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Chemical composition and antibacterial activity of *Lavandula species L.dentata* L. , *L. pedunculata* Mill and *Lavandula abrialis* essential oils from Morocco against food-borne and nosocomial pathogens

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ABSTRACT: For antiinfectious fight, Aromatic and Medicinal Plants, constitute resources to valorize. Six germs belonging to *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* frequently encountered in hospital have been selected to assess antibacterial activity of *Lavandula abrialis, Lavandula dentata* L and *Lavandula pedunculata* Mill. Essential Oils (EO). EOs obtained by hydrodistillation were analyzed by Gas Chromatography/Mass Spectrometry and their antibacterial activity was assessed through discs-diffusion tests. EOs analyses revealed monoterpenes-rich oils. Camphor (49.75%) and 1.8 cineole (39.84%) were found in *L. dentata* EO while camphor (46.36%) ,fenchone (13.19%) and α-pinene(10.74%) were observed in *L. pedunculata*. Linalool (25%), camphor (16.06%), linalool acetate (13.66%) and borneol (11.94%) were the main components in *L. abrialis.* Biological tests proved EOs' antibacterial power against germs despite resistance to Beta-lactamin antibiotics. The highest inhibition was obtained with *L. pedunculata* EO.

KEYWORDS: Volatile compounds; *Lavandula sp*; Antibacterial Activity; Multiresistant Bacteria.

1 INTRODUCTION

Damages from infectious diseases are in continuous growth because of the emergence of more virulent bacteria and the occurrence of multiresistant bacteria towards antibiotics. These facts could be explained by the existence of bacterial communication system like "quorum sensing system" that utilizes such molecules as mediator of information. This mediator could enhance virulent genes expression [1],[2] and biofilm formation to protect bacteria from antibacterial agents' action [3]. This is a highly important problem that industrial firms are facing. Numerous studies screening vegetal and animal biodiversity are done in order to find more efficient molecules [4],[5],[6] to eradicate this widespread problem.

Although phytotherapy had been used traditionally from generations, recent studies prove that it would be a good way to fight efficiently against infectious germs. Thanks to active principles contained in EO of Aromatic and Medicinal plants (AMP), the use of EOs all around the world has proved their antibacterial properties[7],[8],[9],[10]. They are often used alone or in combination with antibiotics[11]. They can also be used as a mixture of EOs in order to strengthen their action [12].

Plants from Lavandula genus are among the most used in the world. They are used in garden to purify atmosphere and in cosmetics [13], [14] for maintenance of home and personal goods. In Moroccan pharmacopeia, they are registered among the most important. Their volatile and non volatile compounds are used by local population to treat wounds, cough,

diabetes, cold, vaginal infection, asthma, respiratory and digestive diseases. They also have antilithiasic, hypoglycemic, anti rheumatism, anti inflammatory, antioxidant and antimicrobial activities [15],[16],[17],[18],[19],[20], [21],[22].

Thus, we chose to work on lavandula species namely: *Lavandula abrialis, Lavandula dentata L., Lavandula pedunculata* Mill from less studied arid and semi-arid Moroccan regions. In this study we compare volatiles compounds of these lavandula species. Then, we focused on antibacterial ability of each plant's EO against food-borne and nosocomial pathogens: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from University Healthcare Centre of Fès (Morocco) with a comprehension approach between chemical composition and antibacterial activity.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND EO EXTRACTION

The plants were collected in the full-blooming stage in Anti Atlas and Middle Atlas regions (Morocco). The species were confirmed by Pr. Ibn Tattou at the scientific institute of Rabat.

Aerial parts of the plants were protected from sunlight and dried at room temperature for 13 days. EOs were obtained by hydrodistillation with Clevenger-type apparatus for two hours and half (2h30). The process was repeated three times for each sample of 100g. EO was dried with anhydrous sodium sulfate then stored in darkness at 4°C for further use.

2.2 EO ANALYSES BY GC/MS AND COMPOUNDS IDENTIFICATION

Gas chromatography (GC) and mass spectrometry (MS) were used to study the chemical composition of the EOs. We used a THERMO ELECTRON gas chromatograph: Trace GC Ultra equipped with DB-5 capillary column (5% phenyl-methyl-siloxan) (inner diameter 30m x0. 25mm, thickness: 0.25µm), flame ionization detector feed with H_2/Air gas and PVT (Programmed Vaporization Temperature), injector with split /splitless-mode. Split-mode injection is used (splitting ratio: 1/50 flow rate: 66mL/min) and the injected volume is 1 µl of 10% EO in purified hexane. Nitrogen is the carrier gas at a flow rate of 1ml/min. The temperature rises from 50°C to 200°C at 4°C/min.

Mass spectrometry carried out with a gas chromatograph THERMO ELECTRON Trace MS System (THERMO ELECTRON: Trace GC Ultra; Polaris Q MS). Fragmentation is done by electronic impact 70eV intensity, capillary column DB-5MS (5% phenyl-methyl-siloxan) (30m x 0, 25mm, 0.25µm).Temperature rises from 50 to 200°C at 4°C/min. Helium is the carrier gas at a flow rate of 1.5 mL/min. Split-mode injection is used with a splitting ratio of 1/70, flow ml/min. The apparatus is linked to a computer with NIST 98 mass spectra library.

The composition was reported as relative percentage of the total peak area.

To identify compounds, Kovats Indices were calculated by comparison with retention time of aliphatic hydrocarbons (C7-C40). Indices and each compound mass spectrum were compared to Adams [23] reference and NIST [24] mass spectra library.

2.3 BACTERIAL STRAINS AND ANTIBIOTICS SUSCEPTIBILITY TESTS

The set of bacteria used for this study were isolated from University Healthcare Centre (Hassan II) of Fès. They were identified and coded as *Escherichia coli (Ec*01, *Ec*02*)*, *Klebsiella pneumoniae (Kp*01, *Kp*02*)*, *Pseudomonas aeruginosa (Ps*01, *Ps*02*)*.

The antibiotic susceptibility tests were performed according to French Microbiology Society recommendations [25],[26]. It includes the synergy test on Muëller- Hinton (M-H) standard medium and M-H with Cloxacillin (250 mg/l).

2.4 DISCS-DIFFUSION TESTS

Bacteria inocula (0.5 Mc Farlan) are prepared in ISS from 24 hours -bacteria culture on Trypton Soya Agar (TSA). 90 mm-Petri dishes containing TSA medium are inoculated by inundation method. 6 mm diameter-sterile discs of filter paper loaded with two (2) µL of EO were placed in the centre of inoculated Petri dishes. EO's activity is assessed by measuring inhibition zone diameter in millimeters (mm) after 24 hours of incubation at 37°C. Isotonic saline sterile water (ISS) was used as negative control and 10µg antibiotic discs of Meropenem (Mel) and Ciprofloxacin (CIP) were used as positive control respectively for Enterobacteria and *P. aeruginosa* strains. Experiments are done at least three times.

2.5 STATISTICAL ANALYSIS

Inhibition diameters are presented as the average of three tests and their standard deviation. This was calculated with Excel 2007 software.

3 RESULTS AND DISCUSSION

3.1 EXTRACTION YIELD

Hydrodistillation of these lavandula species yielded 2.89%, 2.18% and 1.97% of EO for 100g of dried matter respectively for *Lavandula abrialis, Lavandula dentata* and *Lavandula pedunculata*. .

These results are quite higher compared to those of other researchers. Zrira [27] evokes a yield of 2,3 % obtained for *L. abrialis* from Morocco that is slightly inferior to the one obtained in our study. For *L. dentata*'s yield, Imelouane et al [28] report 1.41% from Eastern Morocco. Gamez et al [29] report 0.8% and Bousmaha et al [30] in Algeria obtained 0.24 to 1.34%. Mothana et al [31] reveal 0.58% in Yemen while Msaada et al.[32] found 1.96% in Tunisia as *L. dentata* EO's yields. The important yields in this study are very interesting since they would allow a better industrial exploitation of the species. However, important consideration must be given to the distillation time since changes in yield and chemical composition of EOs may occur [33].

3.2 CHEMICAL COMPOSITION OF EO

Gas Chromatography and Mass spectrometry of EOs revealed for *L abrialis* EO, 31 compounds representing 99.59% (Table1). This EO is dominated by monoterpenes (93.51%). Sesquiterpenes of this EO consist only in 6.08% with 4.08% of α-Terpinol. The major components are linalool (25.86%), camphor (16.06%), 1.8 cineol (16.04%), linalool acetate (13.66%) and borneol (11.94%).This analysis revealed linalool chemotype.

Compared to the published chemical compositions, the chemical profile described here has similarities but also revealed differences.

Harborne and Williams reported linalool 20-23%, linalyl acetate 19-26 % , 1,8cineol 10%, camphor 12% [18]. The chemical composition of *L. Abrialis* describe by Zrira: linalool (33.7%), camphor (17.6%), 1,8-cineol (14.5%) and linalyl acetate (13.5%) is quite the same of ours with small differences in percentages [27]. The same chemotype was found and the other major compounds were the same in both essential oils. Bellahkhdar mentioned that Moroccan middle Atlas EO is dominated by lavandulol (48%) while the French species contains Linalool (35%), linalyle acetate (19 %), camphor (7%) and1,8-cineol (6%) [16]. In general, it seems that Moroccan culture conditions facilitate the development of camphor and cineol while French conditions facilitate linalool production. In *L. angustifolia's* OE and *L. Latifolia*, the parents of *L. abrialis,* the most important compounds are linalool (10-50%), linalyl acetate(12-54%), camphor (0-0.2%) for L *angustifolia's EO* and linalool (26-44%), linalyl acetate (0-1.5%) and camphor (5-14%) for *L. Latifolia*'s EO [15],[16],[18],[34]. *L. abrialis* seems to inherit major components from its both parents.

L. dentata EO contains 27 identified compounds forming 99.52% of total components. Its composition is also characterized by a high level of monoterpenes (99.08%). Sesquiterpenes are in a very weak proportion (0.44%). The most important components of this EO are camphor 49.75% and 1.8 cineole 39.84%. Camphor/cineole chemotype was revealed.

Imelouane et al 28 found β-pinene (27.08%) as the chemotype of eastern Morocco species and other components such as: pincarveol (14.77%) and myrtenal (8.18%). The same authors found that the population from Taforalt, Talazart(Morocco) contains:1, 8 cineol (41.28%), sabinene (13.69%), bicycle [3.1.0] hexan-3-Ol, 4-methylene-1-(1-methylethyl) (6.76%), myrtenal (5.11%) and α-pinene (4.05%)[35].

In Tunisia, for the cultivated species, linalool was found as chemotype (47.30 %) and linalyl acetate (28.65%), bicyclogermacrene (3.40%), camphor (2.32%) and δ-terpineol (1.47%) were registered by Msaada et al. as major compounds [32]. Thus it appears that the chemical composition of *L. dentata*'s EO is greatly influenced by the growing conditions.

Concerning *L. pedunculata,* 30 compounds (98.69% of total compounds) were identified. This EO is dominated by monoterpenes (88.43%) including: α-pinene (10.74%), fenchone (13.19%) and camphor (46.36%).In this camphor-chemotype essential oil, sesquiterpenes reached 10.26% with 7.69% of cubenol. According to Bellakhdar, *L. pedunculata* composition is generally dominated by Fenchone and camphor. Their percentages (together) vary from 47 to 83% with alternation of the

dominant compound [16]. Harborne and Williams confirm this information but cite camphor (24%) as the dominant compound and fenchone (20%) as the second one [18]. Costa et al found Camphor 40.6 % and Fenchone 38% in EO of Portagual species [36].

EO of the close species *Lavandula stoechas,* is dominated by α- thujone, L-camphor and 1.8-cineole [37] or by camphor and fenchone[38]. *L. pedunculata* EOs compositions seem to change very slightly and great similarities are observed between *Lavandula stoechas* and *L. pedunculata* essential oils.

Seven compounds were found in all EOs but with variable percentages (α-pinene, camphene, β-pinene, linalool, camphor, borneol and verbenone) showing clearly that the EO components vary greatly among the species despite their belonging to the same genus. Only camphor was found with significant percentages in all Eos (16.06% in *L. abrialis*, 46.36% in *L. pedunculata* and 49.75 *%* in *L. dentata*). 1.8 cineole was not found only in *L. pedunculata*.

Table 1. Chemical composition of Essential Oils of L. abrialis , L. dentata and L.pedunculata.[a]

Compounds	***% L. abrialis***	***%L. pedunculata***	***% L. dentata***	**KI**
Tricyclene	**-**	0.61	0.05	926
α-Pinène	**0.49**	**10.74**	**0.47**	**939**
Camphene	**0.43**	**5.74**	**0.9**	**954**
Thuja-2,4(10)-diene	-	0.19	0.08	960
Sabinene	0.05	-	-	975
β-Pinène	**0.59**	**0.18**	**0.9**	**979**
Dehydro-1,8-cineole	0.04	-	0.09	991
δ-2 carène	0.03	-	-	1002
δ-3 carène	0.05	4.06	-	1011
O-Cymene	0.07	0.24	-	1026
1,8 cineole	16.04	-	39.84	1031
Z-β-Ocimene	0.05	-	-	1037
E-β-Ocimene	0.03		-	1050
Cis-Sabinene hydrate	-	-	-	1070
Cis linalool oxide	0.5	-	1.17	1072
Cis-vertocitral C	-	0.29	0.06	1080
Fenchone	0.23	13.19	-	1086
Trans linalool oxide	0.61	-	0.98	1086
Linalool	**25.86**	**0.57**	**0.66**	**1096**
<cis> thujone	-	-	0.06	1102
6-Camphenol	-	-	0.14	1113
Fenchol<endo>	-	0.71	-	1116
Trans pinene hydrate	0.55	-	-	1122
Camphenal<α>	-	0.39	-	1126
Iso-3Thujanol	0.09	-	-	1138
Nopinone	-	-	0.39	1140
Camphor	**16.06**	**46.36**	**49.75**	**1141**
Trans sabinol	0.08	-	-	1142
Z-Tagetone	-	-	0.06	1152
Pinocarvone	-	-	0.52	1164
Borneol	**11.94**	**3.04**	**1.04**	**1165**
Pinocamphone	-	0.07	-	1175
Terpinol-4-ol	0.71	0.32	-	1177
E-isocitral	-	-	0.13	1180
Thuj-3-en-10-al	-	0.09	-	1184
α-Terpinol	4.38	-	0.13	1186
Myrtenal	-	-	0.8	1195
myrtenol	-	-	0.61	1195

Verbenone	**0.17**	**0.60**	**0.25**	**1205**
Neo-iso-dihydrocarveol	0.31	0.09	-	1229
Linalool acetate	13.66	-	-	1257
isobornyl acetate	-	0.95	-	1285
Bornyl acetate	0.24	-	-	1285
3-Thujanol acetate	0.25	-	-	1295
Total monoterpene	**93.51**	**88.43**	**99.08**	
A-himachalene	0.5	-	-	1451
β-Selinene	-	-	0.08	
γ-Cadinene	0.36	-	-	1513
Trans Calamenene	-	0.18	-	1522
α-cadinene	-	0.14	-	1538
Selina-3,7(11)-diene	-	0.86	-	1546
Trans Dauca-4(11),7diene	-	0.09	-	1557
Caryophyllene oxide	0.51	-	0.19	1583
Isovelleral	-	0.13	-	1587
1,10-di-epi-cubenol	0.22	7.69	-	1619
epi-α-Cadinol	4.49	-	-	1639
Hinesol	-	0.48	-	1641
β-Eudesmol	-		0.17	1650
α-Cadinol	-	0.58	-	1654
Eudesm7(11)-en-4-ol	-	0.11		1700
Total sesquiterpene	**6.08**	**10.26**	**0.44**	
total	**99.59**	**98.69**	**99.52**	

a.Compounds are listed in their elution order on DB-5 column. Kovats indices (KI) are relative to C_7-C_{40} n-alkanes.

3.3 ANTIBIOTICS SUSCEPTIBILITY TEST

The susceptibility tests to antibiotics (Table 2) proved that the strain *Ec*01 is resistant to Amoxicillin and Amoxicillin with clavulanate, it is also resistant to Chloramphenicol. *Ec*01 produces CTX-M ESBL.

*Ec*02 is resistant to Amoxicillin and Amoxicillin + clavulanate. This strain produces a chromosomal cephalosporinase.

Concerning *K.pneumoniae*'s strains, both are naturally resistant to Ticarcillin and amoxicillin (penicillin) thanks to a natural penicillase highly expressed. *Kp*01 is also CTX-M ESBL productive while *Kp*02 produce a plasmid cephalosporin enzyme.

Pseudomonas strains *Ps*01 and *Ps*02 are naturally resistant to Trimethoprim associated with sulfamethoxazole. This test revealed their resistance to colistin as an exceptional profile. Both strains have developed resistant mechanism to Ticarcillin, Ciprofloxacin, Amikacin, Imipenem, Ceftazidime and Piperacillin. Thus *Pseudomonas* strains are resistant to Beta-lactamin, aminosid and polymixyn.

Table 2. Susceptibility test of bacterial strains to antibiotics. [b]

Test bacteria ATB [c]	***Ec*01**	***Ec*02**	***Kp*01**	***Kp*02**		**Test bacteria ATB**	***Psa*01**	***Psa*02**
Mel_{10} [d]	S[3]	S	S	I		**TIC_{75}**	R	R
CTX_{30}	I	I	I	R		**CT_{50}**	R	R
TIC_{75}	S	S	R	R		**CIP_{10}**	R	R
AX_{25}	R	R	R	R		**AK_{30}**	R	R
FOX_{30}	I	S	I	I		**IPM_{10}**	R	R
C_{30}	R	S	S	S		**CAZ_{30}**	R	R
CT_{50}	S	S	S	S		**PRL_{30}**	R	R
AMC_{30}	R	R	R	R		**SXT_{25}**	R	R

b. The result of the susceptibility test is presented as follow S: Susceptible, I: Intermediate, R: Resistant
c. Antibiotic (ATB) Meropenem (MEL), Cefotaxime (CTX), Ticarcillin (TIC), Amoxicillin (AX), Cefoxitin (FOX), Chloramphenicol (C), Colistin (CT), Amoxicillin+ clavulanate (AMC), Ciprofloxacin (CIP), Amikacin (AK), Imipenem (IPM), Ceftazidime (CAZ), Piperacillin (PRL), Trimethoprime+sulfomethoxazole(SXT).
d. Charge of antibiotic' Discs in µg

3.4 DISCS DIFFUSION TESTS

The discs diffusion tests with 2µL of EOs have proved that the substances are active against *Ec*01, *Ec*02 and *Kp*02. EOs, at this dose, were inactive against *Pseudomonas* strains and *Kp*01. The most susceptible strain was *Ec*01. Antibacterial activity of EO seems to be independent to strains antibiotic profile since the two ESBL bacteria *Kp*01 and *Ec*01 react differently towards the EOs. The inhibition diameters obtained against *Ec*01 were 11±0.8, 12±2.1 and 12.3±2.3 mm respectively with EOs of *L. abrialis, L. dentata* and *L. pedunculata*. The best antibacterial activity was obtained by the use of *L.pedunculata*'s EO. Inhibition diameters were 12.3±2.3, 9.3±0.9, 9±1.2 and 6 mm against *Ec*01, *Kp02*, *Ec*02 and *Kp*01 respectively (Table 3). We remark that in general all EOs seem to have similar activities (small differences) despite the differences in their chemical compositions. We found only seven compounds in all EOs studied as mentioned in chemical composition part. Thus with the hypothesis that the antibacterial activity is probably due to the major compound, this compound would be camphor since it was the only one compound found with significant percentages in all EOs (16.06% in *L.abrialis*, 46.36% in *L. pedunculata* and 49.75 *%* in *L.dentata*). It would also signify that its antibacterial activity doesn't need too high percentage.

However, as mentioned by Mahboubi and Kazampour, activity of pure compounds (such as cineole and camphor) appears to be higher than the activity demonstrated by the whole essential oil [39]. Thus, the hypothesis that antibacterial activity of these EO is due to interactions among all the constituents (or the most important constituents) seems to be better. We could for further information isolate the most important components of each essential namely 1. 8cineole, camphor, linalool and try to highlight interaction type by testing them alone, then combined with different ratio.

Then, we compare the activity found here with other works on some Lavandula species. Mohammedi et al.[20] worked against the same bacterial species with *Lavandula stoechas* and found no effect on *P. aeruginosa.* A weak activity was observed *E .coli* and *K. Pneumoniae* 8.19± 1.49 mm and 5.88±0.57mm. Imelouane et al show that *L. dentata* EO dominated by 1.8 cineole (41.29%) had no effect on *pseudomonas aeruginosa* but led to 11,11,15,20 mm as inhibition against E.coli strains. Against *K.pneumoniae* strains the same EO led to 20 and 38 mm 35. Prabusonivasan et al working with *L.angustifolia* found no activity against *K. pneumoniae* and *E.coli* but against *P.aeruginosa* strains 9.9, 12mm [40].

Antibacterial activity of EO depends on the components interactions but also on the bacterial strains since bacterial strains of the same species may react differently towards the same EO.

Table 3. Antibacterial activity of Lavandula abrialis, Lavandula dentata L. and Lavandula pedunculata Mill. essential oil through discs diffusion tests against Escherichia coli (Ec01, Ec02), Pseudomonas aeruginosa (Psa01, Psa02) and Klebsiella pneumoniae (Kp01, Kp02).

	***Ec*01**	***Ec*02**	***Kp*01**	***Kp*02**	***Psa*01**	***Psa*02**
L. abrialis	11±0.8	8±0	6±0	8.3±0.4	0	0
L.dentata	12±2.1	9.6±0.1	6±0	8.3±0.9	0	0
L.pedunculata	12.3±2.3	9±1.2	6±0	9.3±0.9	0	0
Mel/CIP	24±0.2	34±0.5	22±0.5	17±0.3.	0±0	0±0

f. Diameters of inhibition zone are expressed in mm as means ± standard deviation of triplicate

4 CONCLUSION

This study show that volatiles compounds of Lavandula genus vary greatly. Differences in chemical compounds are according the species. Chemical profile of *L.abrialis* seems to be less influenced by environmental conditions while *L. dentata* profile shows more differences. Thanks to these variable profiles, these species can be considered as a source of active principles which may lead to various biological activities. All the tested EOs showed an interesting antibacterial activity except against *P.aeruginosa* strains. *L. pedunculata* appears to be the most effective. This action seems to be independent to the antibiotic susceptibility profile of the bacterial strains. Further studies will be important to understand chemical compounds' interactions and their mechanism of action.

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