Phytothérapie
DOI 10.3166/phyto-2019-0171

PHARMACOGNOSIE

The Antibacterial Effect of Essential Oils of *Satureja calamintha* subsp. *nepeta* (L.) Briq, *Lavandula multifida* L., and *Mentha pulegium* L., Tested Against some Multiresistant Strains that Are Involved in Nosocomial Infections

Effet des huiles essentielles de *Satureja calamintha* subsp. *nepeta* (L.) Briq, *Lavandula multifida* L. et *Mentha pulegium* L. contre des souches multirésistantes incriminées dans les infections nosocomiales

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Abstract In infectiology, some essential oils (EOs) are able to compete with antibiotic therapy and even surpass it; it is in this context that a study of the chemical composition and the antibacterial power of the EOs of three aromatic and widely used medicinal plants in traditional medicine was conducted which are *Satureja calamintha* subsp. *nepeta* (L.) Briq, *Lavandula multifida* L., and *Mentha pulegium* L. The extracted EO yields were of the order of 2.6%, 0.12%, and 5.29% for *Satureja calamintha*, *Lavandula multifida*, and *Mentha pulegium*, respectively. The obtained EOs were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The results of these analyses showed that *Lavandula multifida* contains carvacrol as a majority compound (70.65%); by contrast, the pulegone is the majority compound of *Satureja calamintha* and *Mentha pulegium* that contain about 87.04% and 71.97%, respectively. The EOs with the pulegone as predominant compound (*Mentha pulegium* and *Satureja calamintha*) showed higher antibacterial activity when tested against some multiresistant strains, compared to those with the carvacrol as major compound. In conclusion, this study may open up prospects for the formulation of phytomedicines against resistant and nonresistant bacteria incriminated in nosocomial infections.

Keywords Essential oil · Chemical composition · Antibacterial effect · Multiresistance · Nosocomial infection

Résumé En infectiologie, certaines huiles essentielles (HE) sont capables de rivaliser avec l'antibiothérapie, et même de la surpasser souvent. C'est dans ce cadre qu'une étude de la composition chimique et du pouvoir antibactérien des HE de trois plantes aromatiques et médicinales marocaines très utilisées en médecine traditionnelle, *Satureja calamintha* subsp. *nepeta* (L.) Briq, *Lavandula multifida* L. et *Mentha pulegium* L., a été réalisée. Les rendements en HE extraites par hydrodistillation sont de l'ordre de 2,6, 0,12, et 5,29 % respectivement pour *Satureja calamintha*, *Lavandula multifida* et *Mentha pulegium*. Les résultats des analyses des HE par chromatographie en phase gazeuse couplée à la spectrométrie de masse ont montré que *Lavandula multifida* contient le carvacrol comme composé majoritaire (70,65 %), par contre la pulégone est le composé majoritaire de *Satureja calamantha* et *Mentha pulegium* avec respectivement des teneurs de l'ordre de 87,04 et 71,97 %. Le pouvoir antibactérien des HE contre des souches multirésistantes a montré que celles à composé majoritaire, la pulégone (*Mentha pulegium* et *Satureja calamintha*), ont une activité antibactérienne plus élevée que celles à carvacrol comme composé majoritaire. En conclusion, cette étude peut ouvrir des perspectives de formulation de phytomédicaments contre les bactéries résistantes et non résistantes incriminées dans les infections nosocomiales.

Mots clés Huile essentielle · Composition chimique · Pouvoir antibactérien · Multirésistance · Infection nosocomiale

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Introduction

In Morocco, as in the rest of the world, infectious diseases in the hospital environment (nosocomial infections) constitute

an important public health problem, because of their serious consequences on human life. At any moment, more than 1.4 million people around the world suffer from infectious complications acquired in the hospital [1]. These infections are caused essentially by bacteria, but viruses, fungi, parasites, and unconventional transmissible agents can also cause these infections [2].

In addition, there is the appearance of antibiotic-resistant microbial strains and the emergence of uncommon diseases [3]. These antibiotic-resistant microbial strains inhibit the effectiveness of existing remedies, hence, the need to search for new natural substances with large spectrum of action in order to study their chemical compositions, especially as people are moving more and more toward natural antibiotics, which showed no risk of resistance. Today, it has been estimated that active ingredients from plants account for 25% of the prescribed drugs that represent a total of 120 natural compounds from 90 different plants [4].

The main purpose of this study is the valorization of aromatic and medicinal plants (AMP), which are very exploited by Moroccans in traditional medicine. In fact, traditional medicine resorts to valuable therapies involving the effective use of the active ingredients of medicinal plants. Moreover, these plant species that are of great importance for the health of the population deserve to be scientifically studied through phytochemical and biological studies so as to ensure their best use. In addition, Morocco is very rich in AMP; however, they remain scarcely exploited scientifically. This study deals with the valorization of the essential oils (EOs) of three species of AMP that are widely used in the treatment of a lot of infectious diseases, which are *Satureja calamintha subsp. nepeta* (L.) Briq, *Lavandula multifida* L., and *Mentha pulegium* L.

Indeed, the use of EOs is extremely topical. The number of products and indications has multiplied. For example, EOs can be used to clean the air in ventilation systems in the hospital environment in order to limit the expansion of microbial germs [5].

From previous studies, these three species of the family Lamiaceae have shown remarkable therapeutic powers. Indeed *Satureja calamintha* subsp. *nepeta* is a plant species known for its carminative, tonic, antispasmodic, sudorific, and stomachic properties [6,7] as well as its antimicrobial activity [8]. The flowers of the aerial parts of *Mentha pulegium* have been used traditionally for their antiseptic properties to treat cold, sinusitis, cholera, food poisoning, bronchitis, and tuberculosis [9], while the herbal tea of *Lavandula multifida's* flower tops are used against cough, asthma, cystitis, and bloating [10]. The antimicrobial tests (carried out on three bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) showed that the EO of *Lavandula multifida* is active on all the studied strains [11].

The aim of this work is to study the chemical composition and the effects of the EOs of these three plants on the in vitro growth of certain germs involved in nosocomial infections (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*). The selection of these three plants was based on an ethnobotanical survey conducted in 2015 [12] in the Masmouda in Morocco region that showed that *Mentha pulegium* is used in the treatment of respiratory diseases, while *Lavandula multifida* is recommended in the case of digestive diseases, and *Satureja calamintha* subsp. *nepeta* is used mainly for respiratory and gastric diseases.

Materials and Methods

Plant Materials

Samples of the aerial parts (stems, leaves, and flowers) of *Lavandula multifida* were collected in May (2015) in Meknes (northern Morocco). Those of *Satureja calamintha* subsp. *nepeta* were collected in April (2015) in Azrou (Middle Atlas of Morocco), and those of *Mentha pulegium* were collected in June (2015) in M'Rirt which is located in Khenifra region in the Middle Atlas of Morocco.

The identification of the three plant species was carried out at the Laboratory of Botany and Plant Ecology of the Scientific Institute of Rabat (Morocco) by Professor M. Ibn Tatou.

The aerial parts of the three plants are dried at room temperature in a shady place for 13 days, and then treated and stored away from light and moisture.

The Extraction of the Essential Oils

The extraction of the EOs from the three plants was carried out by hydrodistillation with the Clevenger-type apparatus [13].

The Determination of the Chemical Composition of the Extracted Essential Oils by Gas Chromatography Coupled with Mass Spectrometry (GC/MS)

The EOs that were extracted from the aerial parts of the three plants were analyzed by thermo-electron-type gas chromatography (Trace GC Ultra) coupled to a thermo-electron trace MS system mass spectrometer (Thermo Electron: Trace GC Ultra, Polaris Q MS). Fragmentation is performed by electron impact intensity 70 eV. The chromatograph is equipped with a column of type DB-5 (5% phenyl-methyl-siloxane) (30 m × 0.25 mm × 0.25 μm film thickness) and a flame ionization detector (FID) powered by a mixture of H2/air gas. The temperature of the column is programmed at the rate of one mounted of 4 °C/min from 50 to 200 °C for 5 min.

The injection mode is split (leakage ratio: 1/70 ml/min flow rate), and the carrier gas used is nitrogen with a flow rate of 1 ml/min.

The identification of the chemical composition of the three plants' EOs was performed based on the comparison of their Kovat's (IK) and Adams' indices, with those of known reference products in the literature [14,15]. It has been supplemented by a comparison of indices and mass spectra with different references [14,16].

The Kovat's indices compare the retention time of any product with that of a linear alkane of the same carbon number. They are determined by injecting a mixture of alkanes (standard C7–C40) under the same operating conditions.

The Antibacterial Tests

The Sensitivity Test

Three bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were chosen for their pathogenicity and incrimination in nosocomial infections. The three bacteria belong to the collection of the Laboratory of Ecology and Biodiversity of Wetlands, in Moulay Ismail University, Faculty of Sciences in Meknes.

The antibiotic sensitivity profiles of the strains indicated in table 1 are made according to the recommendations of the **French Society of Microbiology and EUCAST** [17,18].

The Disk Diffusion Method

The aromatogram is a simple, qualitative method, applied to any bacteria that are considered pathogenic. This method makes it possible to explore a large number of the EOs effect against each strain.

The antibacterial assays that we used derive from those used by (Remmal et al., 1993) [19].

At first, we filtered our oils with a millipore filter with a pore of 0.45 µm. Then we used dimethyl sulfoxide amide (DMSO) as an emulsifying agent (E) because of its effectiveness as a solubilizing agent for EOs with a low concentration (2 µl), which does not influence the antibacterial quality of the tested EO.

The bacterial suspension was prepared from a pure bacterial culture, which is in exponential growth phase on MHA medium, with a concentration of 105 CFU/ml in sterile physiological saline (EρS). Then from the prepared inoculum, we take 1 ml of the bacterial suspension, and we flood it on the surface of a petri dish containing MHA agar where the excess liquid is sucked. We keep the Petri dish in the septic area of the Bunsen burner until it becomes dry, and then we place the sterile disks of blotting paper in the center and on the peripheries of the Petri dish. On each of these disks, we deposit the volumes of 2 µl, 4 µl, 6 µl, 8 µl, 10 µl, 12 µl, and 14 µl of the EO to be tested and for each EO, we perform two replicates. A sterile disk flooded with 6 µl of EρS was used as a negative control as well as another negative control disk impregnated with 2 µl of DMSO.

The dishes are then incubated at a temperature of 37 °C for 18–24 hours. After incubation, the reading is made by measuring the diameter of the inhibition zone.

Table 1 The EO yields of the plants: *Lavandula multifida*, *Mentha pulegium*, and *Satureja calamintha* subsp. *nepeta*

AMP	Yields (in %)
Lavandula multifida L.	0.12 ± 0.01
Mentha pulegium L.	5.29 ± 0.07
Satureja calamintha subsp. *nepeta* (L.) Briq	2.61 ± 0.03

The Minimum Inhibitory Concentration (MIC)

This technique involves inoculating, by means of a standardized inoculum (105 CFU/ml), a growing volume range into EO. After incubation, the observation of the range makes it possible to determine the minimum inhibitory concentration (MIC), which corresponds to the lowest concentration of the EO for which the bacterial growth is no longer visible in vitro (no growth but 100% of the surviving bacteria). 2 µl of DMSO are transferred into the 10 hemolysis tubes containing the culture medium (1 ml) for each bacterial strain. Each volume of the EO is introduced into a hemolysis tube. The 10 volumes chosen are 2 µl, 4 µl, 6 µl, 10 µl, 12 µl, 14 µl, 16 µl, 18 µl, 20 µl, and 22 µl; two replicates were made for each volume. A volume of 6 µl of the bacterial suspension of a concentration of 105 CFU/ml is taken and then deposited in each of the preceding tubes and then 2 µl of DMSO are added for each bacterial strain.

For each oil, two control tests were made: one containing the culture medium (1 ml) plus one bacterial strain and the other containing the culture medium (1 ml) plus 2 µl of the EO.

The Minimal Bactericidal Concentration (MBC)

Nutritive agar poured into petri dishes is cultured with the contents of the tubes having a concentration greater than or equal to the MIC in the series of the previous concentrations. MBC is determined after incubation for 24 h at 37 °C. It is defined as the smallest concentration that totally inhibits bacterial growth.

Results and Discussion

The Essential Oil Yields

Table 1 summarizes the results of the EO yields of the three studied plants.

The yield is expressed as a percentage of the EO volume (mL) relative to the mass of the vegetable matter (g). The calculation formulas are as follows (Fig. 1):

% TH = m1 – m2/m1 × 100

% R = V/m – (m × % TH) ± Standard deviation

With

% TH = Moisture content of the plant material (moisture content).

% R = The extracted yield of the EO expressed as a volume of EO per mass of vegetable matter (V/m) (average of three yields).

m1 = Initial mass of the plant material in g introduced into the oven at time (t0).

m2 = Final mass of the vegetable matter in g removed from the oven at time (tx).

m = Mass of the plant material used for hydrodistillation (in the Clevenger).

V = Volume of the collected EO (in mL).

The hydrodistillation of *Mentha pulegium* gave a high yield of EO. For 100 g of dry matter, the obtained yield is of the order of 5.29%.

These results are quite high compared to those obtained by several other researchers [20], a study claimed a yield of 2.34% obtained for *Mentha pulegium* from Algeria, which is lower than that obtained in our study. In Morocco, a researcher reported a yield of 1.9% for the species of northern Morocco [21].

For *Lavandula multifida*, the obtained average yield is about 0.12%. Another yield of 0.7% was obtained for the Moroccan species of the region of Errachidia [22]. Other researchers [23] who have worked on samples of the Algerian species (Tlemcen) have noted a yield of 0.09%, while another study reported a yield of 2.01% for the species collected in Morocco in the region of TATA (Anti-Atlas) [11].

The yield of the EO of *Satureja calamintha* is 2.61%; this value is higher than that obtained in another study that was conducted (0.082%) in the region of Sefrou in Morocco [24]. The same species collected in different stations in Corsica gave a low yield compared to our results of the order of 0.17–1.2% [25,26]. Moreover, another research project has shown that this plant species is very poor in EO, with a yield of the order of 0.22% [27].

The Chemical Composition of the EO

The chemical composition of the EO was determined by gas chromatography coupled with mass spectrometry. This technique consists of the separation of the constituents. It allowed us, based on GC-MS in combination with IK, to identify the chemical species of the EOs of *Satureja calamintha*, *Lavandula multifida* L., and *Mentha pulegium* L.

Tables 2–4 summarize the chemical composition of the three EOs.

The analysis of *Lavandula multifida's* EO by gas chromatography coupled with mass spectrometry revealed the presence of 62 compounds representing 99.71% of the total of the compounds (Table 2). Indeed, the monoterpene oxygenated fraction is larger, and it represents about 76.56% of the overall chemical composition of the oil, while the oxygen monoterpenes represent 75.5% and the hydrocarbon monoterpenes represent 1.06%. The main major constituents that characterize this EO are the phenolic compounds of which carvacrol is the majority compound (70.65%), followed by bisabolene <E) – ⅄> (13.14%), spathulenol (3.53%), and caryophyllene oxide (2.43%). However, the sesquiterpene fraction appeared in medium proportion (23.15%). It is mainly composed of oxygenated sesquiterpenes with a percentage of 8.3% and non-oxygenated sesquiterpenes with a percentage of 14.85%. The chemical composition of this EO is different from those described in the literature. In Morocco, in the region of Errachidia, the main major constituents that characterize this EO are the phenolic compounds (69.6%) of which carvacrol is the majority compound (57.9%), followed by carvacrol methyl ether (7.6%), paracymen-8-ol (3.9%), and eugenol (0.2) [28], while the EO of *Lavandula multifida* collected from Tetouan is characterized mainly by carvacrol (47.62%), β-bisabolene (9%), linalool (7.42%), menthone (4.98%), and caryophyllene (3.34%) [29]. *Lavandula multifida's* EO collected in Tunisia contains 21 compounds representing 81.42% of the total oil, of which carvacrol (34.95%) and germacrene D (15.19%) are the major compounds [30]. An analysis of the EO of *Lavandula multifida* from Spain gave as major compounds carvacrol and β-bisabolene [31].

The analysis of the EO of *Satureja calamintha* carried out by CG/SM allowed us to identify 28 compounds, representing in total 99.84% of the overall composition of the EO (Table 3).

$$\%\,\mathrm{TH} = \frac{\mathrm{m1} - \mathrm{m2}}{\mathrm{m1}} * 100$$

$$\%\,\mathrm{R} = \frac{\mathrm{V}}{\mathrm{m} - (\mathrm{m} * \%\,\mathrm{TH})} \pm ecart - type$$

Fig. 1 The essential oil yields, calculation formulas

Table 2 The chemical composition of the essential oil of *Lavandula multifida* L

IK	Area %	Nomenclature
762	0.29	Toluene
979	0.16	β-purene
1024	0.09	*p*-cymene
1031	0.03	1,8-cineole
1037	0.43	Ocimene<(Z)-β>
1059	0.09	<γ>terpurene
1086	0.35	Fenchone
1096	0.48	Linalool
1106	0.10	Vertocitral c <Trans>
1114	0.14	Thujone<Trans>
1121	0.88	Fenchol<eno>
1132	0.08	Epoxy-ocimene<Z>
1142	0.03	sabunol<Trans>
1146	0.12	Camphor
1151	0.06	Thuyanol<neoiso-3>
1153	0.04	Thuyanol<neo-3>
1160	0.13	Isoborneol
1161	0.09	Menthol<neo>
1182	0.36	Cymene-8-ol<p>
1186	0.27	terpineol<α>
1192	0.14	dihydrocarvone<cis>
1195	0.09	Myrtenol
1196	0.05	caranone<trans-4>
1223	0.35	Citronellol
1233	0.57	Thymol, methyl ether
1256	0.09	Crysanthenylacetate<cis>
1299	**70.65**	**Carvacrol**
1384	0.08	damascenone<E)β>
1386	0.05	damascone<Z)-β>
1390	0.90	Elemene<β>
1400	0.05	longipinene<β>
1412	0.08	<β>funebrene
1419	0.11	Caryophyllene<E>
1436	0.06	Humelene<β>
1462	0.03	Aromadendrane<dehydro>
1488	0.13	ionone<E)-β>
1505	0.08	bisabolene<β>
1522	0.06	Sesquiphellandrene<β>
1531	**13.14**	**bisabolene<E)-γ>**
1538	0.03	cadinene<α>
1546	0.13	Selina-3,7(11)-diene
1557	0.18	Dauca-4(11),7-diene<trans>
1578	**3.53**	**Spathulenol**
1583	**2.43**	**Caryophyllene oxide**
1594	0.07	Carotol
1608	0.04	atlantol<β>
1613	0.34	biotol<β>
1631	0.12	Muurola-4,10(14)-dien-1-β-ol

(Suite page suivante)

Table 2 *(suite)*

IK	Area %	Nomenclature
1640	0.08	Caryophylla-4(12),8(13)-dien-5β-ol
1644	0.42	Selina-3,11-dien-α-ol
1648	0.31	Guara-3,9-dien-11-ol<cis>
1650	0.12	Valerianol
1666	0.02	Lyral
1682	0.08	Occidentalol acetate
1685	0.32	Longiborneol acetate
1688	0.04	Eudesma-4(15),7-dien-1(3-ol)
1746	0.24	costol<Ɣ>
1761	0.14	lanceol<(Z)->
1763	0.03	acoradienol<β>
1776	0.07	Amorpha-4,7(11)diene<2α-hydroxy>
1784	0.04	Eudesmol acetate
Non-oxygenated monoterpenes		**1.06**
Oxygen monoterpenes		**75.5**
Non-oxygenated sesquiterpenes		**14.85**
Oxygenated sesquiterpenes		**8.3**
Total		**99.71**

We can note that out of 28 identified compounds, 17 are monoterpenes, with in particular a strong preponderance of oxygenated monoterpenes (98.79%) dominated by pulegone (87.04%). As for sesquiterpenes, they are generally minority in this EO (0.12%).

The EO of *Satureja calamintha* has a very important chemical polymorphism. Indeed, a study has shown that the chemical profile of this plant is different from our result. The yields and the nature of the majority compounds vary considerably from one sample to another depending on the origin of the plant. This study revealed that the EO of *Satureja calamintha* is characterized by the presence of p-cymene (20.9%), γ-terpinene (18.7%), and thymol (34.9%) as main chemical constituents [8]. Another Moroccan study showed that *Satureja calamintha* is composed mainly of terminalol (34.52%), α-campholenic aldehyde (14.26%), cedren-13-ol (6.45%), and manoyloxide (3.78%) accompanied by other components at relatively low levels: diepicedrene-1-oxide (2.05%), spathulenol (2.15%), aristolene epoxide (2.42%), and (−) spathulenol (2.63%), totaling 68.26% [24].

These variations in the chemical composition of the EOs from the qualitative and quantitative point of view may be due to certain ecological factors, to the used part of the plant, to the age of the plant, and to the period of the vegetative cycle or even to genetic factors [32].

For *Mentha pulegium* L., the GC-MS technique allowed us to identify 12 chemical species representing a total of 99.787% of the EO compounds (Table 4).

The results of this study showed that monoterpenes represent the major fraction (99.66%) of the EO of *Mentha pulegium*, with a predominance of oxygenated compounds (99.39%) dominated by pulegone (71.97%) and the piperitenone (26.04%) (Table 4). Hydrocarbon monoterpenes in particular (0.27%) and sesquiterpenes in general (0.12%) are minor in this EO.

The chemical composition of the EO of *Mentha pulegium* L. has been the subject of numerous publications. It is characterized by the majority presence of ketones possessing a menthenic skeleton. Indeed, the compositions described are dominated by the pulegone which represents 80.3% in Morocco [9], 65.9–83.1% in India [33], 73.4% in Uruguay [34], and 43.5% in Egypt [35]; and by piperitenone which represents 83.7–97.2% in Greece [36] or piperitone that represents 70.0% in Austria [37].

The Antibacterial Activity of EO of *Satureja calamintha* subsp. *nepeta* (L.) Briq, *Lavandula multifida* L., and *Mentha pulegium* L.

The Sensitivity Test

The antibiotic sensitivity profiles of the strains indicated in table 5 are made according to the recommendations of the French Society of Microbiology and EUCA.

The result of the sensitivity test is presented as follows:

S: Sensitive, I: Intermediate, R: Resistant b. Antibiotics (ATB), Ceftriaxone (CRO), Tobramycin (TOB), Ticarcillin (TIC), Amoxicillin (AX), Cefoxitin (FOX), Chloramphenicol (C), Colistin (CT), Amoxicillin + Clavulanic acid (CMA), ciprofloxacin (CIP), amikacin (AK), imipenem

Table 3 The chemical composition of the essential oil of *Satureja calamintha* subsp. *nepeta* (L.) Briq

IK Adams	Area %	Nomenclature
930	0.06	α-thujone
939	**4.43**	**α-pinene**
952	0.03	α-fenchene
979	0.26	β-pinene
983	**1.14**	**Mentha-2,8-diene<trans-meta>**
987	0.02	Mentha-2,8-diene<cis-meta>
1000	1.72	Mentha-1(7),8-diene<meta>
1072	0.09	Mentha-3,8-diene<p>
1088	0.09	Mentha-2,4(8)-diene<p>
1113	0.03	CAMPHENOL<6>
1144	0.07	Verbenol-trans
1152	**1.87**	**Menthone**
1159	1.50	Pinene oxide<β>
1188	0.04	TERPINEOL<α>
1205	0.08	Verbenone
1237	**87.04**	**Pulegone**
1275	0.32	Menth-1-en-7-al<p>
Total of monoterpenes	**98.79%**	
1376	0.03	Isoledene
1388	0.13	β-bourbonene
1390	0.09	β-elemene
1414	0.03	Funebrene<β>
1422	0.05	Carvylproponoate<cis>
1578	0.23	Spathulenol
1631	0.03	Nuurola-4,10(14)-dien-1-β-ol
1242	0.12	Muurolol<epi-α>
1654	0.20	Cardinol<α>
1697	0.04	Menthol-1'(butin-3-on-1-yl
1715	0.10	Nootkatol
Total sesquiterpenes	**1.05%**	
Total	**99.84**	

(IPM), ceftazidime (CAZ), Piperacillin (PRL), trimethoprim + sulfamethoxazole (SXT). Vancomycin (VA), Tetracycline (TE), Cefalexin (CN), Lincomycin (L) .c: Loading of the antibiotic disk in µg.

According to the antibiogram, we can conclude that the strains of *Staphylococcus aureus* and *Escherichia coli* are sensitive to the majority of antibiotics, while *Pseudomonas aeruginosa* is multiresistant.

Table 4 The chemical composition of the essential oil of *Mentha pulegium* L

Nomenclature	IK Adams	Area %
α-pinene	939	0.14
β-pinene	979	0.13
1,8-cineole	1031	0.01
Trans-p-menth-2-en 1-ol	1140	0.28
Chrysanthenol(Cis)	1164	0.80
α–Terpineol	1188	0.10
Trans-pulegol	1214	0.06
Pulegone	1237	**71.97**
Thymol	1290	0.04
Piperitenone	1343	**26.04**
α–Guaiene	1439	0.06
Himachal-4-en-1-β-ol (11-α H)	1699	0.06
Oxygenated monoterpenes	99.39	
Hydrocarbon monoterpenes	0.27	
Oxygenated sesquiterpenes	0.06	
Hydrocarbon sesquiterpenes	0.06	
TOTAL	**99.78**	

Determination of the Antibacterial Activity of the Essential Oils Using the Disk Diffusion Method (Aromatogram)

We report, in table 6, the diameters of the inhibition zones of the EOs of *Satureja calamintha*, *Lavandula multifida*, and *Mentha pulegium* according to the tested bacterial strains.

Essential Oils with Majority Compound: Pulegone

Based on previous works, we considered that an EO has a bactericidal action if its inhibition diameter is greater than 12 mm [38,39]. Thus, the EOs of *Satureja calamintha* and *Mentha pulegium* act actively at low concentration (2 µl/ml) on *Escherichia coli* and *Staphylococcus aureus*. These bacteria are generally sensitive to the phenolic-rich EOs [40]. *Pseudomonas aeruginosa* is particularly very resistant even when tested to large concentrations (40 µl/ml) for *Satureja calamintha*, and it shows inhibition when tested with *Mentha pulegium* EO beginning from the volume of 36 µl.

Strains of *Pseudomonas aeruginosa* have always been resistant to these EOs which have as a major compound the pulegone [41,42].

The EOs of *Mentha pulegium* and *Satureja calamintha* are rich in pulegone which represent 71.97% and 87.04%, respectively. Indeed, it has been demonstrated by some researchers [43] that the antimicrobial activity of the polegon contained in the EO of *Satureja calamintha* and its majority

Table 5 The test of sensitivity of bacterial strains toward some antibiotics

Tested ATBc	*Staphylococcus aureus*	Tested ATBc	*Escherichia coli*	Tested ATB_c	*Pseudomonas aeruginosa*
CIP_5	S	AK_{30}	S	TIC_{75}	R
VA_{30}	S	C_{30}	S	CAZ_{30}	I
TE_{30}	S	PRL_{75}	S	CT_{50}	R
CN_{15}	S	IPM_{10}	S	CIP_{10}	R
L_{15}	S	AMC_{30}	S	IPM_{10}	S
AX_{25}	R	AX_{25}	R	AK_{30}	R
TOB_{10}	S	CRO_{30}	S	SXT_{30}	R

The result of the sensitivity test is presented as follows:
S: Sensitive; I: Intermediate; R: Resistant b. Antibiotics (ATB); Ceftriaxone (CRO); Tobramycin (TOB); Ticarcillin (TIC); Amoxicillin (AX); Cefoxitin (FOX); Chloramphenicol (C); Colistin (CT); Amoxicillin + Clavulanic acid (CMA); ciprofloxacin (CIP); amikacin (AK); imipenem (IPM); ceftazidime (CAZ); Piperacillin (PRL); trimethoprim + sulfamethoxazole (SXT); Vancomycin (VA); Tetracycline (TE); Cefalexin (CN); Lincomycin (L) .c: Loading of the antibiotic disk in µg
According to the antibiogram, we can conclude that the strains of *Staphylococcus aureus* and *Escherichia coli* are sensitive to the majority of antibiotics, while *Pseudomonas aeruginosa* is multiresistant

Table 6 Diameters of the inhibition zones of the strains of P.a, S.a, and E.c tested against the plants' three essential oils studied by the disk diffusion method (aromatogram)

EO's concentration (µl/ml)	Inhibition diameter (mm)								
	EO of *Lavandula multifida*			EO of *Mentha pulegium*			EO of *Satureja calamintha nepeta*		
	E.c	*S.a*	*P.a*	*E.c*	*S.a*	*P.a*	*E.c*	*S.a*	*P.a*
2	14 ± 1.41	15.5 ± 0.7	06 ± 00	15 ± 1.41	15 ± 00	06 ± 00	14.5 ± 0.7	14.5 ± 0.7	06 ± 00
4	16.5 ± 0.7	15.5 ± 0.7	06 ± 00	17.5 ± 0.7	15.5 ± 0.7	06 ± 00	17.5 ± 0.7	15 ± 00	06 ± 00
6	16.5 ± 0.7	16.5 ± 0.7	06 ± 00	20.5 ± 0.7	15.5 ± 0.7	06 ± 00	17.5 ± 0.7	14.5 ± 0.7	06 ± 00
8	17 ± 1.41	17 ± 00	06 ± 00	21.5 ± 0.7	16 ± 00	06 ± 00	17.5 ± 0.7	14.5 ± 0.7	06 ± 00
10	16.50 ± 0.7	18.5 ± 0.7	06 ± 00	22 ± 00	16.5 ± 0.7	06 ± 00	18.5 ± 0.7	14.5 ± 0.7	06 ± 00
12	18 ± 1.41	18.5 ± 0.7	06 ± 00	23.5 ± 0.7	17.5 ± 0.7	06 ± 00	21.5 ± 0.7	15.5 ± 0.7	06 ± 00
36			12.5 ± 00			14 ± 00			06 ± 00
40			13 ± 00			15 ± 00			06 ± 00
6 (DMSO)	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00
6 (ED)	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00	06 ± 00

constituents when tested against several bacteria and molds is the same.

In 2011, a lot of researches have been conducted on the EO of *Satureja calamintha* and on the pulegone as its main constituent; they discovered a microbial and antifungal activity [44].

Essential Oils with Majority Compound: Carvacrol

These results also showed a strong antibacterial activity of the EO of *Lavandula multifida*. We note that *Lavandula multifida's* EO is effective on *Staphylococcus aureus* and *Escherichia coli* bacteria tested at low concentrations (2 µl/ml). However, this species is inactive on *Pseudomonas aeruginosa's* strain.

We also note that the bacterial strains of *Staphylococcus aureus* and *Escherichia coli* have approximately the same inhibition diameter when tested against the EO of *Lavandula multifida*. As an example, if we take the dose of (2 µl/ml), we have 14.00 ± 1.41 mm (*Staphylococcus aureus*) and 15.5 ± 0.7 (*Escherichia coli*); this is not the case for pulegone plants. *Escherichia coli* strain was more sensitive than the *Staphylococcus aureus* strain.

These results show that carvacrol also has an antibacterial activity such as that of pulegone. This has been proven by some researchers who tested 5 µL of a species of *Lavandula multifida* with a chemical composition that is

Table 7 The evaluation of the MIC and MBC of the essential oils of *Lavandula multifida, Satureja calamintha* and *Mentha pulegium* when tested against *Escherichia coli* and *Staphylococcus aureus* strains

	Lavandula multifida's EO		***Mentha pulegium's EO***		***Satureja calamintha nepeta's EO***	
	MIC µl/ml	**MBC µl/ml**	**MIC µl/ml**	**MBC µl/ml**	**MIC µl/ml**	**MBC µl/ml**
Escherichia coli	2	2	2	2	2	2
Staphylococcus aureus	12	18	2	3	2	6

dominated by carvacrol (66.2%) on the same bacterial strains that we studied. They found 17 mm of inhibition diameter when the EO was tested against *Escherichia coli* strain, 7.5 mm when tested against *Pseudomonas aeruginosa*, and 28 mm when tested against *Staphylococcus aureus* [22]. These results were reinforced by other studies confirming this hypothesis of the antibacterial activity of carvacrol, with an EO dominated by carvacrol (47.62%), and they found 9.5 mm inhibition diameter when the EO was tested against *Escherichia coli* strains and no effect when tested against *Pseudomonas aeruginosa* [29].

Carvacrol is also considered as a biocide with its precursor called *p*-cymene which has a weak antibacterial effect and synergic effect when associated to the carvacrol; this effect is translated by the expansion of the membrane which means disabling this membrane [45–47].

MIC and MBC Tests

Only the oil-sensitive strains were tested, the results of which are shown in table 7.

The determination of the MIC of the EO, with a concentration ranging from 2 to 22 µl/ml, showed that the *Escherichia coli* strain is sensitive to the three EOs, *Satureja calamintha*, *Lavandula multifida*, and *Mentha pulegium;* the MIC is manifested in the volume of 2 µl/ml. The MICs recorded for the *Staphylococcus aureus* strain are variable depending on the used EO. Indeed, we distinguished the 12 µl/ml, 2 µl/ml, and 2 µl/ml, respectively, for *Lavandula multifida*, *Mentha pulegium*, and *Satureja calamintha*.

The obtained CMBs vary according to the tested bacterial species. *Escherichia coli* is the most sensitive. Indeed, the above three EOs eliminated completely *Escherichia coli* (100%) to 2 µl/ml; as for *Staphylococcus aureus*, the CMBs are respectively 18 µl/ml, 2 µl/ml, and 6 µl/ml for *Lavandula multifida*, *Mentha pulegium*, and *Satureja calamintha*.

These results could be attributed to the nature of the chemical composition of the EO. According to other studies, the biological activity of an EO is related to its chemical composition, the functional groups of its major compounds (alcohols, phenols, aldehydes), and the synergistic effects between its components [48]. Thus the most effective chemical compounds with a wide spectrum of antimicrobial action are phenols (thymol, carvacrol and eugenol), alcohols (α-terpineol, terpinen-4-ol, menthol, geraniol, linalool), aldehydes (geraniol, citral and neral), and ketones (carvone, pulegone, and camphor) [48–50]. Finally, although the antimicrobial activity of an EO is mainly attributed to its major compound, the synergistic or antagonistic effect of each of its constituents present in low concentrations is also taken into consideration [51-53].

The absence of antimicrobial activity could be explained by the resistance developed by a large number of strains which react differently to the various types of EOs. Among the strains studied, *Pseudomonas aeruginosa* was resistant. In fact, this bacterium has an intrinsic resistance to biocidal agents, in relation to the nature of its outer membrane. The latter is composed of lipopolysaccharides which form a barrier impermeable to hydrophobic compounds. In the presence of permeabilizing agents of the outer membrane, inactive substances against *Pseudomonas aeruginosa* become active [54]. It appears that this strain is resistant to a very large number of EOs [55,56].

Conclusion

It is possible to provide patients and health practitioners with a new drug formulated with EOs tested against nosocomial infections; these EOs can also be used to clean the air or in ventilation systems in the hospital environment so as to limit the expansion of microbial germs.

These findings are among the discoveries conducted on these species that confirm their use in traditional medicine and open perspectives for their valorization in a scientific way.

Acknowledgements I would like to express my sincere and faithful thanks to all participants who contributed to the accomplishment of my research project for their support, advice, and continuous encouragement. Without their help, this work would not be possible

I would also express my thanks to Mr. Ibn Tatou who provided me with great help in the identification of the three plant species

My sincere thanks also go to Miss. Faiq Hind for her valuable collaboration and help in the translation of this article

Conflicts of Interests: the authors have no conflicts of interests to declare.

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