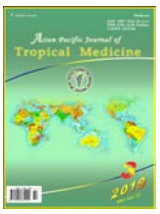

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Chemical composition of *Mentha suaveolens* and *Pinus halepensis* essential oils and their antibacterial and antioxidant activities

Abdelhakim Bouyahya[1]✉, Omar Belmehdi[2], Jamal Abrini[2], Nadia Dakka[1], Youssef Bakri[1]

[1]*Laboratory of Human Pathologies Biology, Department of Biology, Faculty of Sciences, and Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco*

[2]*Biology and Health Laboratory, Department of Biology, Faculty of Sciences, Abdelmalek Essaadi University, Tetouan, Morocco*

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ABSTRACT

Objective: To determin the chemical compounds of *Mentha suaveolens* (*M. suaveolens*) and *Pinus halepensis* (*P. halepensis*) essential oils (Eos) and evaluate their antioxidant and antibacterial activities.

Methods: The chemical composition of *P. halepensis* and *M. suaveolens* EOs was determined by GC-MS analysis. The antioxidant activity was evaluated using DPPH, ABTS and FRAP assays. The antibacterial effect was tested against 6 bacterial strains using the well diffusion method and micro-dilution assay.

Results: The major components of *P. halepensis* EOs were β-caryophyllene (28.04%), myrcene (23.81%) and α-pinene (12.02%). However, piperitenone oxid (56.28%), piperitenone (11.64%) and pulegone (6.16%) were the major components of *M. suaveolens* EOs. *M. suaveolens* EOs showed remarkable antioxidant activities compared with *P. halepensis* EOs, showing antioxidant capacity values of IC_{50}=(64.76±2.24) μg/mL, IC_{50}=(82.73±3.34) μg/mL, and IC_{50}=(93.35±4.45) μg/mL, revealed by DPPH, ABTS and FRAP assays, respectively. However, *P. halepensis* EOs showed interesting antibacterial effects against all bacterial strains. The most sensible strains to *P. halepensis* EOs were *Staphylococcus aureus* [(34.00±0.50) mm], *Listeria monocytogenes* [(31.00±1.50)] mm and *Proteus mirabilis* [(29.00±2.25)mm]. Furthermore, the lowest minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were revealed by *P. halepensis* EOs against *Staphylococcus aureus* [MIC=MBC=0.125% (v/v)] and *Listeria monocytogenes* [MIC=MBC=0.25% (v/v)].

Conclusions: *P. halepensis* and *M. suaveolens* EOs contain bioactive compounds that could have potential applications against bacterial infections and oxidative stress related diseases as well as for food conservation. However, further investigations are necessary to isolate and investigate the action mechanisms of these bioactive compounds.

1. Introduction

Oxidative stress results from unbalancing between generation and degradation of free radicals in cells. Currently, several data showed that the oxidative stress is a major risk factor of genesis for several diseases such as inflammation, diabetes, and cancer[1]. Synthetic antioxidant drugs have shown several side effects that require the screening of natural antioxidants. In this context, several studies currently focused on the secondary metabolites of medicinal plants such as essential oils(EOs) as source of novel antioxidants.

On the other hand, the bacteria continue to develop sophisticated

✉First and corresponding author: Bouyahya Abdelhakim, Laboratory of Human Pathologies Biology, Faculty of Sciences of Rabat, University Mohammed V of Rabat, Morocco.
Tel: +212601350878
E-mail : boyahyaa-90@hotmail.fr
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mechanisms to resist against antibiotic actions[2]. The resistance developed by some bacteria towards a large number of antibiotics challenges the search of new alternative molecules, especially those extracted from natural sources including medicinal plants.

Among the bioactive compounds containing in medicinal plants, we found EOs, which exhibited various biological activities such as antimicrobial, antioxidant, antitumor and anti-inflammatory effects[3,4]. These effects are certainly attributed to terpenoids bioactive compounds containing in EOs.

Mentha suaveolens (*M. suaveolens*) and *Pinus halepensis* (*P. halepensis*) are two medicinal plants used traditionally for the treatment of some diseases in several countries including Morocco. In fact, *M. suaveolens* is used by Moroccan population to treat pathologies of digestive system, allergy and diabetes, while *P. halepensis* is used against stomach disorders[5].

M. suaveolens and *P. halepensis* EOs showed some biological properties such as antimicrobial and antioxidant effects[6]. However, in Morocco, EOs of these two species have not been largely studied. This study aimed to determine the chemical composition of *P. halepensis* and *M. suaveolens* EOs, and evaluate their antibacterial and antioxidant properties.

2. Materials and methods

2.1. Plant material and EOs extraction

The leaves of *P. halepensis* (voucher specimen: RAB16) and the flowery tops of *M. suaveolens* (voucher specimen: RAB08) were collected during the summer months (June) from the North-West of Morocco. The samples were air dried at room temperature under dark condition. The extraction of EOs was carried out by hydrodistillation method using clevenger-type apparatus. The obtained oils were dried, weighed and stored at 4 ℃ until use.

2.2. Gas Chromatography-mass spectrometry(GC-MS) analysis EOs

The volatile components of *P. halepensis* EOs and *M. suaveolens* EOs were analyzed by GC-MS using the same parameters previously published[6]. The identification of volatile compounds was carried based on the literature and the retention index. The confirmation of each compound was made by comparison of its mass spectra with those of NIST02 library data.

2.3. Antioxidant activity

2.3.1. The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical-scavenging assay

DPPH was used for the determination of free radical-scavenging activities of *P. halepensis* and *M. suaveolens* EOs[6,7]. Briefly, 0.2 mL aliquots of various concentrations of methanol dissolved EO's samples were added to 1.8 mL of a 0.004% DPPH methanolic solution. After 30 min incubation time at room temperature, the absorbance was measured at 517 nm as optical density[6]. The EOs antioxidant activities expressed as the percent of scavenging DPPH radical was calculated by the following formula:

$$\text{DPPH scavenging activity } (\%) = [(A_c - A_t)/A_c] \times 100.$$

A_c is the absorbance of the control (without oil), and A_t is the absorbance of the sample. Trolox and ascorbic acid were used as positive controls, and EOs concentration providing 50% inhibition (IC_{50}) was calculated by plotting the inhibition degrees against the sample concentrations. The test was carried out in triplicate, and the IC_{50} values were reported as means±standard deviation (SD).

2.3.2. Reducing ferric power determination

Reductive ability was investigated by the Fe^{3+} to Fe^{2+} transformations in presence of *P. halepensis* and *M. suaveolens* EOs[6,7]. EOs and control (ascorbic acid and trolox) were mixed separately and successively with 2.5 mL volumes of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%). After incubating the mixture at 50 ℃ for 20 min, 2.5 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged for 10 min at 3 000 rpm. Finally, a volume of 2.5 mL was collected from the upper layer of the obtained solution and mixed with an equal volume of distilled water and 0.5 mL $FeCl_3$ (0.1%). The absorbencies were measured at 700 nm by a spectrophotometer and plotted against the corresponding sample concentrations to deduce the EO concentration providing 50% of absorbance decrease (IC_{50}). Trolox and ascorbic acid were used as positive controls. The test was carried out in triplicate and IC_{50} values were reported as means±SD.

2.3.3. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

ABTS radical-scavenging activity of EO was determined according to the method described by El Bouzidi *et al.*[6] with some modifications. The ABTS radical cation was produced by the reaction between 5 mL of ABTS stock solution and 5 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, this solution was diluted with water to get an absorbance of (0.700±0.015) at 734 nm and equilibrated at 30 ℃. EOs at various concentrations were diluted with dimethyl sulfoxide to get sample solution. A volume of 5 mL sample solution was homogenized with 195 mL of ABTS solution, the mixture was incubated at room temperature for 6 min and its absorbance was recorded at 734 nm. Blanks were run in each assay. ABTS scavenging ability was expressed as IC_{50} (μg/mL) and the inhibition percentage was calculated using the following formula:

$$\text{ABTS scavenging activity } (\%) = (A_0 - A_1)/A_0 \times 100.$$

Where: A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

2.4. Antibacterial activity

2.4.1. Bacteria strains

The antibacterial effects of *M. suaveolens* and *P. halepensis* EOs were evaluated against *Escherichia coli* (*E. coli*) K12 (Laboratory of Food Microbiology, UCL, Belgium: MBLA), *Staphylococcus aureus* CECT 994(*S. aureus*), *Listeria monocytogenes* serovar 4b CECT 4032 (*L.*

monocytogenes) and *Proteus mirabilis* (Spanish type culture collection: CECT)(*P. mirabilis*), *Pseudomonas aeruginosa* IH (*P. aeruginosa*)(Institute of hygiene, rabat, Morocco: IH), and *Bacillus subtilis* 6633 (*B. subtilis*) (German collection of microorganisms: DSM). Strains were stored in inclined agar medium at 4 ℃. Before use, the bacteria were revived by two subcultures in an appropriate culture medium; lysogeny broth (LB) (Biokar Diagnostics, Beauvais, France) at 37 ℃ for (18-24) h. For the test, final inoculum concentrations of 10^6 CFU/mL bacteria were used according to the National Committee for Clinical Laboratory Standards, USA.

2.4.2. Determination of inhibition diameters

To determine the inhibition diameters of *P. halepensis* and *M. suaveolens* EOs against the tested bacteria, we have used agar-well diffusion assay as described by Bouhdid *et al.*[8]. This method is mostly used as preliminary screening assay to further quantitative investigations. Briefly, a basal layer medium was made by cooling Muller-Hinton agar on Petri dishes to solidify. An 8 mm diameter cylinder was deposited under aseptic conditions on Muller-Hinton agar layer. A total of 6 mL LB medium containing 0.8% agar and inoculated by a fresh culture of the tested bacterial strain was used as a top medium layer. The wells formed were filled with 50 µL of *P. halepensis* and *M. suaveolens* EOs after enriched LB medium solidification. After 24 h incubation time at an appropriate temperature for each tested strain, all cultures were examined and the diameters of the appeared inhibition zones were measured in millimeters. All the bacterial growth inhibition assays were performed in triplicate.

2.4.3. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC of *M. suaveolens* and *P. halepensis* EOs against the tested bacteria were determined according to the broth micro-dilution assay described by Bouhdid *et al.*[9] and Bouyahya *et al.*[6]. The test was carried out on liquid medium using a 96-wells polypropylene microtitre plate. After deposing a 50 µL (different concentrations) of the tested oil in each well, 50 µL (final concentration of 10^6 CFU/mL) of a bacterial suspension was added to each well. After incubation at 37 ℃ for 18 h, the resazurin (10 µL) was added to each well to assess bacterial growth. Bacterial growth was detected after incubation at 37 ℃ for 2 h by reduction the blue dye resazurin to pink resorufin, and then the MIC was determined as the lowest EO concentration that prevented resazurin color changes. To determine the MBC, 10 µL were transferred from negative subcultures to on plat count agar medium and then the MBC were determined after 24 h incubation time at 37 ℃[8].

2.5. Statistical analysis

The comparison of means between groups was performed with one-way analysis of variance (ANOVA) followed by Tukey test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Chemical composition

The chemical composition of *P. halepensis* and *M. suaveolens* EOs was identified using GC-MS (Figure 1 and 2). The obtained results are listed in Table 1. As shown, 20 compounds were identified in *M. suaveolens* EOs, while *P. halepensis* EOs contain 23 volatile compounds. The main compounds of *P. halepensis* EOs were β-caryophyllene (28.04%), myrcene (23.81%), α-pinene (12.02%), and caryophyllene oxide (6.78%). However, for *M. suaveolens* EOs, the main compounds were piperitenone oxide (56.28%), piperitenone (11.64%), and pulegone (6.16%).

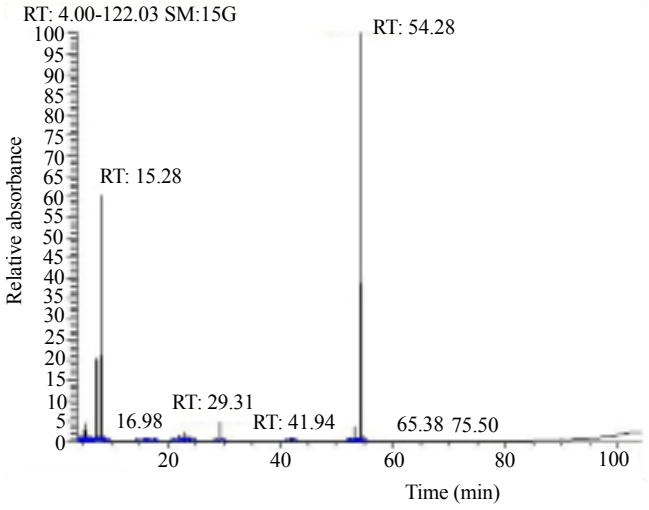

Figure 1. GC-MS analysis of *P. halepensis* essential oils.

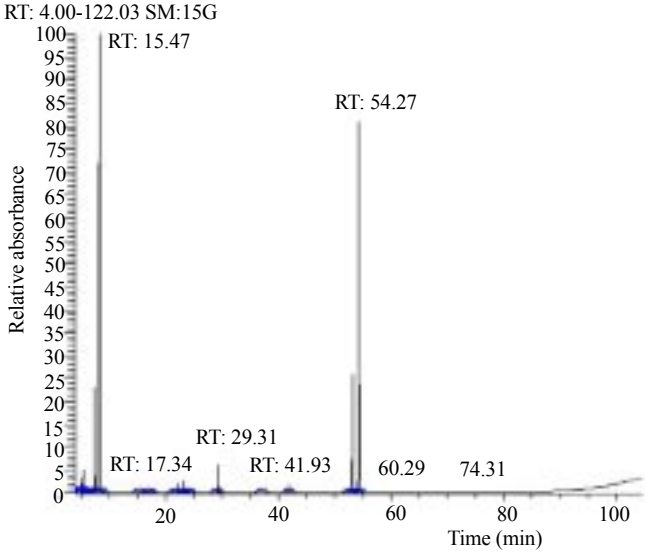

Figure 2. GC-MS analysis of *M. suaveolens* essential oils.

3.2. Antioxidant activity

To evaluate the antioxidant effects of *P. halepensis* and *M. suaveolens* EOs, 3 complementarily assays were used: DPPH scavenging activity assay, ABTS and FRAP tests, and in order to compare the obtained data, the results were expressed as IC_{50} values (Table 2). As listed in the table, *M. suaveolens* EOs revealed higher antioxidant capacities than *P. halepensis* EOs. Indeed, *M. suaveolens* EOs showed antioxidant capacity values at IC_{50}=(64.76±2.24) µg/mL, IC_{50}=(82.73±3.34) µg/mL, and IC_{50}=(93.35±4.45) µg/mL using DPPH, FRAP and ABTS assays, respectively. Moreover, it has been noticed that the DPPH assay was more sensitive than FRAP and ABTS tests.

Table 1. Chemical composition of *P. halepensis* and *M. suaveolens* EOs.

N	Retention index (RI)	Compounds	*P. halepensis* (%)	*M. suaveolens* (%)
1	891	Tricyclene	0.71	-
2	919	α-thujene	0.63	-
3	931	α-pinene	12.02	1.47
4	946	Camphene	1.23	0.80
5	960	Sabinene	0.73	1.05
6	974	β-pinene	-	2.03
7	987	Myrcene	23.81	3.26
8	1 004	α-Phellandrene	1.27	-
9	1 012	α-Terpinene	2.34	-
10	1 018	*p*-Cymene	0.70	-
11	1 023	Limonene	1.02	1.98
12	1 029	1.8-Cineole	-	0.74
13	1 051	γ-Terpinene	0.64	-
14	1 084	Terpinolene	1.28	-
15	1 102	Linalol	3.62	
16	1 143	Campher	0.81	-
17	1 162	Borneol	3.92	-
18	1 179	*p*-Cymen-8-ol	2.62	-
19	1 193	α-Terpineol	1.27	-
20	1 211	Menthone	-	2.35
21	1 245	Pulegone	-	6.16
22	1 294	*cis*-Piperitone epoxide	-	1.82
23	1 321	Piperitenone	-	11.64
24	1 348	Piperitenone oxide	-	56.28
25	1 372	*p*-Menthane-1,2,3-triol	-	0.71
26	1 397	α-Caryophyllene	1.92	1.39
27	1 412	β-Caryophyllene	28.04	0.84
28	1 444	α-Humulene	0.85	0.56
29	1 474	Germacrene D	0.74	1.03
30	1 501	d-Cadinene	0.52	0.67
31	1 547	Spathulenol	-	1.02
32	1 586	Caryophyllene oxide	6.78	1.64
Total			97.47	97.44

Table 2. Antioxidant activities of *P. halepensis* and *M. suaveolens* EOs (μg/mL).

Assays	Essential oils		Controls	
	P. halepensis	*M. suaveolens*	Ascorbic acid	Trolox
DPPH	113.25±7.18	64.76±2.24	22.61±1.08	34.12±2.13
FRAP	135.14±6.82	82.73±3.34	31.63±1.42	55.25±4.19
ABTS	143.60±7.21	93.35±4.45	44.37±2.32	54.74±3.85

Values represent means±SD for triplicate experiments.

3.3. Antibacterial activity

The antibacterial activity of *P. halepensis* and *M. suaveolens* EOs was evaluated against 6 bacterial strains using agar well diffusion assay. The obtained results were expressed as the diameters of inhibition around the wells (Table 3). As summarized in the table, *P. halepensis* EOs showed a higher antibacterial effect than *M. suaveolens* EOs against some strains. Indeed, *P. halepensis* EOs exhibited remarkable inhibition of *S. aureus*, *L. monocytogenes* and *P. mirabilis* with inhibition zones of (34.00±0.50) mm, (31.00±1.50) mm and (29.00±2.25) mm, respectively. These effects were higher than those obtained with chloramphenicol. For the determination of the MIC and MBC, the micro-dilution assay was used. The obtained results were listed in Table 4. As shown, *P. halepensis* EOs demonstrated remarkable inhibition of *S. aureus* [MIC=MBC=0.125% (v/v)] and *L. monocytogenes* [MIC=MBC=0.25% (v/v)]. However, *M. suaveolens* EOs showed an antibacterial inhibition at moderate concentrations.

Table 3. Antibacterial activity of *P. halepensis* and *M. suaveolens* EOs using agar-well diffusion assay (mm).

Microorganisms	Inhibition zone diameter*		
	P. halepensis	*M. suaveolens*	Chloramphenicol
S. aureus 994	34.00±0.50[a]	26.00±1.50[b]	26.00±3.00[b]
P. aeruginosa	12.00±1.25	13.00±0.50	NA
L. monocytogenes	31.00±1.50[a]	24.00±0.66[b]	24.00±1.66[b]
B. subtilis 6633	16.00±0.45	15.00±1.25	NA
P. mirabilis	29.00±2.25	23.00±1.50	NA
E. coli K12	14.00±1.25[b]	13.00±0.33[b]	25.00±2.66[a]

Chloramphenicol was used as positive control. Values represent means±SD for triplicate experiments; NA: not active; in each line, differenet letters (a, b) indicate significant differences(P<0.05, n=3); *Diameter of inhibition zone included well diameter of 8 mm, by the agar-well diffusion method at a concentration of 50 μL of oil/well. Final bacterial density was around 10^6 CFU/mL.

Table 4. *P. halepensis*, *M. suaveolens* EOs, and chloramphenicol (μg/mL) against 6 human pathogen bacteria strains tested by microdilution assay.

Microorganisms	*P. halepensis*		*M. suaveolens*		Chloramphenicol	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus 994	0.125	0.125	0.250	0.500	8.000	32.000
P. aeruginosa	1.000	>1.000	>1.000	>1.000	64.000	64.000
L. monocytogenes	0.250	0.250	0.500	1.000	16.000	32.000
B. subtilis 6633	1.000	1.000	1.000	>1.000	64.000	128.000
P. mirabilis	0.500	1.000	1.000	1.000	64.000	64.000
E. coli K12	1.000	>1.000	>1.000	>1.000	8.000	32.000

MIC and MBC are expressed as% (v/v). Final bacterial density was around 10^6 CFU/mL.

4. Discussion

Several studies have shown that EOs possess remarkable biological properties such as anticancer, anti-inflammatory, antioxidant and antimicrobial effects[3,4]. Morocco, as a Mediterranean country, is rich in vegetation, in particularly medicinal plants. The chemical and biological valorization of Moroccan medicinal plant secondary metabolites may be an important strategy to develop alternative drugs. Our study focused on the characterization of *P. halepensis* and *M. suaveolens* EOs and the investigation of their antibacterial and antioxidant activities.

P. halepensis is a woody plant, so for the EOs extraction the leaves were used. However, *M. suaveolens* is an herbaceous plant, and in this case the flowery tops were used to extract the EOs. It has been noticed that the major compounds of *M. suaveolens* EOs (pulegone, piperitenone and piperitenone oxide) were totally absent in *P. halepensis* EOs. On the other hand, the major compounds of *P. halepensis* EOs existed in *M. suaveolens* EOs but in low concentrations.

Several studies have reported the chemical composition of *P. halepensis* and *M. suaveolens* EOs[10-13]. Our findings are not completely in accordance with the literatures. These differences are attributed certainly to the variability of ecological conditions between the areas of collection. Indeed, Llorens-Molina *et al.*[14]

showed that the chemical composition of *M. suaveolens* EOs is variable between several regions. Moreover, the chemical variability between *P. halepensis* EOs was also proved by Dob *et al.*[15], which determined the volatile compounds of *P. halepensis* EOs in several areas from Algeria and revealed different results[15]. Other several studies currently showed that the chemical composition of same species is influenced by various factors particularly climatic conditions[14,16]. For this reason, the comparison of the essential oil composition described in different studies is sometimes difficult, if not impossible, not only because different methodologies of isolation and analysis may have been used, but also when different plant parts and various developmental stages are involved. Recently, some studies have reported that chemical flexibility of secondary metabolites of *P. halepensis* is due essentially to response against induced stress by climatic changes[17,18].

Generally, the synthesis of secondary metabolites in medicinal plants is affected by various internal and external factors, which influence the regulation of these metabolites in space and time *via* several epigenetic pathways such as DNA methylation, histones modifications and remodelage of chromatin in order to respond against specific physiological functions[19,20].

Piperitenone oxide, the major compound of *M. suaveolens* EOs, is known for its cardiovascular and anti-nociceptive effects[21,22] and as an insect repellent. However, pulegone, one of the main components of this plant, is known for its antimicrobial activity[23], as well as for its hepatoprotective effect[24]. On the other hand, β-caryophyllene the main component of *P. halepensis* EOs, is known for its various biological activities such as anticancer and analgesic properties[25], local anesthesic activity[26], anti-arthritic and anti inflammatory effects[27]. Moreover, α-pinene and myrcene also possess numerous biological activities such as antimicrobial[28]. These various activities of these biomolecules explain its importance as alternative for medical purposes. In addition, as it has been shown that the major components vary between these two studied plants; these EOs can be complementary as bioactive compounds sources.

The evaluation of antioxidant activity revealed that the IC_{50} values obtained with the DPPH test were lower than those obtained with the FRAP and ABTS tests. The differences between the two results can be explained by the mechanism of the reaction involved. The major compounds of *P. halepensis* EOs are β-caryophyllene, α-pinene, myrcene and *p*-cymene, while the major compounds of *M. suaveolens* EOs are piperitenone oxid and piperitenone, which could explain the differences in the antioxidant activity between them. In addition, the oxygenated monoterpenes found in these EOs may act as antioxidant agents.

The antiradical activity of our *P. halepensis* EOs using DPPH test [IC_{50}=(113.25±7.18) μg/mL] was more important than that of Algerian *P. halepensis* EOs evaluated at different vegetative stages [IC_{50} values ranging from (201.28±2.75) to (236.18±3.73) μg/mL][13]. Comparatively, this DPPH-radical scavenging activity was lower than that of the synthetics antioxidants ascorbic acid and trolox [IC_{50}=(22.61±1.08) μg/mL and (34.12±2.13) μg/mL, respectively].

The antioxidant properties of *P. halepensis* EOs and MSEO are certainly attributed to the phenolic compounds present in these EOs, which are able to neutralize the free radicals *via* different mechanisms. However, the use of three experimental methods to estimate the antioxidant activity showed that the antioxidant effect of *P. halepensis* and *M. suaveolens* EOs does not only depend on the chemical composition, but also on the medium where reaction takes place. Our results are in accordance with other studies carried out on the antioxidant activities of *P. halepensis* and *M. suaveolens* EOs[13,29].

The antibacterial activity of *M. suaveolens* and *P. halepensis* EOs was determined against 6 strains of reference using agar well diffusion assay and microtitration assays. The results revealed that *P. halepensis* EOs showed remarkable inhibitory effects on all tested microorganisms. It would be related to their major components specially β-caryophyllene which is known by its antibacterial effect[30]. However, it is difficult to attribute the activity of a complex mixture to a single or particular constituent. In fact, there is some evidence that minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components. The variation in chemical composition of essential oil might be responsible for the different antibacterial effects.

The mechanisms of action of plant EOs have been mostly studied. Nevertheless, the studies about the antimicrobial action of essential oil and their components still remains. It has been reported that the antimicrobial actions of essential oil are linked to one of the most important EOs characteristics, its hydrophobicity resulting in increased cell permeability and consequent leaking of cell constituents. The most known targets of EOs are: cell wall and membrane disturbance, alteration of ATP production and protein synthesis, pH disturbance, intra-cytoplasmic changes, DNA damage, and quorum sensing inhibition[31]. Moreover, this study revealed that Gram-negative bacteria are more resistant to *P. halepensis* and *M. suaveolens* EOs than the Gram-positive ones. The resistance of Gram-negative bacteria can be attributed to the presence of outer membrane surrounding the cell wall[32].

In this study, the antioxidant and antibacterial activities of *M. suaveolens* and *P. halepensis* EOs were evaluated. The analysis of the chemical composition showed considerable qualitative variability in the composition of *P. halepensis* and *M. suaveolens* EOs. The variability in essential oil composition is the results of an adaptive process to particular ecologic conditions (geographical regions, climate conditions, altitude), period of collection of the plant, the studied part (leaves or flowers), state of plant (fresh or dry) and method of extraction of the essential oil. The antioxidant effects were showed mostly by *M. suaveolens* EOs, while *P. halepensis* EOs showed interesting antibacterial properties. The results revealed the eventually possibilities to use of *M. suaveolens* EOs as source of antioxidant compounds and *P. halepensis* EOs as source of antibacterial compounds. However, further studies are required to isolate bioactive components and to elucidate their antioxidant and antibacterial mechanisms.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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