Antimicrobial activity of essential oils extracted from leaves of native Moroccan plants against clinical bacterial isolates

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Abstract

Introduction: Medicinal plants possess plentiful of bioactive molecules effective against multi-resistance bacteria. The aim of this study is to assess the *in vitro* antimicrobial activities of essential oils extracted from three Moroccan aromatic plants.

Method: Analysis of essential oils of *Origanum compactum*, *Rosmarinus officinalis* and *Pelargonium asperum*, collected from different localities in Morocco was performed using a GC-MS spectrophotometry. Antibacterial activity by disc diffusion assay was evaluated *in vitro* against five clinical multi-resistant isolates.

Results: *Origanum* showed strong antibacterial activity against tested strains except *Pseudomonas aeruginosa* while *Rosmarinum* showed a bactericidal effect against *Acinetobacter baumannii*, *Escherichia coli* and *Staphylococcus aureus*. *Pelargonium* presented only slight growth inhibition of *Staphylococcus aureus* on solid medium. Interestingly, fractions F7 and F8 of *Pelargonium* which represented only 0.3% and 0.1% respectively of the total mass were found to be bactericidal against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Conclusions: Ours results showed that the antimicrobial activities were variables depending on the chemical composition of essential oils, the fraction used and the microorganism tested.

Essential oils fractionation allows detection of bioactive substances, especially those owning antimicrobial activity and present in small quantities.

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Introduction

The overuse of antibiotics to treat infectious diseases resulted in the emergence of multidrug resistance (MDR) bacteria. Actually, this increased phenomenon is currently considered as a major threat to global public health. This has prompted the World Health Organization (WHO) to direct research into new alternatives and especially to plants that have always constituted a source of ongoing bioactive molecules and current inspiration for new medicines. Delacroix in 1881 showed for the first time the essential oils (Eos) action against bacteria [1]. Since then, numerous studies have been reported the *in vitro* antimicrobial activities of certain chemical compounds of various plant extracts [2]. The capacity of certain essential oils to neutralize germs is now irrefutable [3].

Origanum compactum, *Rosmarinus officinalis* and *Pelargonium asperum* are widely used plants over the world and particularly in Mediterranean countries. They are known for their aromatic, gastronomic and phytotherapeutic features. Hence, EO extracted from these plants is actively investigated for antimicrobial effect [4], as they could substitute antibiotics in the treatment of infectious diseases caused by multi-resistant bacteria (MRB).

The mode of action of EOs on bacteria is not clearly elucidated, but for the variety of molecules in these oils, it seems that their effect results from a combination of several modes of action that involve various cell targets [5].

This study was carried out aiming to perform qualitative and quantitative analyses and to assess the *in vitro* antimicrobial activities of EOs extracted from *O. compactum*, *R. officinalis* and *P. asperum* in order to contribute to the development of the Moroccan native flora by identifying new potentially interesting therapeutic molecules.

Method

Phytochemical analysis

Plant material and essential oil extraction

Plant specimens used in this study were harvested in March 2013 from different Moroccan localities. *O. compactum* collected from Taounate, *R. officinalis* from Debdou and *P. asperum* from Rabat. Authentication and extraction were made by the National Institute of Plant Medicinal and Aromatic (NIPMA) in Taounate (Morocco). Selected fresh leaves were oven dried at 40°C for 15 h, ground into fine powder, then passed through an 80 mesh sieve with 0.177 mm of diameter and stored at 20°C in sealed plastic bags for future use.

Large-scale extraction was performed by vapo hydro-distillation in the Technological Hall of NIPMA. Small scale extraction was done in the laboratory of chemistry at the NIPMA-Taounate by hydro-distillation (Clevenger). 100 g of plant material were extracted with 500 ml distilled water for 3 h. Obtained EOs were stored at 4°C and protected from light. The yield of EOs was calculated using the following formula: $R = (Px/Py) \times 100$ [6]

R: Oil yield (%); Px: Oil weight (g); Py: Plant oil weight (g).

Identification of essential oils compounds by GC-MS spectrophotometry

Qualitative and quantitative analysis of EOs chemical composition was conducted at the Plant Biotechnology Laboratory applied to aromatic and medicinal plants (LBVpam) in Faculty of Sciences and Techniques, Saint Etienne, France. Analytical study was carried out using Gas Chromatography Mass Spectrometer (GC-MS Agilent 6850 GC 5973) equipped with a capillary column DB-5 (30 m x 0.25 mm id. x 0.25 µm film thickness) by injecting 2 µl of extract using hexane as solvent. Carrier gas was helium at 1 ml/min. Oven temperature programmed from 60°C to 245°C at 3°C/min. The system is connected to

a MSDCHEM program and a Wiley data bank to identify compounds.

Pre-fractionation of the EOs

Each EO was fractionated away from light and air stream at room temperature into 10 fractions by chromatography on silica Type 60 column as previously described [7]. Purification and concentration of each fraction eluted in 350 ml of the mobile phase was carried out by vacuum distillation using a rotary evaporator BUCHI type Rotavapor R -205. The house- bath temperature was set at 33°C.

Evaluation of antibacterial activity

Microorganisms

EO antibacterial activity was performed on five clinical isolates resistant to at least five antibiotics including methicillin resistant *Staphylococcus aureus* PN15 (MRSA) and *Escherichia coli* extended spectrum beta-lactamase producing TF2 (ESBL). Resistance profiles were previously determined following Clinical and Laboratory Standards Institute (CLSI) and are shown in Table 1.

Determination of antibacterial activity by disc diffusion assay

Bacteria was cultured on Plate Count Agar (PCA, Biokar) and incubated overnight at 37°C to obtain individual colonies. Colonies were suspended in distilled water to achieve turbidity equal to 0.5 McFarland standard (10^6 - 10^8 CFU/ml). Petri dish containing 20 ml of Mueller Hinton Agar (MHA,

Biokar) was flooded with the bacterial suspension and excess inoculum was removed. 10 µl of each EO were applied to 6 mm diameter sterile paper Wattman discs and were then carefully placed onto the surface of Petri dish containing MHA medium. An empty disc soaked with distilled water was used as negative control and a 30 µg amikacin disc as positive control (Sigma-Aldrich). The dishes were maintained at 4°C for 15 to 20 min to allow diffusion of EOs and then, incubated at 37°C for 18 to 22 h under normal atmosphere [8]. Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm) and interpreted [9]. Experiments were carried out in triplicate and the average zone of inhibition was calculated.

Determination of Minimal Inhibitory Concentration (MIC) by broth microdilution assay [10]

The MIC was determined by broth micro-dilution assay [10] using resazurin (Sigma-Aldrich) as a survival indicator. Resazurin was prepared by dissolving 1 mg per 1 ml of sterile distilled water and then filtered through a micro-filter (0.2 µm, Millipore).

A twofold serial dilution (ranging from 16 - 0.05 µl/ml) for each EO was performed in Mueller Hinton Broth (MHB, Biokar) containing 3% of ethanol and 50 µl of each dilution were distributed in a 96-well plate. Fresh bacterial colonies were selected and suspended in Mueller Hinton broth at turbidity equal to 0.5 McFarland and mixed with an equal volume of the previous serial dilution of each EO.

Table 1. Resistance profiles of the tested bacteria species

Gram	Strains	Antibiotic resistance profile*
Negative	<i>Escherichia coli</i> - TF2 (ESBL)	AMC. ATM. CTX. CAZ. CIP. OFX. GN. TOB
	<i>Klebsiella pneumonia</i> CT59R	AMC. ATM. CAZ. CTX. CIP. OFX. GN. TOB. CS
	<i>Pseudomonas aeruginosa</i> -P134	AMC. ATM. CTX. TIC. TCC. CIP. OFX. GN. TOB. TZP
	<i>Acinetobacter baumannii</i> -P142	AMX. ATM. CAZ. TIC. TCC. CIP. OFX. GN. TOB. TZP
Positive	<i>S. aureus</i> -SARM, PN15	P. CM. OX. FOX. TE. GN

* AMC: Amoxicillin; ATM: Astreonom; P: Penicillin; CTX: Ceftriaxon; CAZ: Ceftazidim; CIP: Ciprofloxacin; OFX: Ofloxacin; TIC: Ticarcillin; TCC: Ticarcillin-Clavulanic acid; GN: Gentamicin; TOB: Tobramycin; CS: Colistin; TZP: Tazobactam-piperacillin; CM: Clindamycin; OX: Oxacillin; FOX: Cefoxitin; TE: Tetracyclin.

After an overnight incubation at 37°C, 5 µl of resazurin (1 mg/ml) were added and further incubated for 2 h at 37°C to visualize bacterial growth. The MIC value corresponds to the lowest concentration of EO which produces no resazurin discoloration.

Determination of the Minimal Bactericidal Concentration (MBC) [10]

On PCA plates, 5 µl of bacterial culture picked up from wells were no resazurin discoloration observed was streaked. Plates were incubated for 18 to 20 h at 37°C and MBC corresponds to the lowest EO concentration which gives no bacterial growth.

Determination of the inhibitory fraction by broth dilution assay

Test was performed using 0.5 ml Eppendorf tube with a final volume of 200 µl MHB mixture containing 3% of ethanol; 20 µl bacterial suspension (10^6 to 10^8 CFU/ml) and 1.2 µl of fraction extract (6 µl/ml). After incubation at 37°C for 18 to 20 h, bacterial growth was visualized by adding 5 µl of resazurin to each tube and incubated for 2 h at 37°C. Fractions inhibiting bacterial growth showed no resazurin discoloration. To assess the effect of this inhibition, 5 µl picked from no resazurin discoloration mixture was streaked on PCA plates and incubated for 18 to 20 h at 37°C. A fraction has a bactericidal activity when no bacterial growth on Petri dishes was observed. Fractions exhibiting bacterial growth were considered bacteriostatic.

Results

Phytochemical analysis

Essential oil extracts obtained from *O. compactum*, *R. officinalis* and *P. asperum* presented pleasant aromas but variable colors, yields and compositions. Actually, the attained yields were $2.10 \pm 0.07\%$, $1.20 \pm 0.10\%$ and $0.80 \pm 0.10\%$ respectively.

As shown in **Table 2**, *O. compactum* consists of 28 items that represent 99.99% of the identi-

Table 2. Chemical composition of *Origanum compactum* essential oil

Compounds	Area	RT
	%	min*
Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	1.0614	5.2806
1S- α -Pinene	0.6103	5.4844
Camphene	0.0863	5.9296
β -Pinene	0.2157	6.6833
3-Octanone	0.0879	6.8281
β -Myrcene	1.4174	6.9595
α -Phellandrene	0.2405	7.5361
3-Carene	0.0747	7.6085
α -Terpinen	2.1893	7.8740
p-Cymene	8.4457	8.1369
D-Limonene	0.2478	8.2844
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	0.2129	8.3541
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.0614	8.8449
γ -Terpinene	17.2504	9.3035
Cyclohexane, 1-methylene-4-(1-methylethenyl)-	0.1560	9.7541
Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	0.1168	10.2878
Benzene, 1-methyl-4-(1-methylethenyl)-	0.0779	10.4890
1,6-Octadien-3-ol, 3,7-dimethyl-	1.3580	10.8484
Borneol	0.1528	13.7851
2,6-Octadienal, 3,7-dimethyl-, (Z)-	0.0471	14.0399
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	0.4292	14.1257
3-Cyclohexene-1-methanol, α , α 4-trimethyl-	0.1651	14.7453
Cyclohexene, 1,6-dimethyl-	0.0773	14.8472
Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	0.1594	16.5341
p-Thymol	70.05	70.05
Calvacrol	47.8524	19.2563
Phenol, 2-methyl-5-(1-methylethyl)-	0.0153	19.5218
Caryophyllene	1.4410	24.0544
Total: 28 elements	99.99%	---

*: RT: Retention time.

Table 3. Chemical composition of *Rosmarinus officinalis* essential oil.

Compounds	Area	RT
	%	min*
Bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-, (1S)-	0.1129	5.2350
1R- α -Pinene	19.5096	5.4898
Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	2.8712	5.9296
Bicyclo[3.1.0]hex-3-en-2-ol, 2-methyl-5-(1-methylethyl)-, (1 α ,2 α ,5 α)-	0.3512	6.0155
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.6343	6.6940
3-Octanone	0.1462	6.8308
β -Pinene	1.0380	6.9595
(+)-4-Carene	0.1975	7.8741
Benzene, 1-methyl-4-(1-methylethyl)-	3.3604	8.1369
D-Limonene	3.5273	8.2898
Eucalyptol	35.2038	8.4105
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	0.1989	9.2955
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.1700	10.2905
Benzene, 1-methyl-4-(1-methylethenyl)-	0.1822	10.4863
1,6-Octadien-3-ol, 3,7-dimethyl-	2.8668	10.8537
1,3-Cyclopentadiene, 1,2,5,5-tetramethyl-	0.4107	11.6959
1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-ol	0.1281	11.9158
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1 α ,3 α ,5 α)]-	0.0783	12.4924
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1 α ,3 α ,5 α)]-	0.1495	12.5032
(1R)-Camphor	70.05	70.05
7.3225	12.7499	76.19
Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-	0.1279	13.0878
Bicyclo[2.2.1]heptan-3-one, 6,6-dimethyl-2-methylene-	0.8015	13.3533
2-methyl-6-methylene-7-octen-2-ol	0.4659	13.7208
Borneol	4.6807	13.7878
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	1.5234	14.1230
Benzenemethanol, α , α ,4-trimethyl-	0.2400	14.4154
3-Cyclohexene-1-methanol, .alpha.,.alpha.4-trimethyl-	4.7384	14.7506
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	0.3645	15.0671
Verbenone	70.05	70.05
5.6493	15.2119	76.19
6-Octen-1-ol, 3,7-dimethyl-, (R)-	0.2807	16.0675
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	0.1456	16.6414
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	0.2241	16.9230
2,6-Octadien-1-ol, 3,7-dimethyl-, (E)-	1.1567	17.0518
Bornyl acetate	0.6086	18.4625
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	0.1869	20.7046
Caryophyllene	0.2072	24.0571
1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	0.1393	25.5188
Death and Dyeing	70.05	70.05
Total: 37 elements	91,40%	-

*: RT: Retention time.

fied compounds. The major components of this oil are: Carvacrol (47.85%), p-Thymol (15.75%), and γ -terpinene (17.2504%).

Essential Oil of *R. officinalis* consists of 37 elements which represent 91.40% of the identified compounds (**Table 3**). The major components of this oil are: Eucalyptol (35.20%), 1R- α -Pinene (19.51%) and (1R) -Camphor (7.32%).

The essential oil of *P. asperum* consists of 18 items that represent 73.94% of the identified compounds (**Table 4**). The major constituents of this oil are: Citronellol (25.0708%), Citronellyl formate (10.5261%) and Geraniol (10.4604%).

Table 4. Chemical composition of Pelargonium asperum essential oil.

Compounds	Area	RT
	%	min*
1R- α -Pinene	1.2236	5.4846
β -Pinene	0.2669	6.9597
α -Phellandrene	0.1251	7.5390
Benzene, 1-methyl-3-(1-methylethyl)-	0.5503	8.1344
D-Limonene	0.3506	8.2873
β -Phellandrene	0.1436	8.3570
1,3,6-Octatriene, 3,7-dimethyl-, (E)-	0.2452	8.4723
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.2452	8.8451
1,6-Octadien-3-ol, 3,7-dimethyl-	3.6166	10.8512
Rosenoxide	1.5522	11.2401
2H-Pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-	0.5851	11.8811
2-isopropyl-5-methyl-cyclohexanone	5.8805	13.4688
Citronellol	25.0708	16.0945
Geraniol	10.4604	17.0653
Citronellyl formate	10.5261	18.0255
2,6-Octadien-1-ol, 3,7-dimethyl-, formate, (E)-	4.0983	19.0741
(+)-Ledene	3.0623	26.9484
β -Maaliene	5.9391	32.0361
Total: 18 elements	73.94	-

*: RT: Retention time.

Pre-fractionation by liquid chromatography

The fractional separation of essential oil extracts obtained from *O. compactum*, *R. officinalis* and *P. asperum* was performed by vacuum distillation column. EO was separated into 10 fractions (F1, F2... F10) containing different chemical families (Hydrocarbons, Esters, Ethers, Aldehydes, Ketones, Alcohols and Acid). Each fraction exhibits smell and color except fractions F1 and F2 of each EO. Fractions F3 and F4 represented together about 50% of total yield of each essential oil. Yields and densities of crude extracts and their fractions are given in **Table 5**. It should be noted that the total mass of eluted fractions was slightly superior to the initial mass. In addition, density of some fractions was higher than the corresponding total oil.

Antibacterial activity

Antibacterial activity was evaluated against five multi-drug resistant clinical isolates including Gram negative (*E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*) and Gram positive (*S. aureus*) bacteria. The total results obtained using disc diffusion method as well as MIC and MBC are given in **Table 6**.

O. compactum oil showed a very significant antibacterial activity against the strains tested. It is strongly inhibitory a determined zone inhibition diameter ranging from 29.33 ± 1.15 to 48.5 ± 0.7 mm and bactericidal effect in liquid medium at concentrations less or equal to 0.5 μ l/ml. The action of this oil is slightly inhibitory for *P. aeruginosa* in a solid medium with a diameter of inhibition equal to 14.61 ± 1.99 mm and bacteriostatic at a concentration of 1 μ l/ml in liquid medium.

On solid medium, EO of *R. officinalis* had no effect on *P. aeruginosa* (7.67 ± 1.15 mm) and slightly inhibit the other species with inhibition diameters varying between 10.02 ± 0.1 and $14, 75 \pm 0.7$ mm. Dilution method showed bactericidal effect, especially against *A. baumannii*, *S. aureus* and *E. coli*, at concentra-

Table 5. Yield and density of the essential oil fractions.

Fraction	Chemical families eluted	<i>O. compactum</i>			<i>R. officinalis</i>			<i>P. asperum</i>		
		Weight	Yield	Density	Weight	Yield	Density	Weight	Yield	Density
		mg	%	g/cm ³	mg	%	g/cm ³	mg	%	g/cm ³
1	Hydrocarbons	5193.6	16.4	0.84	7049.3	22.5	0.86	3213.6	10.4	1.1
2	Esters + Ethers	1167.9	3.7	0.94	3607.3	11.5	0.88	747.5	2.4	0.92
3	Aldehydes + Ketones	12110.6	38.3	0.7	12947.0	41.3	0.8	7125.7	23.1	0.82
4	Aldehydes + Ketones + Alcohols	7867.6	24.9	0.7	2130.4	6.8	0.76	8431.6	27.3	0.78
5	Aldehydes + Ketones + Alcohols	1339.2	4.2	0.86	728.5	2.3	0.72	2450.2	7.9	0.8
6	Alcohols	921.2	2.9	0.84	305.6	1.0	0.84	426.5	1.4	0.7
7	Alcohols	367.8	1.2	0.84	110.4	0.4	0.82	96.1	0.3	0.8
8	Alcohols	43.2	0.1	0.84	130.2	0.4	0.78	44.6	0.1	0.76
9	Alcohols	1995.6	6.3	1	2393.0	7.6	0.86	7769.5	25.2	0.92
10	Acids	600.4	1.9	0.92	1934.2	6.2	0.92	544.9	1.8	1.3
Total	-	31607	99.9	-	31336	100	-	30850,1	99.9	-
Crude essential oils		30 000	2.1± 07	0.90	30 000	1.2±1	0.96	30 000	0.8±1	0.78

Table 6. Antimicrobial activity of *Origanum compactum*, *Rosmarinus officinalis* and *Pelargonium asperum*.

Bacterial strains	<i>O. compactum</i>			<i>R. officinalis</i>			<i>P. asperum</i>			AN ^b 30µg
	Ø: mm ^a	Interpretatio	MBC/MIC	Ø: mm ^a	Interpretatio	MBC/MIC	Ø: mm ^a	Interpretatio	MBC/MIC	Ø
	10µl/ml	n*	µl/ml	10µl/ml	n*	µl/ml	10µl/ml	n*	µl/ml	mm
<i>A. baumannii</i> : P142	48.5 ± 0.71	Very strong inhibition	0.25/0.25 = 1	14.75 ± 0.70	Slight inhibition	8/4= 2	8.5 ± 2.12	Non inhibition	4/2= 2	23.4 ± 0.8
<i>E. coli</i> (ESBL): TF2	29.33 ± 1.15	Strong inhibition	0.5/0.5 = 1	10.02 ± 0.10	Slight inhibition	4/4= 1	8.67 ± 0.57	Non inhibition	> 16/8> 16	24.5 ± 0.7
<i>K. pneumoniae</i> : CT59R	29.5 ± 2.12	Strong inhibition	0.5/0.5 = 1	13.0 ± 1.41	Slight inhibition	16/2= 8	9.5 ± 0.7	Non inhibition	> 16/2> 16	24.6 ± 0.5
<i>P. aeruginosa</i> : P134	14.61 ± 1.99	Slight inhibition	> 4/1> 4	7.67 ± 1.15	Non inhibition	> 16/>16> 16	7.5 ± 0.5	Non inhibition	>16/> 16>16	22.4 ± 0.3
<i>S. aureus</i> (SARM): PN15	39.0 ± 1.73	Very strong inhibition	0.5/0.25 = 2	10.33 ± 1.52	Slight inhibition	4/2= 2	13.67 ± 1.52	Slight inhibition	2/2= 1	25.2 ± 0.8

^a: diameter of zone inhibition in millimeters including the paper disc; ^b: disc of Amikacin antibiotic; *: Mutai *et al.* (2009).

Table 7. Antibacterial activities of the fractionated EOs on different clinical bacterial strains.

Bacterial strains	<i>O. compactum</i>		<i>R. officinalis</i>		<i>P. asperum</i>	
	6µl/ml		6µl/ml		6µl/ml	
	Inhibiting fractions	Activity; Oussou et al. (2008)	Inhibiting fractions	Activity; Oussou et al. (2008)	Inhibiting fractions	Activity; Oussou et al. (2008)
<i>A. baumannii</i> : P142	F3, F4, F5	Bacteriostatic	F4	Bacteriostatic	F4, F 5, F6, F7	Bacteriostatic
<i>E. coli</i> (ESBL): TF2	F3, F4, F5	Bacteriostatic	F4, F5	Bacteriostatic	F4, F5, F6, F7	Bacteriostatic
<i>K. pneumoniae</i> : CT59R	F3, F4, F5	Bacteriostatic	F4, F5	Bacteriostatic	F4, F5, F6, F7	F7:Bactericidal
<i>P. aeruginosa</i> : P134	F3	Bacteriostatic	F7	Bacteriostatic	F7, F8	F7:Bacteriostatic F8:Bactericidal
<i>S. aureus</i> SARM: PN15	F3, F4, F5	Bacteriostatic	F1, F4, F5	Bacteriostatic	F1, F2, F3, F4, F5, F6, F7	Bacteriostatic

tions less or equal to 8 µl/ml, but it is bacteriostatic against *P. aeruginosa* and *K. pneumoniae*.

P. asperum EO, are considered not inhibitory on solid medium with diameters not exceeding $13.67 \pm 1,52$ mm. Whereas, in liquid medium this oil had a bactericidal effect against *S. aureus* and *A. baumannii* at concentrations less than 4 µl/ml and a bacteriostatic effect even at a concentration of 16 µl/ml for other bacteria.

As shown in **Table 6**, dilution method in liquid medium revealed that activities of certain oils on tested bacteria were bactericidal with MBC equal to MIC and the ratio MBC/MIC = 1, while for others, the MBC value capable of killing the inoculums was twice the MIC value.

The 10 fractions of each EO were assessed for antibacterial activity using MH broth medium. Inhibiting fractions were identified using 6 µl/ml and their antimicrobial effects were interpreted as shown in **Table 7**.

For *O. compactum* only F3, F4 and F5, that represents 67.4% of the total weight of this oil, showed bacterial growth inhibition and with only a bacteriostatic effect.

Fractions F4, F5 and F7 together represent 9.5% of the total mass of *R. officinalis* oil (**Table 5**). Bacterial growth inhibition was detected with F1, F4, F5 and F7 with a bacteriostatic effect.

P. asperum oil had various fractions exhibiting inhibitory effect against tested bacteria (F2 to F9) and which represent 86.2% of the total composition (**Table 5**). All these fractions showed a bacteriostatic effect except fractions F7 and F8 which were respectively bactericidal against *K. pneumoniae*, and *P. aeruginosa* (**Table 7**). The mass fraction of F7 and F8 were respectively 0.3 % and 0.1% of total mass.

Discussion

Three Moroccan native plants (*O. compactum*, *R. officinalis* and *P. asperum*), were subjected to phytochemical and antimicrobial activity studies. Comparing our results with others cited in literature, differences were found and these probably attributed to factors related to genetic, environment and techniques used.

The obtained yields values were more or less comparable to other published studies. Indeed, the same yield (2.1 ± 07) has been reported for *O. compactum* [11], while for *R. officinalis* obtained yield (1.2 ± 10) was lower than various reported values ranging from $1.74 \pm 0.38\%$ to $2.58 \pm 0.75\%$ [12]. Important yield was obtained for *P. asperum* (0.8 ± 10) compared to previous studies that consisted of approximately 0.15 to 0.40% [13].

These differences in performance can be attributed to several intrinsic and extrinsic factors including genotype and environment [7].

Chemical compositions were slightly different from those described in the literature with a predominance of Carvacrol as major component of the *O. compactum* essential oil [11, 14], while for *R. officinalis* our results were different from those described in the literature with the regular presence of the same major components [7, 15]. Regarding *P. asperum*, our values were slightly different from those described in the literature with Citronellol and Geraniol being the main elements [13].

The exact composition of oils is not always specified for plant sections used and generally, EOs chemical compositions are depending on their derivation [16]. This variability may affect the physicochemical properties and bioactivity of these essential oils [13].

From the results shown above (**Table 5**), there is a performance difference between the fractions obtained for the same EO, which may be proportional to a difference in polarity between molecules.

Furthermore, we can underline that 30% of the fractions obtained are denser than *O. compactum* crude extract and that fraction F9 had the same density than water. All of *R. officinalis* fractions showed lower density than of the crude extract. For *P. asperum* 70% of fractions were denser than total extract while fractions F1 and F10 showed higher density than water.

These differences in densities are related to the chemical composition that characterizes each fraction. Density provides information on the quality of the chemical composition, since a density less than 0.9 g/cm³ indicates the presence in this oil of terpene and aliphatic compounds at high rates, whereas a density greater than 1 indicates a highly varied composition polycyclic terpene compounds [17]. The obtained inhibition diameters, CIM and CBM varied depending on the oil used and the bacterial strain tested. Thus, essential oil is considered active if it

produces superior inhibition diameters or equal to 15 mm [18].

The comparison of the numerical values of our results with other publications, often remain qualitative, since the authors come to express the results in case of MIC and MBC with various units, either µl/ml, in % (v / v) (Bourkhis *et al.* 2007) or µg/ml (Oussou *et al.* 2008). This study demonstrated that *O. compactum* have significantly high antimicrobial activity against tested strains except *P. aeruginosa*. This was expressed by large zone inhibition diameters ranging from 29.33 mm to 48.50 mm as well as low as by low MIC values of 0.025% (v/v) and 0.05% (v/v) and MBC/MIC ratios of 1 and 2. On the other hand, only slight inhibition was observed for *P. aeruginosa* with a MIC of 0.1% (v/v) and a MBC superior to 4. Hence, our results correlated with those reported in the literature [7]. Therefore, MIC and MBC values ranging from 0.0625% to 0.125% and from 0.0625 to 0.25% have been reported for *E. coli* and from 0.0078 to 0.031% and from 0.0078 to 0.125% for *S. aureus* [14].

The high activity shown by the *O. compactum* EO is probably associated with its high content of Carvacrol (48%), γ-Terpinen (17%) and Thymol (16%) representing the major constituents of this oil. These compounds are phenols and considered to have a high antibacterial action [5, 21]. In addition, oxygenated terpenes and especially terpene alcohols are highly active antimicrobial agents [22]. Moreover, several studies have shown that the total antimicrobial activity of EOs is not always attributed to the major compounds, but may be due to synergism [23] or antagonism [24] interactions with others minor compounds.

Although *R. officinalis* exhibited only slight inhibitions of bacterial growth on solid medium, MIC values were 0.2% (v/v) and 0.4% (v/v) with bactericidal effect against *A. baumannii*, *E. coli* and *S. aureus*. Previous study reported bactericidal effect of *R. officinalis* against *E. coli* and *S. aureus* [12] which is in agreement with our result. For *K. pneu-*

moniae, a bacteriostatic effect was observed while no antimicrobial activity was detected against *P. aeruginosa*.

The antibacterial activity of *R. officinalis* is provided probably from the major compounds of this Oil as Eucalyptol (35.20%), 1R- α -Pinene (19.51%) and the (1R) -Camphor (7.32%) 62.03% of which are considered to have a high antibacterial action [15, 25].

In the case of *P. asperum*, no inhibition growth could be obtained in solid medium for examined strains except a slight inhibition observed for *S. aureus*. Whereas a MIC value of 0.2% (v/v) was obtained for *A. baumannii* and *S. aureus* with bactericidal effect. Previous study reported a weak effect of *P. asperum* oil from Comoros islands on *Escherichia coli* and *Staphylococcus aureus*. This effect can be attributed to terpene alcohols, mainly Citronellol (25.07%) and Geraniol (10.46%) that is considered to own antibacterial activity [13].

P. aeruginosa has an outer membrane composed of lipopolysaccharides which form an impermeable barrier to hydrophobic compounds which may explain its resistance to EOs tested [3, 26].

The discrepancy between the methods used in microbiology to study the activity of EOs is relatively linked to the degree of solubility and volatility of each EO [27, 28].

Antimicrobial activities of fractionated EOs were performed using directly liquid medium (Table 7). Numerous fractions inhibited bacterial growth but showed often bacteriostatic effect except fractions F7 and F8 of *P. asperum* which were respectively bactericidal against *K. pneumoniae*, and *P. aeruginosa*. The mass fraction for F7 and F8 was respectively 0.3 % and 0.1% of the total mass. This suggests the presence of a certain antagonism between compounds avoiding the expression of any bactericidal effect in mixed oil.

Generally, the difference in the action between these EOs is likely due to the difference in chemical composition, nature and cell wall composition

of microorganisms tested and also the influence of the reaction medium which requires studying each compound alone and in combination to avoid false results [14, 29].

Conclusion

The present study provides additional data of the chemical composition and antimicrobial activities of the essential oils extracted from *O. compactum*, *R. officinalis* and *P. asperum* obtained from Moroccan aromatic plants.

Among the fractions obtained, some of them provided interesting antibacterial activities, especially against *P. aeruginosa* which exhibited high resistance to assayed EOs.

This allows deducing that certain molecules present in small quantities, even in trace in the essential oil can be more active if they are concentrated and purified. Important features illustrated in this work consider these three plants, and generally all medicinal plants, as a heritage to be preserved and developed as source of discovering more effective bioactive molecules. Further studies should be carried out to overcome the limitations of employing essential oils as alternative way for health care.

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