpene hydrocarbons								1.6		
Oxygenated sesquiterpenes		14.2	11.8		3.9	16.8		15.1	13.6	
Apocarotenes		21.6	34.3	19.9	27.2	31.4	55.4	26.2	33.4	44.9
Terpenes		**41.3**	**59.6**	**45.6**	**33.0**	**50.3**	**57.7**	**46.3**	**50.1**	**73.9**
Aromatic hydrocarbon					0.8			1.1	1.5	
Aromatic esters		5.4			12.2			13.0		
Aromatic compounds		**5.4**			**16.0**			**14.1**	**1.5**	
Aldehydes			3.9	23.3	1.1		18.6	0.6		14.7
Nitrogen compounds				15.6						
Fatty acid and its ester				2.4		2.8	3.1			
No aromatic compounds			**3.9**	**41.3**	**1.1**	**2.8**	**21.7**	**0.6**		
Total identified compounds		**95.7**	**80.6**	**95.1**	**94.9**	**84.4**	**97.8**	**91.7**	**87.3**	**88.6**

Discussion

Volatiles from leaves, stems and flowers of Chemlali, Arbequina and Koroneiki cultivars were characterized.

Flowers of all the cultivars were the organs that produced most of the volatiles, with yields reaching 0.024, 0.015 and 0.017%, respectively. Generally, Chemlali produced most volatiles, regardless of the tested organ. Chemlali is of Tunisian origin, while Arbequina and Koroneiki cultivars were introduced for reasons of productivities.

The differences observed for these yields could then be influenced by edaphic and climatic conditions [11], differing in different countries [12]. Additionally, the essential oil content and its composition may vary according to the plant part [13]. To test the effect of

volatiles extracted from the different organs of the three cultivars, they were tested against the most pathogenic bacteria for the olive tree, *Pseudomonas savastanoi* and *Agrobacterium tumefaciens,* by both the diffusion and broth dilution methods. Interaction with some soil bacteria, such as *Pseudomonas aureofaciens*, *Burkholderia glathei* and *Bacillus pumilus* were also noted.

P. savastanoi seemed to be more susceptible to the applied olive volatiles than *A. tumefaciens,* with inhibition zones varying from 10 to 20 mm. All the volatiles seemed to be active against this bacterium, but Chemlali stem volatiles had the best effect. In the case of *A. tumefaciens*, only volatiles extracted from leaves, flowers and stems of Chemlali and Arbequina leaf volatiles exhibited inhibition zones that reached a maximum of 10.5 mm.

Interaction of olive volatiles with soil bacteria was variable according to the tested microorganism, cultivar and organ. Indeed, *B. pumilus* seemed to be the most sensitive, with inhibition diameters similar to that of Ampicillin.

The other bacteria, *P. aureofaciens* and *B. glathei*, presented much smaller inhibition zones (13 and 10 mm, respectively) than those registered for the antibacterial reference drug (30 and 40 mm, respectively). Chemlali volatiles showed inhibition zones against both bacteria and inhibited their visible growth at quite low concentration (125 µg/ml).

Arbequina and Koroneiki volatiles presented inhibition zones against *P. savastanoi* according to diffusion method, but only stem volatiles of Arbequina inhibited visible growth of this bacterium at 125 µg/ml.

Similarly, Koroneiki volatiles, extracted from its flowers and stems, did not show inhibition zones against *A. tumefaciens* but presented nevertheless a visible inhibition growth at 125 and 225 µg/ml, respectively. The negative response of *A. tumefaciens*, when using the diffusion method, may be explained by the high resistance of these Gram-negative bacteria.

Additionally, the diffusion method can greatly vary according to the molecules [14], the organisms tested [15], and the inoculum size. Then, physical and chemical properties of the drugs as well as biological behavior of the bacteria could be put in competition, sometimes with a rather unpredictable outcome [16]. The volatiles were also tested qualitatively and quantitatively against several pathogenic fungi. All the tested volatiles exhibited moderate antifungal activities, with inhibition zones varying from 7.5 to 15 mm. These values were much smaller than those registered for the antifungal reference drug (45 to 57 mm).

However, all the Chemlali volatiles and Arbequina leaf volatiles exhibited a moderate antifungal activity against *P. italicum*, while the antifungal reference drug presented no activity against this species.

Differently, using the dilution method, almost all the olive volatiles exhibited interesting antifungal activities against the majority of fungi at low doses.

Growth of *F. solani* was totally inhibited by volatiles of Chemlali leaves, while growth of *A. niger* was totally inhibited by volatiles of Chemlali leaves and flowers. Additionally, *B. cinerea* was totally inhibited by volatiles extracted from Chemlali flowers. Consequently, Chemlali appeared to be the most active cultivar, totally inhibiting the growth of the three pathogenic fungi. *P. italicum* was totally inhibited by stem volatiles of the three olive cultivars, and by Arbequina flower volatiles. Thus *P. italicum* was the most sensitive species. The antifungal activity of olive volatiles, evaluated by diffusion method, was moderate.

However, this activity was more interesting when micro-dilution method was adopted, with low values of MIC and MFC. This proposed that the size of the inhibition zone does not reflect the real antibacterial efficiency of volatiles, since it is affected by the solubility of the oil, its diffusion in the agar, its evaporation, etc. This point was in agreement with Kim et al. [17] and Cimanga et al. [18] observations.

The essential oil activity is evidently related to the chemical composition of its compounds, their proportions and their interactions each other [19, 20].

Antifungal susceptibility is influenced by the type of medium, the inoculum size, the pH, the temperature and the time of incubation [21]. All tested samples exhibited an interesting antioxidant activity against DPPH radicals, reaching over 80% inhibition.

The most effective volatiles were those from stems of all the cultivars that inhibited 50% of radicals in the range 0.75-0.9 mg/ml.

Similarly, an important antioxidant activity was noted for all the volatiles when tested against the cation radicals $ABTS^{\bullet+}$, reaching 100% of radical inhibition for some of them. This activity depended on the tested organ, the cultivar and the contact time.

Leaf volatiles of Arbequina appeared to be the most active, scavenging the totality of radicals at only 1 mg/ml and in a very short time. Chemlali stems and Chemlali and Arbequina flowers were the most active against $ABTS^{\bullet+}$ when applied at low concentrations and short time of contact. Trolox equivalent antioxidant capacity measured after 30 min of contact presented elevated values, demonstrating the powerful antioxidant activity of these volatiles. Awika et al. [22] reported the advantage of $ABTS^{\bullet+}$ test over DPPH, as $ABTS^{\bullet+}$ test is operable over a wide range of pH, inexpensive and more rapid than the DPPH test. The absorbance of DPPH at 517 nm is depended on light, oxygen, pH and type of solvent [23]. Aruoma [24] mentionned that more than one method of antioxidant testing should be used to gain a perceivable indication of antioxidant efficacy of the tested substances. Chemlali, Arbequina and Koroneiki leaf, flower and stem volatiles exhibited interesting antioxidant and antimicrobial activities.

The chemical analyses evidenced the presence of several bioactive compounds. Indeed, all the volatiles contained hydrocarbons in important proportions (up to 49%). 1-Hexadecene, the main aliphatic hydrocarbon,

Table 5. Bioactive compounds identified in Chemlali, Arbequina and Koroneiki volatiles.

Compounds	Biological activity	References
Hydrocarbons		
Hexadecene	Antioxidant and antimicrobial activities	[26]
Nonane derivate	Antioxidant and antimicrobial activities	[35-36]
Dodecane	Antioxidant activity	[37]
Trimethyldodecane	Antimicrobial activity	[38]
Pentadecane	Antimicrobial activity	[39]
Terpenes		
Oxygenated Monoterpenes		
Linalool	Antioxidant and antimicrobial activities	[40-41]
Terpineol	Antioxidant and antimicrobial activities	[42-43]
Monoterpenes hydrocarbon		
Carene	Antioxidant and antimicrobial activities	[44]
Oxygenated sesquiterpenes		
Nerolidol	Antioxidant and antimicrobial activities	[30; 45]
Caryophyllene oxide	Antioxidant and antimicrobial activities	[46-47]
Ligulyl oxide	Antioxidant activity	[31]
Apocarotenes		
β-Ionone	Antioxidant and antimicrobial activities	[48-49]
Dihydroedulan	Antioxidant and antimicrobial activities	[50-51]
Beta-Damascenone	Antioxidant and antimicrobial activities	[28; 52]
Geranylacetone	Antioxidant and antimicrobial activities	[53]
Theaspirane	Antioxidant activity	[54]
Hexyl benzoate	Antimicrobial activity	[55]
Methyl 4-formylbenzoate	Antimicrobial activity	[56]
Aldehyde		
Nonanal	Antifungal activity	[57]
Fatty acid		
Nonanoic acid	Antifungal activity	[58]

especially in Chemlali leaves (34.3%) is known for both antioxidant and antimicrobial activities [25-27]. Also terpenes were well represented among these olive cultivar volatiles, reaching a maximum of 73.9%.

Among apocarotenes, dihydroedulan IIA was particularly detected in Arbequina flowers (18.4%), while dihydroedulan IA was the major one in Koroneiki flowers (17.8%). Additionally, many other major bioactive apocarotens, exhibiting antioxidant and antimicrobial activities, were characterized, such as *(E)*-β-damascenone (Chemlali leaves, 16.8%) and *(E)*-geranylacetone (Koroneiki stems, 14.2%) [28-29] (Table 5).

Regarding oxygenated sesquiterpenes, the most representative ones were *(E)*-nerolidol (Koroneiki leaves, 13.1%) and liguloxide (Arbequina stems, 11.0%) [30- 31].

Some studies have proved that sometimes the whole volatile extracts have a more powerful biological activity compared to the major component [32 -33]. These authors propose that the compounds present in the greatest proportions were responsible only for a part of the total activity; also, the other components present with smaller amount, contribute to the unregistered activity. As well, a synergistic effect between all components should be considered [34].

Conclusion

Chemlali, Arbequina and Koroneiki volatiles have shown an interesting antibacterial activity against dangerous pathogenic bacteria; in particular, the principal Tunisian cultivar Chemlali exhibited a powerful activity against *P. savastanoi* and *A. tumefaciens* at low concentration (125 µg/ml). Both Chemlali and Arbequina inhibited the visible growth of the majority of the tested fungi at 125 µg/ml through their leaf, stem and flower volatiles and could block completely their growth in many cases. Interestingly, all the tested olive volatiles have an excellent capacity to scavenge radicals. 50% of radicals were inhibited at 100 µg/ml by the Arbequina leaf volatiles which seemed to be the most active. Many bioactive compounds such as hydrocarbons, oxygenated monoterpenes and apocarotenes, have been identified in the olive cultivar volatiles. These components could contribute to the recorded activities, expected to be related to their stereochemistry, to the proportions in which they are present and to the interactions between them. Further research is required to elucidate the exact mode of action of these active principles. Thus, olive tree volatiles might be a prospective source of alternative antimicrobial and antioxidant agents interesting for a potential use in the biological control or the conservation of food products.

Conflict of Interest

The authors declare no conflict of interest.

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