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Chemical Composition and Antifungal Activity of Three Anacardiaceae Species Grown in Tunisia

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ABSTRACT

The essential oil composition of *Pistacia lentiscus*, *Pistacia vera* and *Pistacia terebinthus* was analyzed by GC and GC-MS analysis and their antifungal activity was studied against ten phyto-pathogenic fungi. Qualitative and quantitative differences between oils were observed. All oils were rich in monoterpene hydrocarbons, the major constituents were α-pinene (16-20%), α-terpinene (32-41%) and limonene (4-25%). Principal component analysis and hierarchical cluster analysis separated the three *Pistacia* leaf essential oils into two groups, each constituting a chemotype. The *in vitro* antifungal activity of tested oils has shown a significant antifungal activity against all tested fungi.

Key words: *Pistacia*, essential oils, antifungal activity, principal component analysis

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INTRODUCTION

Crop loss due to fungal species also remains a serious problem[1]. Emerging resistance of these species is seriously decreasing the number of effective antimicrobials[2,3,4,5]. The crop production has tended to reduce the use of chemical in their products due to increasing pressure of consumers or legal authorities or to adopt more natural alternatives for crop protection. Plants and their essential oils are potentially useful sources of antimicrobial compounds. Numerous studies have been published on the antimicrobial activities of plant compounds against many different types of microbes, including phyto-pathogenic fungi[6]. The main constituents of essential oils were mono and sesquiterpenes including carbohydrates, phenols, alcohols, ethers, acetates and ketones which are responsible for their biological activity. Aromatic and medicinal plants produce a wide variety of volatile terpenes and their oxygenated derivatives. Mixtures of these substances, which are known as essential oils can be isolated from diverse parts of plants by steam distillation. The antimicrobial properties of essential oils are well recognized and their preparations are found applications as naturally occurring antimicrobial agents in pharmacology, pharmaceutical botany, phyto-pathology, medical and food preservation. Thus, the discovery of essential oil preparations that possess antimicrobial activities has been the subject of many research investigations of a wide variety of plant species[6,7,8]. In an attempt to reduce the use of synthetic pesticides, extensive investigations into the possible exploitation of plant compounds as natural commercial products, that are safe for humans and the environment. Indeed, the search of natural compounds and management methods alternatives to classical pesticides has become an intense and productive research field [9,10]. The genus of *Pistacia* which belonged to the family of Anacardiaceae there are 11 species which some of them used as ornamentals and some valued as fruit tree. *Pistacia* trees are characteristic for the Mediterranean basin flora. Five species of the genus grow naturally in the Mediterranean basin and Middle East: *P. lentiscus*, *P. atlantica*, *P. palaestina*, *P. terebinthus* and *Pistacia vera* originated in central Asia and is cultivated throughout the Mediterranean region[11]. The aims of this study were, in a first step to assay the main constituents of the essential oil obtained from the leaves of *P. lentiscus*, *P. terebinthus* and *Pistacia vera* growing in Tunisia. In a second step, we assessed their antifungal potential against ten phyto-pathogenic fungi.

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MATERIALS AND METHODS

Plant material: The leaves of *P. lentiscus*, *P. terebinthus* and *Pistacia vera* were collected from the I.N.R.G.R.E.F. arboretums (National Institute of Researches on Rural Engineering, Water and Forests). Five samples collected from more than five different trees were harvested, mixed for homogenization and used in three replicates for essential oil extractions. The specimen of the plant was submitted to the herbarium division of the institute and identification was confirmed in the Laboratory of Forest Ecology.

Isolation of the essential oils: The essential oils were extracted by hydrodistillation of fresh plant material (100 g of each sample in 500 mL of distilled water) using a Clevenger-type apparatus for 3 h according to the standard procedure described in the European Pharmacopoeia.

The oils were dried over using anhydrous sodium sulfate (a pinch 10 mL^{-1}) and stored in sealed glass vials at 4°C before analysis. Yield was calculated based on dried weight of the sample (mean of three replications).

Gas chromatography-mass spectrometry: The composition of the oils was investigated by GC and GC/MS. The analytical GC was carried out on an HP5890-series II gas chromatograph (Agilent Technologies California USA) equipped with Flame Ionization Detectors (FID) under the following conditions: the fused silica capillary column, apolar HP-5 and polar HP Innowax (30 m×0.25 mm ID, film thickness of 0.25 mm). The oven temperature was held at 50°C for 1 min then programmed at rate of 5°C min^{-1} to 240°C and held isothermal for 4 min. The carrier gas was nitrogen at a flow rate of 1.2 mL min^{-1}; injector temperature: 250°C, detector: 280°C; the volume injected: 0.1 mL of 1% solution (diluted in hexane). The percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction. GC/MS was performed in a Hewlette Packard 5972 MSD System. An HP-5 MS capillary column (30 m×0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometry. The carrier gas was helium, with a flow rate of 1.2 mL min^{-1}. Oven temperature was programmed (50°C for 1 min, then 50-240°C at 5°C min^{-1}) and subsequently held isothermal for 4 min. Injector port: 250°C, detector: 280°C, split ratio: 1:50. Volume injected: 0.1 mL of 1% solution (diluted in hexane); mass spectrometer: HP5972 recording at 70 eV; scan time: 1.5 s; mass range: 40-300 amu. Software adopted to handle mass spectra and chromatograms was ChemStation. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library). Further confirmation was done from Retention Index data generated from a series of alkanes retention indices (relatives to C9-C28 on the HP-5 column)[12].

Antifungal activity assays: Eight plant pathogenic fungi were obtained from the culture collection of the Tunisian National Institute of Agronomic Research (INRAT). Cultures of each of the fungi were maintained on Potato Dextrose Agar (PDA) and were stored at 4°C and in 1 mL of glycerol 25% at -20°C. The fungal species used in this study were: *Fusarium culmorum, F. avenaceum*, *F. oxysporum, F. subglutinans, F. verticillioides, F. nygamai*, *F. nygamai, Botrytis cinerea, Microdochium nivale* and *Alternaria* sp.. Antifungal activity was studied by using an in vitro contact assay which produces hyphal growth inhibition[13]. Essential oil was dissolved in 1 mL of Tween 20 (0.1% v/v) and then added into 20 mL PDA at 50°C to obtain a final concentration of 4 µL mL^{-1}. A mycelia disk of 5 mm in diameter, cut from the periphery of a 7 day-old culture, was inoculated in the center of each PDA plate (90 mm diameter) and then incubated at 24°C for 7 days. PDA plates treated with Tween 20 (0.1%) without essential oil were used as control. Tests were repeated in triplicate. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control using the following formula:

$$\% \text{ Inhibition} = \frac{(C - T)}{C} \times 100$$

where, C is an average of three replicates of hyphal extension (mm) of controls and T is an average of three replicates of hyphal extension (mm) of plates treated with essential oil.

Statistical analysis: Data of germination, seedling growth and fungi inhibition assays were subjected to one-way Analysis of Variance (ANOVA), using the SPSS 17.0 software package. Differences between means were tested through Student-Newman-Keuls (SNK) and values of $p \leq 0.05$ were considered significantly different. To evaluate whether the essential oil constituents identified are useful in reflecting the chemical relationships between species, 13 compounds, detected in the oil samples with contents in the essential oils of 3.5% in at last one species, were subjected to PCA and HCA using SPSS 17.0 software[14].

RESULTS AND DISCUSSION

Yield and chemical composition of *Pistacia* species essential oils: The mean yields of the three *Pistacia* leaf oils varied according to the species from 0.14±0.026 for *P. lentiscus* to 0.24±0.03 and 0.28±0.02, respectively for

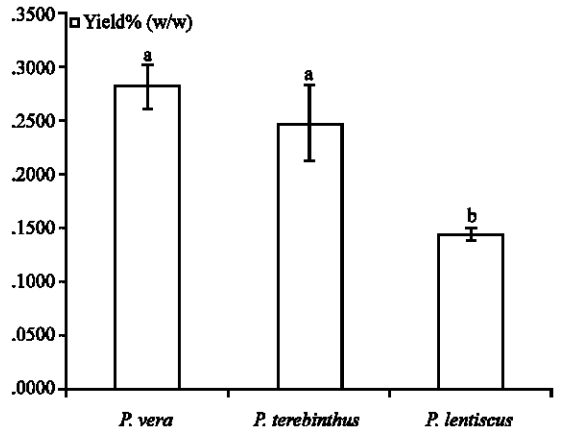

Fig. 1: Average essential oils yield of three *Pistacia* species. Values with different superscripts (a-b) are significantly different at $p = 0.05$ (means of three replicates)

P. terebinthus and *P. vera*. The Analysis of Variance (ANOVA) indicated that the oil yields were significantly different between the species ($p \leq 0.05$). The average classification showed the presence of two overlapping groups (Fig. 1) the first group was constituted by *P. terebinthus* and *P. vera* which have the high yield and the second group was only constituted by *P. lentiscus*.

Similar yield was obtained in Morocco for *P. lentiscus* (0.14%) and in accordance with those obtained in Corsica by Castola *et al.*[15]. Nevertheless, it is less than those reported by Duru *et al.*[16] for the plants collected from Turkey (0.3%). On the other hand, *P. vera* from Tunisia showed higher yield than that from Turkey (0.15%)[16]. The chromatographic analysis (GC (RI) and GC/MS) of the essential oils allowed the identification of 42 components (Table 1), representing 94.73-98.9% of the total oil content.

In the oil from *P. lentiscus*, the hydrocarbonated monoterpenes amounted to 63.9%; on the other hand, the total sesquiterpenic fraction amounted to 31.3% of the total oil. The main compounds are the monoterpenes α-pinene (20.6%), limonene (15.3%) and β-pinene (9.6%), the oxygenated monoterpene terpinen-4-ol (8.2%) and the sesquiterpene germacrene D (8.4%). Other compounds, in a lesser amount, are α-phellandrene (3.85%) and α-terpineol (3.5%). In the literature, Amhamdi *et al.*[17] in Morocco reported β-myrcene (39.2%) limonene (10.3%), β-gurjunene (7.8), germacrene (4.3%), α-pinene (2.9%), as the most abundant compound in *P. lentiscus* essential oils. In a previous study, the chemical composition of essential oils of *Pistacia lentiscus* L. from Tunisian populations have been reported by Douissa *et al.*[18], data revealed from this study showed the abundance of α-pinene 17%, δ-terpinene (9%) and terpen-4-ol (12%). In the oil from *P. terebinthus*, the hydrocarbons monoterpenes amounted to 83.12%, with a total sesquiterpenes amount of 9.4% (7.1% sesquiterpene hydrocarbons and 2.3% of oxygenated sesquiterpenes) of the total oil. α-terpinene (41.34%), α-pinene (19.24%), δ-terpinene (6.99%) and α-terpinolene

Table 1: Chemical composition of the essential oils extracted from the leaves of three *Pistacia* species

N°	Components	RI	*P. lentiscus*	*P. terebinthus*	*P. vera*	Identification
1	tricyclene	926	0,2	-	0.3	MS, RI
2	α-thujene	931	0,65	0.22	-	MS, RI
3	α-pinene	939	20,6	19.21	16.07	MS,RI, Co-GLC
4	camphene	953	0,9	0.19	0.1	MS, RI
5	sabinene	976	1,9	-	0.1	MS, RI
6	β-pinene	980	9,6	1.99	2.32	MS, RI
7	β-myrcene	991	3,4	0.11	1.29	MS, RI
8	α-phellandrene	1005	3,85	0.43	0.1	MS,RI, Co-GLC
9	δ-3-carene	1011	-	0.26	0.13	MS, RI
10	α-terpinene	1018	4,1	41.34	32.44	MS,RI, Co-GLC
11	p-cymene	1023	1,2	-	-	MS, RI
12	limonene	1031	15,3	4.36	25.1	MS,RI, Co-GLC
13	δ-terpinene	1062	-	6.99	-	MS, RI
14	α-terpinolene	1088	2,2	8.02	1.13	MS, RI
15	linalool	1098	-	0.1	-	MS, RI
16	α-campholenal	1125	-	-	0.1	MS, RI
17	pinocarvone	1165	-	0.11	0.12	MS, RI
18	terpen-4-ol	1177	8,2	1.38	0.91	MS,RI, Co-GLC
19	α-terpineol	1189	3,5	2.14	4.52	MS, RI
20	(Z)-piperitol	1205	-	0.22	0.93	MS, RI
21	citronellol	1228	-	0.31	-	MS, RI
22	*iso*-bornyl acetate	1279	-	0.12	1.74	MS, RI
23	α-copaene	1376	1,1	-	0.37	MS, RI
24	β-bourbonene	1384	1,2	0.1	0.23	MS, RI
25	α-cububene	1386	0,6	-	-	MS, RI
26	β-elemene	1387	1,3	-	0.11	MS, RI
27	β-cububene	1390	-	1.92	0.41	MS, RI
28	longifolene	1402	-	-	0.22	MS, RI
29	(Z) -caryophyllene	1418	2,6	3.67	0.14	MS,RI, Co-GLC
30	β-farnasene	1452	-	-	0.34	MS, RI
31	α-humulene	1454	0,9	-	0.13	MS,RI, Co-GLC
32	allo-aromadendiene	1463		-	0.31	MS, RI
33	α-murrolene	1474	1,1	-	-	MS, RI
34	germacrene D	1478	8,4	-	-	MS, RI
35	bicyclogermacrene	1480		-	0.24	MS, RI
36	β-bisabolene	1498	1,6	-	-	MS, RI
37	δ-cadinene	1524	0,8	1.41	0.15	MS,RI, Co-GLC
38	α-calacorene	1542	-	-	0.23	MS, RI
39	caryophyllene oxide	1581	1,1	0.91	1.51	MS, RI
40	cedrol	1596	-	0.43	0.21	MS, RI
41	humulene epoxide	1606	0,7	-	0.61	MS, RI
42	α-cadinol	1653	1,9	0.96	2.12	MS, RI
Total identification (%)		98.9	96.9	94.73		
Monoterpene hydrocarbons (%)			83.12	63.9	79.08	
Oxygenated monoterpenes (%)			11.7	4.38	8.32	
Sesquiterpene hydrocarbons (%)			19.6	7.1	2.88	
Oxygenates sesquiterpenes (%)			3.7	2.3	4.45	

RI: Retention Index on apolar HP-5 MS column, MS: Mass spectrometry, Co-GLC: Co-injection, -: Not detected

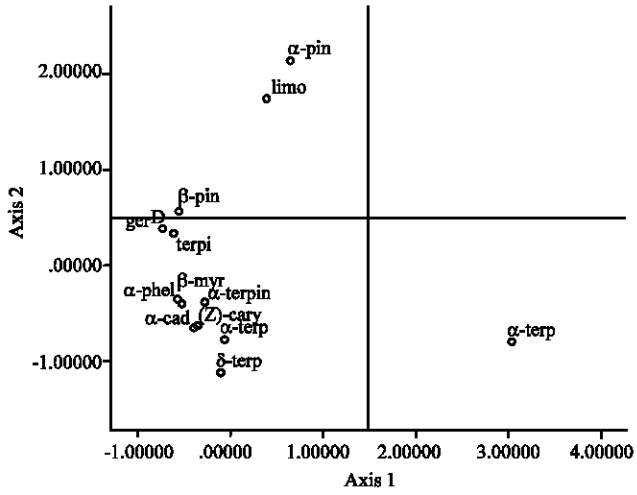

Fig. 2: Principal component analysis of 13 compounds for the leaf essential oils of three *Pistacia* species

(8.02%) were the most abundant among the hydrocarbonated monoterpenes. The global chromatographic analysis of *P. vera* oil showed a complex mixture consisting mainly of monoterpene hydrocarbons and small amounts of oxygenated mono and sesquiterpenes. It was dominated by monoterpene hydrocarbons (79.08%) and oxygenated monoterpenes (8.32%), while oxygenated and sesquiterpenes hydrocarbons were only present in small percentage (respectively, 4.45 and 2.88%). The major components detected in the oil were α-pinene (16.07%) and α-terpinene (32.44%). The chemical composition of *P. vera* and *P. terebinthus* was previously reported in Turkey by Duru *et al.*[16] and it have shown that essential oils of these two species were rich in oxygenated monoterpenes like terpinen-4-ol and α-terpineol. These differences found between the main constituents of oils obtained from *Pistacia* species growing in Tunisia and these from the same species but growing in Turkey and other countries could be related particularly to climate, soils and the genetic background of tree.

Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA), to evaluate whether the identified essential oil components may be useful in reflecting the chemotaxonomic relationships in the three *Pistacia* species, 13 compounds with contents in the essential oils of minimum 3.5% in at last one species (Table 2), were selected for the PCA and the HCA. The PCA horizontal axis explained 57.95% of the total variance and the vertical axis a further 42% (Fig. 2). The HCA based on the Euclidean distances between groups indicated two groups of species (A and B), identified by their essential oil chemotypes with a dissimilarity >20 (Fig. 3 and 4). Group B was further divided into two Subgroups (*P. vera* and *P. terebinthus*) with a dissimilarity

Table 2: Content in the essential oils extracted from the leaves of three *Pistacia* species of the 13 compounds selected for the principal component and the hierarchical cluster analysis

N°	Components	Abbreviations	*P. lentiscus*	*P. terebinthus*	*P. vera*
1	α-pinene	a_pin	20.60	19.21	16.07
2	β-pinene	β-pin	9.60	1.99	2.32
3	β-myrcene	β-myr	3.40	0.11	1.29
4	α-phellandrene	α-phel	3.85	0.43	0.10
5	α-terpinene	α-terp	4.10	41.34	32.44
6	limonene	limo	15.30	4.36	25.10
7	δ-terpinene	δ-terp	0.00	6.99	0.00
8	α-terpinolene	α-terp	2.20	8.02	1.13
9	terpinen-4-ol	terpi	8.20	1.38	0.91
10	α-terpineol	α-terpin	3.50	2.14	4.52
11	(Z)-caryophyllene	(Z)-cary	2.60	3.67	0.14
12	germacrene D	ger D	8.40	0.00	0.00
13	α-cadinol	α-cad	1.90	0.96	2.12

<1. *P. lentiscus* stand out in both HCA and PCA analyses, forming separate group A. Since the essential oil components within the same group were significantly correlated and tend to vary in the same way, we considered each group as a chemotype (Fig. 3 and 4). In fact group A was reduced to *P. lentiscus* which was highly positively correlated with the vertical axis with an essential oil distinguished by high contents of α-pinene, limonene, β-pinene and terpinen-4-ol. *P. terebinthus*, forming the subgroup B1, which was highly positively correlated with the horizontal axis, shared with *P. vera* forming the subgroup B 2 a high content of α-terpinene (32.44-41.34%), however, it was specified by a considerable percentage of limonene (25.1%).

Antifungal activity of essential oils of three *Pistacia* species: Essential oils isolated from leaves of *P. lentiscus*, *P. terebinthus* and *Pistacia vera* were tested for their antifungal activity against ten plant pathogenic fungal

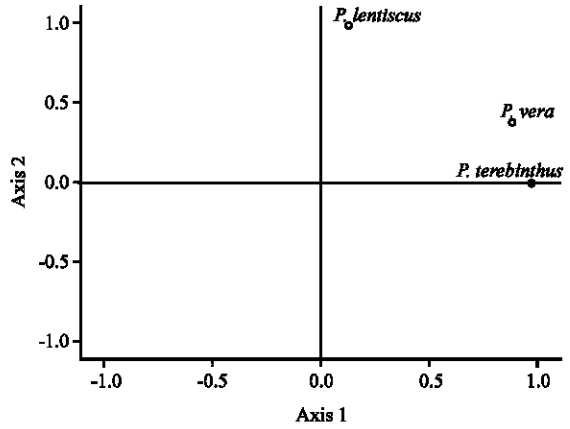

Fig. 3: Principal component analysis of three *Pistacia* species based on their chemical composition

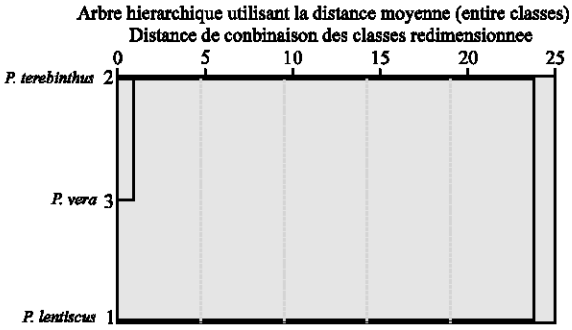

Fig. 4: Dendrogram obtained by cluster analysis based on the Euclidean distances between groups of the leaf essential oils of three Tunisian *Pistacia* species

species. According to obtained results in Table 3, all essential oils showed significant inhibition of fungal growth, this study also indicated that the antifungal activity is variable depending on the fungal strain and tested oils. Essential oils of *P. terebinthus* were most effective against *F. avenaceum* and *F. verticillioides* when compared with *P. lentiscus* and *P. vera.* Our results are in agreement with the literature, in fact, the antimicrobial activity of essential oils and extracts from species belonging Anacardiaceae family were reported. Essential oils of *Pistacia* species collected from Turkey have been reported to have antifungal activity against *Fusarium sambucinum*, *Rhizoctonia solani* and *Pythium ultimum*[16]; essential oils of *Pistacia lentiscus* grown in Tunisia have been studied by Douissa *et al.*[18] and data reveled from this study show an important antimicrobial activity. In general, there was a correlation between the antifungal activity and percentage of some major components. Table 1 indicated that tested oils contain similar major components like α-pinene, limonene and α-terpinene

Table 3: Antifungal activity of essential oil extracted from leaves of three *Pistacia* species

	Essential oils inhibition % at 4 μL mL^{-1}		
Fungus	*P. lentiscus*	*P. vera*	*P. terebinthus*
F. avenaceum	44.43±8.96 a A	63.18±8.54 abc AB	67.97±0.39 cd B
F. culmorum	68.09±7.40 c A	47.33±10.37 a A	63.52±3.09 cd A
F. oxysporum	57.95±7.71 abc A	59.22±4.20 abc A	58.67±3.64 abc A
F. subglutinans	54.70±0.41 abc A	65.29±7.48 bc A	54.26±8.11 abc A
F. verticillioides	52.48±7.36 ab A	49.65±5.93 ab A	47.72±3.21 a A
F. nygamai	59.19±0.41 abc A	66.57±4.64 c AB	70.47±3.00 d B
Botrytis cinerea	51.92±1.56 ab A	60.50±4.42 abc A	48.58±8.43 ab A
Microdochium nivale	63.18±7.86 bc A	72.17±6.19 c A	56.60±10.54 abcA
Alternaria sp.	61.72±4.10 bc A	64.58±6.82 bc A	57.41±5.31 abc A
Bipolaris sorokiniana	56.51±5.85 abc A	63.76±4.87 bc A	55.72±6.97 abc A

Small letters compare means in the lines and capital letters in the columns. Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$). (Mean of three replicates), Means in the same line by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$)

which are known by their antimicrobial activity. Indeed, the antimicrobial activity of monoterpenes suggests that they diffuse into pathogens and damage cell membrane structures[19]. α-pinene, which was found in appreciable amounts in the oils of this study, has been reported to be the cause of the antifungal activity of oil from *Pistacia lentiscus*[20]. In another report it was demonstrated that the antimicrobial activity of essential oil was associated with phytochemical components such as monoterpenes[21]. Sokovic kand Griensven[22], described antifungal activity of limonene and α-pinene against *Verticillium fungicola* and *Trichoderma harzianum.* Thus, the antifungal activity of the oil in this study is not attributed only to the high proportions of hydrocarbonated monoterpenes, however, other major or trace components in the oil could give rise to its antifungal activity. Indeed, there are synergistic and antagonistic interactions between oil components. The mode of action of essential oils was investigated by many authors who suggested that the antimicrobial activity is produced by interactions provoked by terpenes in the enzymatic systems related with energy production and in the synthesis of structural components of the microbial cells[23]. Other reports suggested that the components of the essential oils cross the cell membrane, interacting with the enzymes and proteins of the membrane such as the H^+/ATPase pumping membrane, so producing a flux of protons toward the cell, exterior which induces changes in the cells and ultimately their death. Besides, several authors[24,25,26] reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. In addition, Daferera *et al.*[27] reported

that the fungitoxic activity of essential oils may have been due to formation of hydrogen bonds between the hydroxyl group of oil components and active sites of target enzymes.

CONCLUSION

New trends in crop protection lead to a reduction in the levels of pesticides or/and to the use of "naturally-derived" pesticide from plants, animal or microbial origin. Among natural substances, essential oils and extracts from several types of plants used as flavouring agents are known to possess many biological activities and seem to be suitable for different types of products as bio-herbicide. Our study could give the solution, which in its first part had focused on the correlation between the chemical composition and the effectiveness as antifungal agents of essential oils extracted from Tunisian *Pistacia* species.

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