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Chemical composition and Antibacterial power of *Lavandula multifida* L. essential oil against multiresistant strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolated in hospital

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ABSTRACT: Bacterial infectious diseases continue causing havoc while pharmaceutical firms produce less inhibitor against pathogens. In the frame of this anti infectious fight, we chose to work on *Lavandula multifida* L. (*Lm*) from Southern Morocco. Steam-distillation of the plant was done with Clevenger-type apparatus and Essential Oil (EO) obtained was analyzed by gas chromatography (GC) and mass spectrometry (MS). A phytochemical study of the species was also performed through standard qualitative reactions with specific reagents. Antibacterial activity of EO was first achieved through discs-diffusion tests against *Escherichia coli (Ec*0A*, Ec*0B), *Pseudomonas aeruginosa (Psa*0A, *Psa*0B) and *Klebsiella pneumoniae (Kp*0A, *Kp*0B). Then Minimal Inhibitory Concentrations (MIC) were determined by macrodilution method and Minimal Bactericidal Concentrations (MBC) were determined. Steam-distillation of the species yielded 2.01 ml of EO for 100g of dried matter and analyses revealed Phenol 2,3,5,6 tetramethyl as major component with 89.97%. Phytochemical tests proved the existence of polyphénols, triterpenes, sterols, mucilage and saponosids. Biological tests proved that this EO has a significant antibacterial power against the germs despite their resistance to Beta-lactamin, aminosid and polymyxin antibiotics. Extreme inhibition diameters were 37.3±4.4mm for *Ec*0B and 10.6±0.5mm for *Psa*0A. 0.6µl/ml was the MBC against *Ec*0A, 9.6µl/ml was the MBC obtained against *Psa*0A and *Psa*0B. This study, among the rare found about this species, confirms its utilization by local people to treat digestive and respiratory infections. Discovery of a chemotype is a good way to valorize the species. It could be easily isolated to serve as a model for new antibacterial drug.

KEYWORDS: Chemical study; *Lavandula multifida* L; Antibacterial Activity; Multiresistant Bacteria.

1 INTRODUCTION

Through generations, aromatic and medicinal plants have proved their benefits in human health management. These plants can be considered as a source of active principles since many of them had been the basis of drug manufacture. Among volatiles compounds, thymol isolated from *Thymus vulgaris* is well-known for its antimicrobial activity [1]. As volatile isolated compounds, essential oils which are a mixture of volatile compounds are used as complementary medicine. In this frame, essential oils from *Lavandula* genus are wide used by people and firms to provide well-being feeling and products.

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Lavandula species are among the most used by Moroccan people. They have many activities such as anxiolytic, anti inflammatory and antioxidant activities [2],[3],[4],[5].

Lavandula multifida L. is a Mediterranean native species wide spread in Morocco. It is used by local population to treat wounds, diabetes, cold, respiratory and digestive diseases [6],[7],[8],[9]. Additionally, *L.multifida* has hypoglycemic, hypolipidemic and antifongal virtues[10],[11],[12]. However, its essential oil has been little studied for antimicrobial ability since only two reports on Moroccan population of the species can be found[13],[14]. Thus, we focused our work on antibacterial activity of *L.multifida* essential oil from Moroccan arid region.

In this paper, we report chemical study (volatile and non volatile extracts) of the species then, we assess antibacterial power of *L.multifida*'s essential oil against gastrointestinal and respiratory tract pathogens *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from University Healthcare Centre of Fès (Morocco).

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND EO EXTRACTION

The plant was collected after the flowering stage in the Anti Atlas region. The species was confirmed by Pr. Ibn Tatou at the scientific institute of Rabat.

The aerial parts of the plant were protected from sunlight and dried at room temperature for 13 days. EO from this part was obtained by steam distillation 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 ANALYSIS (GC AND GC/MS) AND COMPOUNDS IDENTIFICATION

Gas chromatography (GC) and mass spectrometry (MS) were used to study the chemical composition of the EO. 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 injected volume is 1 µl sample of 10% EO solution 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 reference [15] and NIST[16] mass spectra library.

2.3 PHYOTCHEMICAL SCREENING

Phytochemical study was achieved on aerial part of *L.multifida*. Dried sample of the plant was ground into fine powder. Specific extracts were prepared for each family of searched compounds. Extracts were obtained by extraction with solvents (ether and distilled water). This study is a set of qualitative tests based on standard colour and / or precipitation reactions. We used methodology described by Bruneton, Bidié et al. and Karumi et al. [2], [17], [18].

2.4 ANTIMICROBIAL ACTIVITY

2.4.1 BACTERIAL STRAINS

The set of six bacteria used for this study were isolated from University Healthcare Centre (Hassan II) of Fès. They were identified and coded as *Escherichia coli (*Ec0A ESBL, Ec0B*)*, *Klebsiella pneumoniae (*Kp0A ESBL, Kp0B*)*, multiresistant strains of

*Pseudomonas aeruginosa (*Ps0A, Ps0B*)*. The antibiotic susceptibility profiles of the strains shown in table 1 are performed according to recommendations of French Microbiological society[19] and [20] EUCAST .

Table 1: Susceptibility test of bacterial strains to some antibiotics. [a]

Test bacteria ATB[b]	*Ec*0A	*Ec*0B	*Kp*0A	*Kp*0B		Test bacteria ATB	*Psa*0A	*Psa*0B
Mel_{10} [c]	S	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

a. The result of the susceptibility test is presented as follow S: Susceptible, I: Intermediate, R: Resistant
b. 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).
c. Charge of antibiotic' Disc in µg

2.4.2 DISCS-DIFFUSION TESTS

Bacteria inocula (0.5 Mc Farlan) are prepared in ISS from 24h 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 2µL of EO were placed in the centre of inoculated Petri dishes. EO's activity is assessed by measuring inhibition zone diameter in mm after 24 hours of incubation at 37°C.Isotonic saline sterile water (ISS) was used as negative control and10µg 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.

2.4.3 MIC AND MBC

MIC is determined by macrodilution method in Brain Heart infusion broth. EO is diluted in DMSO to a concentration of 600µL/mL. Then two fold-dilutions are done over the range of 0.15µL/mL to 19.2µL/mL. Final volume of the tests-tubes is 4mL of liquid medium. The tubes were inoculated with 10^5 CFU/mL [21], [22] and the different concentrations of the testing oil were added. The final concentration of DMSO in each tubes was <1.3%.

Tubes were incubated at 37°C for 24hours. The test was performed in duplicate. Several controls were used: culture medium plus DMSO without the extract, culture medium without DMSO and extract, culture medium with amoxicillin.

After MIC determination, 100µL of all the tubes without visible growth were spread on TSA and incubated for 24hours à 37°C. The MBC corresponds to the lowest concentration at which bacteria are killed.

3 RESULTS AND DISCUSSION

3.1 EXTRACTION YIELD

Steam distillation of 100g (dried matter) of Lm yields 2.01 mL of EO. This yield of 2.01% (mL/100g), is better than 0.7% found by Sellam et al[13] as yield with Moroccan sample from Errachidia. Benbelaid et al. [23] who worked on fresh aerial part of *L. multifida* from Tlemcen region in Algeria obtained 0.09%.

The yield obtained in our study is acceptable, but according to Costa et al. Danh et al. and Da Porto et al.[24],[25],[26], it would be 6 to 18 times higher by using supercritical fluid method instead of steam distillation. However, supercritical fluid process leads to a modification in EO' composition. Steam distillation seems to be the best process to have a good overview on plants volatile compounds [24].

3.2 CHEMICAL COMPOSITION OF EO

Analysis of EO by Gas chromatography and Mass spectrometry revealed 22 compounds representing 99.59% of total compounds (Table2). The five most important compounds which cover 96.91% are Durenol (Phenol 2,3,5,6 tetramethyl) (89.97%), caryophyllene oxide (2.43%), sphatulenol (1.83%), aromadendrene (1.68%) and carvacrol methyl ether (1%).

EO composition can be regrouped in monoterpenes (92.83%) and sesquiterpenes (6.48%). Chemical composition of this EO is different from those in the literature. Sellam et al.[13] in Moroccan population from Errachidia found 66.2% of carvacrol, p-cymene-8-ol (4.2%), caryophyllene oxide (2.7%), terpinolene (2.6%) while Douhri et al.[14] found in Moroccan population from Tetouan, carvacrol (47.62%), β-bisabolene (9%), linalool (7.42%), menthone (4.98%), caryophyllene (3.34%).

In Tunisia Chograni et al.[27] studied 11 samples of Lm' EOs and found 35 compounds. Carvacrol was the main one: 21.14 to 47.02%, then ester acrylic acid dodecanyl (8.96 to 14.06 %) and β-bisabolene (12.96 to 19%). Zuzarte et al.[12]worked on two samples of Lm from Portugal and found also that carvacrol (42.8 and 41.5%) was the main compound followed cis-β-ocimene (27.4 and 27%).

Our work reports for the first time the presence durenol (89.97%), in this species. It is also the first report of a so high percentage of a major component in *L.multifida*. Therefore, this compound can easily be isolated and purified for further studies. Furthermore, we notice that, all the works on *L.multifida* report phenol-type molecule as main compounds.

We chose to compare the chemical composition of our sample with other Lavandula species: EO of *L. Coronopifolia* is dominated by trans-β-ocimene (26.9%), carvacrol (18.5%), β-bisabolene (13.1%) and myrcene (7.5%)[28]. *Lavandula stoechas* EO is dominated by α- thujone, L-camphor and 1.8-cineole[29] (Sertkaya et al.,2010) or by camphor and fenchone [30].

In *L. angustifolia* OE, the most important compounds are linalool and linalyl acetate[25],[26],[31]. We notice that our OE's composition is different and chemical composition vary greatly in the same genus Lavandula and in the same species *Lavandula multifida* L. according to the region.

Table 2: Composition of Lavandula multifida L. Essential Oil from southern Morocco[d]

Compounds	%	KI
α-pinène	0.03	939
β-pinène	0.03	979
δ-3 carène	0.09	1011
1,8 cineol	0.06	1031
Terpinolene	0.1	1088
<cis> thujone	0.36	1102
Camphor	0.37	1141
Cymen-8-ol<p>	0.04	1182
carvacrol methyl ether	1	1215
Durenol[e]	89.97	1361
Carvacrol acetate	0.78	1372
Total monoterpene	92.83	
Aromadendrene	1.68	1451
<α>patchoulene	0.14	1456
Viridiflorene	0.10	1496
Silfiperfolan-6-α-ol	0.04	1507
Coehene epoxide<α>	0.04	1574
Spathulenol	1.83	1578
Caryophyllene oxide	2.43	1583
Hinesol	0.23	1641
Total sesquiterpene	6.49	
Manool oxide	0.08	1987
Isomethyl-β-ionone	0.14	2070
Abietol dehydro	0.05	2368
others	0.31	

d.Compounds are listed in their elution order on DB-5 column. Kovats indices (KI) are relative to C7-C40 n-alkanes.
e.The compound was confirm by comparison of the spectrum with NIST mass spectra library

3.3 PHYTOCHEMICAL SCREENING

Phytochemical study showed abundance of polyphenols including tannins and flavonoids. The presence of flavonoids was manifested by the development of a pink-orange coloration that proved the existence of flavones. The presence of tannins was proved by a bluish coloration that specifies gallic tannins. Anthocyanes, leucoanthocyanes and catechic tannins were not found. The species also contains sterols, triterpenes mucilage and a small proportion of saponosids. The search of alkaloids led to a negative result (Table 3).

Table 3: Result of phytochemical study of Lavandula multifida L. from Southern Morocco

Chemical groups		Reagents or reaction name	Results
tannins	global	FeCl3	++ (Dark green color)
	catechic	Stiasny reagent	--- (red precipitate)
	gallic	Reaction with sodium acetate	++ (bleue coloration)
flavonoids	anthocyanes	Acido-basic reaction	---
	flavones	Cyanidin reaction	+ +(Pink-orange color)
	leucoanthocyanes	Cyanidin reaction without Mg shavings	---
alkaloids		- Valser - Mayer Reagent - Dragendorff Reagent	---
mucilage		precipitation Reaction	++(flake)
saponosids			+
Sterols et triterpenes		Liebermann Buchard Reaction	+ (Red ring and brownish violet color of the supernatant layer)

++ = abundant - = absent + = weak reaction

3.4 ANTIMICROBIAL ACTIVITY

3.4.1 ANTIBIOTICS SUSCEPTIBILITY TEST

The susceptibility tests to antibiotics (Table 2) proved that the strain Ec01 is resistant to Amoxicillin and Amoxicillin with clavulanate, it is also resistant to Chloramphenicol. Ec0A produces CTX-M ESBL.

Ec0B 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. Kp0A is also CTX-M ESBL productive. While Kp0B produce a plasmid cephalosporin enzyme.

Pseudomonas strains Ps0A and Ps0B are naturally resistant to Trimethoprim associated with sulfamethoxazole. They are also resistant to colistin that is exceptional in *P. aeruginosa*'s strains. 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.

3.4.2 DISCS DIFFUSION TESTS

The discs diffusion tests with 2µL of EO have proved that the substance is active on all the strains. The lowest activities are obtained with Psa0A: 10.6±0.5mm and Psa0B:14.5±0.7.

The best activities are showed by 37.3±4.4 mm inhibition diameter zone against Ec0B and 36.3±2 mm against Kp0A.

Ec0A and Kp0B are averagely susceptible to EO, their inhibition diameters zones are respectively 28.6±1.5 and 24.5±4.9 mm(Table 4). These results seem to show that durenol lead to a better antibacterial activity compared to carvacrol since sellam et al. [13] found 17mm of inhibition against *E.coli* strains and 7.5mm against *P. aeruginosa* with 5µl of *L.multifida* EO dominated by Carvacrol(66.2%). The results of Douhri et al, [14] who found 9.5mm of inhibition against *E.coli* strains and no effect against *P. aeruginosa*, with an EO dominated by carvoacrol (47.62%) reinforce this hypothesis.

Our results are different from those of Benbelaid et al. [23] they proved that inhibition diameters are proportional to EO's quantity. In their work, for 2µl they obtain: 17mm for Escherichia coli, 13mm for *Klebsiella pneumoniae* and 7mm for *Pseudomonas aeruginosa*. It is only with 5µl that inhibition zone reached 22, 18 and 8 mm for the same bacterial strains.

We now compare this activity with other EOs. Mohammedi and Attik [32] worked on the same bacterial species with *Lavandula stoechas*, they found no effect on *P. Aeruginosa* .A weak activity was observed for *E .Coli* and *K. Pneumoniae* 8.19± 1.49mm and 5.88±0.57mm.

Lavandula stoechas is dominated by camphor, thujone and fenchone and the results show that *L.multifida* is more active than *Lavandula stoechas*. So the difference in EO composition even in the same genus leads to a significant difference in the activity. The remarkable activity of *L.multifida* is probably due to the presence of phenol-type molecule (Durenol) at a very high proportion. To strengthen this hypothesis we cite three thymus' EOs: *Thymus capitatus* with carvacrol chemotype, T*hymus ciliatus* with thymol chemotype and *Thymus algeriensis* with camphor.

In this study phenolic-rich EOs had similar activity and they were more active than camphor-rich EO [33],[34]. The second hypothesis is that caryophyllene oxide, known for his antibacterial activity at a low percentage could be responsible of the antibacterial activity. The last one is synergy between these two active compounds.

Table 4 : Antibacterial activity of Lavandula multifida L. essential oil through discs diffusion tests for Escherichia coli (Ec0A, Ec0B), Pseudomonas aeruginosa (Psa0A, Psa0B) and Klebsiella pneumoniae (Kp0A, Kp0B).

Test Bacteria	Ec0A	Ec0B	Kp0A	Kp0B	Psa0A	Psa0B
Lm' EO	28.6±1.5[f]	37.3±4.4	36.3±2	24.5±4.9	10.6±0.5	14.5±0.7
Mel/CIP	24±0.2	34±0.5	22±0.5	17±03.	0±0	0±0

f. Diameters of inhibition zone are expressed in mm as means ± standard deviation of triplicate, (MEL) Meropenem, (CIP) Ciprofloxacin

3.4.3 MIC AND MBC DETERMINATION

MIC determination of EO, with a spectrum range from 0.15 to 19.2µL/mL, shows that Ec0A is the most susceptible strain. Its MIC is between 0.33 and 0.6µL/mL. The highest MIC is 4.8µl/ml obtained for Psa0B. The same MIC of 2.4µl/ml is observed for Ps0Aand Kp0B (Table 5).

The lowest MBC is 0.6µL/mL for Ec0A and the highest is 9.6µL/mL for Pseudomonas Psa0A and Psa0B.The bactericidal concentrations can be ranged as follow Ec0A (0.6µl/ml) <Kp0A (1.2µL/ml) <Ec0B (2.4µL/mL) <Kp0B (4.8µL/mL) <Psa0A=Psa0B (9.6µL/mL).

Determination of MIC by macrodilution method shows that EO antimicrobial activity differs with strains as discs diffusion tests showed. Some strains are more susceptible than others. However, when comparing the two methods, we notice that the susceptibility of the strains is not ranged in the same order since the two methods differ in running conditions[35],[36].

The results proved that the species has a great potential to help fighting against microbial agents. The MIC determine here could be significantly reduced by purifying the bioactive compounds. One the one hand, if antimicrobial activity is due to durenol, thanks to the high percentage (89.97%), the compound could be easily purified and tested alone. It could be also easy to add radicals in the aim to improve its effectiveness and lower its MIC and MBC. On the other, if carophyllene oxide (2.46%) is the antimicrobial agent, it means that the MIC and MBC can be very lower than those observed in this experiment.

Table 5: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of Lavandula multifida L. essential oil for Escherichia coli (Ec0A, Ec0B), Pseudomonas aeruginosa (Psa0A, Psa0B) and Klebsiella pneumoniae (Kp0A, Kp0B)g.

	Test bacteria					
	Ec0A	Ec0B	Kp0A	Kp0B	Psa0A	Psa0B
MIC (µL/mL)7	0.6	1.2	1.2	2.4	2.4	4.8
MBC (µL/mL)	0.6	2.4	1.2	4.8	9.6	9.6

g. MIC and MBC are expressed in µL/mL. MIC is determined by macrodilution method

4 CONCLUSION

Lavandula multifida L. essential oil from southern Morocco possesses an antibacterial activity despite multiresistance of germs to antibiotics. This study among the rare found about this species confirms its utilization by local people to treat microbial infections. The discovery of durenol, as major component is a good way to valorize the species. It also seems to the most effective compounds in this essential oil.This molecule could be easily isolated to serve as a model for pharmaceutical firms to produce new drug against resistant and non resistant bacteria.

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