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Phytochemical Analysis and Antibacterial Activity of Essential Oil Of *Lavandula multifida* L.

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ABSTRACT: Essential oil from the aerial parts of *Lavandula multifida L.* (Lamiaceae), used in the Moroccan traditional medicine, was extracted by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). 34 constituents, representing 95.25 **%** of the total oil were identified. The major components at the species level were Carvacrol (47.62%), β- bisabolene (9.01%), and Dodecyl Acrylate (8.37%), Linalol (7.42%), Menthone (4.98%), β- Caryophyllene (3.34%), β- Pinene (3.21%). Antibacterial activity of this oil was tested against human pathogenic bacteria: gram-negative and gram-positive bacteria by the agar diffusion method. The minimum inhibitory concentrations (MIC) of the oil were determined by the microdilution technique. The oil showed significant inhibitory activity against the bacteria, *S. Aureus* (14.330 ± 0.577 mm), *B. subtilis* (12.670± 0.577 mm), *P. mirabilis* (12± 1 mm), *P. vulgaris* (11.67± 0.577 mm), *L. innocua* (10.660 ± 0.577 mm), *L. monocytogenes* (8.667± 0.577 mm), *E. coli CECT* (9.667± 0.577 mm) and *E. coli K12* (9.333 ± 0.577 mm). No inhibitory activity was observed against the bacterium *Ps. aeruginosa*.

KEYWORDS: Lavandula multifida, Lamiaceae, Hydrodistillation, GC/MS Analysis, Essential oil, Antibacterial activity.

1 INTRODUCTION

Many plants have been used for centuries as remedies for human diseases, because they contain natural bioactive components as therapeutic value. Various plant extracts and oils have been reported, in the literature, to have antioxidant or antimicrobial properties [1].

Lavandula (lavender, Lamiaceae) is a genus of 39 species, it's used in folk medicine for their analgesic, relaxing and sedative effects. And it's also used as stomachic agent [2-3-4], to treat depression and diabetes [5-6]. Several studies revealed that those plants are rich in wide variety of secondary metabolites that proves its antimicrobial properties [7] as monoterpenes, diterpenes [8], triterpenes [9], sesquiterpenes [10], coumarins [11] and phenolic compounds [12- 13- 14].

Lavandula multifida L. is a small perennial shrub native of the South-Western Europe, Mediterranean and North Africa from Morocco to Egypt [15]. Mature specimens have stiff, upright branches. Its evergreen leaves are ferny. The violet-blue flowers are borne in three-parted clusters held atop tall, fine stems that rise above the foliage.

However, a few studies were released to support these therapeutic properties; two documents on pharmacological investigation on *L. multifida* evaluated its hypoglycemic action [5], and its anti-inflammatory activity [16].

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The aims of this study were to investigate the chemical composition of the Moroccan *Lavandula multifida L.* essential oil and its antibacterial effect.

Fig. 1. Lavandula multifida L.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE AND PLANT MATERIAL

The aerial parts of *Lavandula multifida* used in this research were collected at the full flowering stage in January 2011 Northwest of Morocco from Nekkata region at an altitude up to 64 m (Tetuan, Morocco) and authenticated by Prof. Mohamed KADIRI (Laboratory of Algology and Mycology, Department of Biology, Faculty of Science, Abdelmalek Essaadi University, Tetuan, Morocco). Samples were further transported to the laboratory.

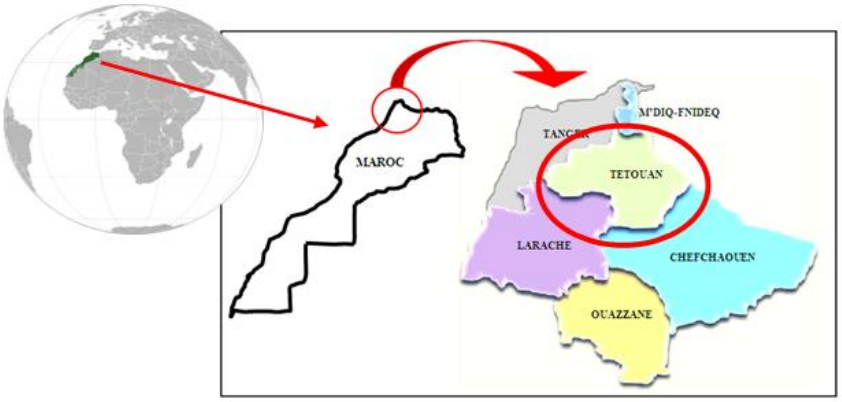


Fig. 2. Map showing the geographical location of Tetuan, Morocco

2.2 ESSENTIAL OIL EXTRACTION

Fresh mature flowers of *L. multifida* were collected from the study area. Essential oil extraction was obtained from 500 g Air-dried plant by hydro-distillation during 3 h using a Clevenger-type apparatus. The oil obtained was stored at 4°C until test and chemical analysis in appropriate dark flasks. The content of essential oil (g) was calculated just after the extraction experiment, it was expressed as mean value of triplicate extractions and used to calculate the essential oil yield which expressed in percentage relative to the mass of dry matter (g of oil for 100 g of dry plant matter).

Yield (%) = (weight of essential oil (g) / 100 g dry plant) x 100

2.3 CHEMICAL ANALYSIS OF THE ESSENTIAL OIL

The essential oil was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

2.3.1 GAS CHROMATOGRAPHY ANALYSIS (GC)

GC analyses were performed on a Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a HP-5 capillary column (30 m x 0.25 mm x 0.25 µm). The temperature was programmed from 50°C after 5 min initial hold to 250°C at 4°C/min. Gas chromatography conditions were as follows: N2 as carrier gas (1.8 ml/min); split mode was used (Flow: 72.1 ml/min, ratio: 1/50); temperature of injector and detector was 275°C. The machine was led by a computer system type "HP ChemStation", managing the functioning of the machine and allowing to follow the evolution of chromatographic analyses. Diluted samples (1/50 in hexane) of 1.2 µl were injected manually.

2.3.2 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

GC/MS analyses were performed on a Hewlett-Packard equipped with a HP-5MS (Crosslinked 5% PHME Siloxane) capillary column (30 m x 0.25 mm i.d, 0.25 µm film thickness) and coupled with a mass spectrometer (HP 5973). The temperature was programmed 50 to 250°C at 2°C/min. The carrier gas was He (1.5 ml/min) and used split mode (Flow: 112 ml/min, ratio: 1/74.7). The different compounds were confirmed by reference to their MS identities (Library of NIST98 Spectra). MS operating parameters were: ionization voltages 70eV, ion source temperature 230°C, scan mass range 35-450 amu.

2.4 PREPARATION OF MICROORGANISMS

In order to evaluate the antimicrobial activity of essential oil, 10 bacteria were used, a gram positive and gram negative. The bacterial organisms were standard isolates from ATCC: American Type Culture Collection, CECT: Spanish Type Culture Collection, DCM: the German Collection of Microorganisms, IH: Institute of Hygiene, Rabat, Morocco and MBLA: Laboratory of Food Microbiology, UCL, Belgium. Table 1 shows the bacterial strains used and their origins.

Table 1. List of bacteria used in the study

Bacteria	Origin	Type of organism
Staphylococcus aureus	MBLA	G-positive
Staphylococcus aureus 25923	ATCC	G-positive
Bacillus subtilis 6633	DCM	G-positive
Listeria innucua 4030	CECT	G-positive
Listeria monocytogenes 4032	CECT	G-positive
Escherichia coli 4076	CECT	G-negative
*Escherichia coli K*12	MBLA	G-negative
Proteus vulgaris 484	CECT	G-negative
Proteus mirabilis	IH	G-negative
Pseudomonas aeruginosa	IH	G-negative

The bacterial strains used in the present study are preserved in a mixture of glycerol and Brain Heart broth (BHI, Biokar Diagnostics, Beauvais, France). For conservation for short-term, strains are maintained on an inclined agar medium at 4 ° C. Before use, the bacteria were revived by two subcultures in an appropriate culture medium: Luria-Bertoni (LB) broth (Biokar Diagnostics, Beauvais, France) at 37 ° C for 18 to 24 hours. For the test, final inoculums concentrations of 10^6 CFU/ml bacteria were used according to the National Committee for Clinical Laboratory Standards, USA (NCCLS 1999).

2.5 IN VITRO ANTIBACTERIAL ACTIVITY

2.5.1 INHIBITORY ZONE ASSAY

The antibacterial activity of the *L. multifida* L. was evaluated by agar-well diffusion method [17]. Fifteen milliliters of the Mueller-Hinton Agar (MHA Biokar Diagnostics, Beauvais, France) at 45 °C were poured into sterile Petri dishes. After solidification, sterile 8 mm diameter cylinders were deposited. The bacterial strains used in this work were prepared in Six ml of LB medium containing 0.8 % agar at a final concentration of 10^6 CFU/ml then was evenly spreaded onto the surface of the agar plates of MHA. After solidification, the wells were filled with 50 µl of pure essential oil. Plates were kept at 4 ºC for 2h and then incubated at 37 ºC for 24 h. Antibacterial activity was evaluated by measuring the diameter of circular inhibition zones around the well, in millimeters (including disc diameter of 8 mm). The absence of such a zone was interpreted as the absence of inhibitory activity. Tests were performed in triplicate.

2.5.2 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC)

Determination of minimal inhibitory concentration was evaluated only with bacteria that displayed inhibitory zones by a modified resazurin microtitre-plate (Costar; Corning Inc, Corning, NY, USA) assay as reported by Sarker et al [18] with a final volume in each microplate well of 100 µl. For susceptibility testing, 50 µl of LB broth supplemented with bacteriological agar (0.15% (w/v)) was distributed from the second (B) to the eighth (H) test wells.

The first well (line A) of the microplate were prepared by dispensing 100 µl of the essential oil and then 50 µl of serial two fold dilutions were pipette from the first (A) to the seventh well (G) of each microtitre line, ranging from 4 to 0.0625% (v/v). The eighth well (H) was considered as positive growth control because no essential oil was added, containing just LB agar (0.15% (w/v)). Then, 50 µl of a bacterial suspension were added to each well of a 96-well. MIC was defined as the lowest concentration that inhibited the visible bacterial growth. The experiments were repeated at least twice. The plates were incubated for 24 h at 37°C. After incubation time, 10 µl of 0.1% resazurin solution were added per well, coloring them blue as suggested by Mann and Markham [19]. Plates were incubated at 37°C for additional 2 h. than they were read for color change from blue to pink in live-bacterial strains containing wells. Oil that showed preliminary fast change of resazurin color on microtitre-plate assay, reveled that it does not have possessed antibacterial potential. So the bioactivity of the oil was screened by its inhibition of the dye reduction [20]. For determination of Minimum Bactericidal Concentration (MBC) both negative and positive controls were set at 37ºC for 24 h and bacterial colony number was counted. The least concentration of essential oil at which no visible growth in sub-culture was obtained in PCA medium plates was considered as MBC. The determinations of MBC values were done in triplicate.

2.6 KINETICS OF BACTERIAL GROWTH

Inoculums were prepared by inoculating medium Tryptone Soy Broth (TSB, Biokar Diagnostics, Beauvais, France) with an overnight culture of *S. aureus* MBLA and *P. mirabilis* IH incubating for three hours. 1 ml of aliquot of inoculums was added to 9 ml of medium of TSB containing 0.15% of agar. The LMEO was added to each tube to achieve final concentrations of essential oil of 2 MIC, MIC, MIC/2 and MIC/4. The bacterial culture used without essential oil was considered as negative control. The tubes were incubated at 37º C in an incubator/shaker. The optical density at a wavelength of 660 nm was measured every hour during the period of growth in order to monitor the bacterial growth. Experiments were performed in triplicate.

3 RESULT

This study favors the report that the essential oils, with high monoterpenes hydrocarbons, are very active against bacteria [21].

3.1 CHEMICAL COMPOSITION

The yield of *Lavandula multifida* L. essential oil obtained by hydro-distillation of dry material was 0.097% (w/w).The composition of essential oil and its relative percentages are given in Table 2. Thirty four compounds, representing 95.25% of the essential oil were identified, Carvacrol (47.62%), β-bisabolene (9.01%), and Dodecyl Acrylate (8.37%), Linalol (7.42%) are as the major constituents and this oil contains the minor constituents like Menthone (4.98%), β- Caryophyllene (3.34%), β-Pinene (3.21%) and Terpinolene (2.41%).

Table 2. Chemical composition of Lavandula multifida L. essential oil (GC and GC-MS analysis)

KI	Compounds	%	Identification techniques
	Monoterpene hydrocarbons		
931	α-Thujene	0.12	MS, KI
939	α-Pinene	1.21	MS, KI
943	Camphene	0.24	MS, KI
971	Sabinene	072	MS, KI
980	β- Pinene	3.21	MS, KI
986	β-Myrcene	0.32	MS, KI
1001	δ-2- Carene	0.04	MS, kI
1006	α-Phellandrene	0.09	MS, KI
1019	α-3-Carene	0.08	MS, KI
1023	p-Cymene	0.67	MS, KI
1031	Limonene	0.08	MS, KI
1032	β-Phellandrene	0.01	MS, KI
1042	(Z)-β-Ocimene	1.02	MS, KI
1050	(E)-β-Ocimene	0.87	MS, KI
1081	Terpinolene	2.41	MS, KI
	Oxygenated monoterpenes		
978	3-Octanol	0.18	MS, KI
1033	1,8-Cineole	0.41	MS, KI
1098	Linalol	7.42	MS, KI
1102	α-Thujone	0.16	MS, KI
1116	Trans-Pinene hydrate	0.21	MS, KI
1143	Camphre	1.31	MS, KI
1154	Menthone	4.98	MS, KI
1193	α-Terpineol	0.02	MS, KI
1230	Piperitone oxide	0.03	MS, KI
1298	Carvacrol	47.62	MS, KI
1358	Eugenol	0.17	MS, KI
	Sesquiterpene hydrocarbons		
1418	β- Caryophyllene	3.34	MS, KI
1460	Allo-aromadendrene	0.03	MS, KI
1509	β-Bisabolene	9.01	MS, KI
	Oxygenated sesquiterpenes		
1556	Caryophyllene alcohol	0.41	MS, KI
1581	β- Caryophyllene oxide	0.07	MS, KI
1649	β-Eudesmol	0.35	MS, KI
1617	5-epi-7-epi-α-Eudesmol	0.07	MS, KI
1675	Dodecyl Acrylate	**8.37**	MS, KI
	TOTAL	**95,25**	

KI: Identification based on kovàts indices

MS: identification based on comparison of mass spectrometry.

3.2 IN VITRO ANTIBACTERIAL ACTIVITY

The in vitro antibacterial activity of the *L. multifida* L. essential oil (LMEO) against the tested bacteria was qualitatively and quantitatively assessed by the presence or absence of inhibition zones. According to the results shown in Table 2, a significant inhibitory effect against bacteria was exhibited by the LMEO (*S. aureus* MBLA, *B. subtilis* DCM 6633, *L. innocua* CECT 4030, *P. mirabilis* CECT 484, *P. vulgaris* IH), with diameter of inhibition zones ranging from 10.660 ± 0,577 to 14.330 ± 0,577 mm. the largest inhibition zones were on *S. aureus* ATCC 25923, *S. aureus* MBLA, *B. subtilis* DCM 6633 and *P. mirabilis* CECT 484 with the diameter of 16.000 ± 2,828, 14.500 ± 0,707, 12.500± 0,707 and 12.500 ± 0.707 mm, respectively. We note

reduced zone sizes around *L. monocytogenes* CECT 4032, *E. coli* K_{12} MBLA and *E. coli* CECT 4076 with 8.500± 0,707; 9.500 ± 0,707 and 9.500± 0,707mm, respectively. On the other hand, this essential oil was found to be non effective against *Ps. aeruginosa* IH.

Table 3. Antibacterial activity of essential oil of L. multifida L. against the tested bacteria

Bacteria	Inhibition zone (mm)
Gram-positive	
B. subtilis DCM 6633	12.500± 0,707
S. Aureus MBLA	14.500 ± 0,707
S. Aureus ATCC 25923	16.000 ± 2,828
L. innocua CECT 4030	11.000 ± 1,414
L. monocytogenes CECT 4032	8.500± 0,707
Gram-negative	
E. coli K_{12} MBLA	9.500 ± 0,707
E. coli CECT 4076	9.500± 0,707
P. mirabilis IH	12.500 ± 0,707
P. vulgaris CECT 484	11.500± 0,707
Ps. aeruginosa IH	0

Each value is expressed as means ± SD (n = 3).
Diameter of inhibition zone including well diameter of 8 mm (P< 0.0001)

3.3 MIC AND MBC

The MIC of the essential oil was determined at concentrations ranging from 2% to >4%. The *S. aureus* MBLA and *B. subtilis* DCM 6633 were more sensitive than other tested bacteria and had a MIC values between 1 and 2% (v/v) while MBC values were between 2 and >4% respectively. Although *L. innocua* CECT4030*, L. monocytogenes* CECT4032 and *E. coli* K_{12} MBLA had a MIC= MBC >4% (v/v), those were the most resistant bacteria. All the results are given in Table 4.

Table 4. Antibacterial activity of essential oil of L. multifida L. in liquid medium: micro-dilution method

Bacteria	Strain	Essential oil (v/v)	
		MIC	MBC
B. subtilis	DCM 6633	2%	>4%
S. Aureus	MBLA	1%	2%
S. Aureus	ATCC 25923	1%	>4%
L. innocua	CECT 4030	>4%	>4%
L. monocytogenes	CECT 4032	>4%	>4%
E. coli K12	MBLA	>4%	>4%
E. coli	CECT 4076	4%	>4%
P. mirabilis	IH	4%	>4%
P. vulgaris	CECT 484	4%	>4%

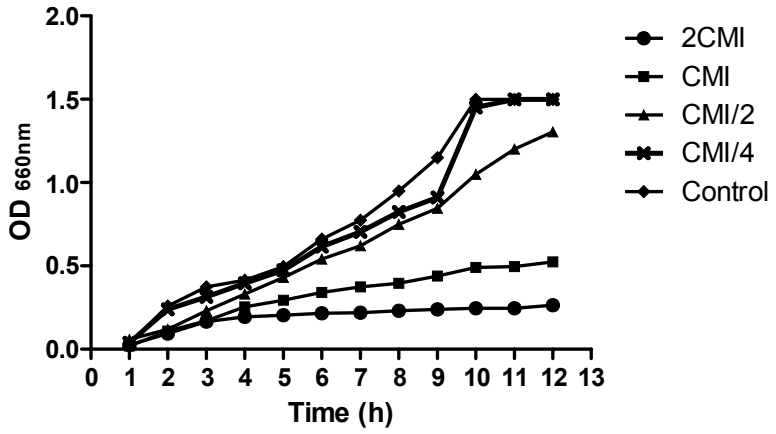
MIC : Minimum inhibitory concentrations.
MBC : Minimum bactericidal concentrations.

The essential oil of *L. multifida* L. was found to be equally effective against *E. coli* K12 MBLA, *E. coli* CECT 4076, *P. mirabilis* IH and *P. vulgaris* CECT 484 showed the MIC value was 4% (v/v) and MBC >4% (v/v).

3.4 KINETICS OF BACTERIAL GROWTH

This essential oil showed a weak antibacterial activity. This study was conducted to evaluate its effect on the kinetics of bacterial growth. Growth curves of bacteria in the presence of different concentrations of this oil are shown in fig 3

a)



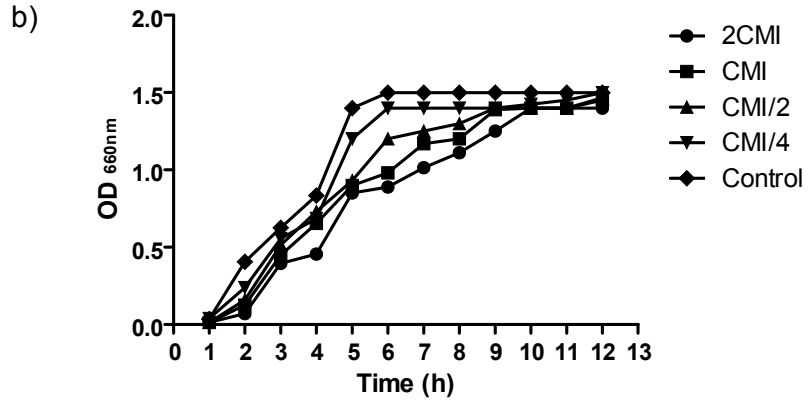


Fig 3: Kinetics of bacterial growth of S. aureus (a) and P. mirabilis (b) of different concentrations of essential oil of Lavandula multifida L. (●) 2 CMI, (■) CMI, (▲) CMI/2, (X) CMI/4, (♦) Control without EO. Experiments were performed in duplicate.

S. aureus MBLA the most sensitive of all the tested strains, there was a reduction of maximum growth rate and final optical density (OD) to concentrations of essential oil more or equal to MIC (1% (v/v)). This effect was even more evident as oil concentration increased (2 MIC = 2% (v/v)). Therefore the MIC found for this strain was confirmed. For concentrations lower than MIC (MIC/2= 0.5%, MIC/4= 0.25% (v/v)), the bacterial growth was not completely inhibited but the maximum growth rate and final OD was reduced remarkably. Nevertheless, for *P. mirabilis* IH the growth kinetic was not affected by the oil concentrations tested. Indeed, the MIC corresponding to this strain was 4% (v/v). For this bacterium, it was not able to test doses above 2 MIC because the absorption of oil interfered with the bacterial growth monitored by OD measurement.

4 DISCUSSION

To explore the biological activity of some medicinal plant, extraction of bioactive compounds had been used. Thus it also facilitates pharmacology studies leading to synthesis of a more effective drug with reduced toxicity [22]. Some traditional herbal remedies had an important role in the discovery of the physiological activities of many compounds such as those with anti-inflammatory effect or those effective against antibiotic-resistant strains of bacteria [23-24].

Phytochemical analysis of this oil isolate 15 monoterpene hydrocarbons (α-thujene, α-pinene, camphene, β- pinene, β-myrcene, α-phellandrene, β-phellandrene, limonene, terpinolene, …), 11 oxygenated monoterpenes (1,8-cineole, carvacrol, linalol, 3- octanol, α- terpineol, menthone, …), 3 sesquiterpene hydrocarbons (β- caryophyllene, allo-aromadendrene, β-bisabolene) and 5 oxygenated sesquiterpenes (Caryophyllene oxide, Caryophyllene alcohol, β- Eudesmol, 5- epi-7- epi-α-Eudesmol, Dodecyl acrylate). The strong antimicrobial activity of the EOLM against the tested bacteria could be attributed to the presence of high percentage of monoterpene hydrocarbons (11.09%), oxygenated monoterpene (62.51%), Sesquiterpene hydrocarbons (12.38%) and oxygenated sesquiterpenes (9.27%), as reported by Sivropoulou et *al.* [25] and Yangui et *al.* [26]. It was mentioned by Carson et *al*. [27] that α-terpineol and 1.8-cineole cause on *S. aureus* the leakage of absorbing material and make cells susceptible to sodium chloride. Also that 1,8-cineole, it was considered to have antimicrobial activity by permeabilization of bacterial membranes and facilitate the entry of others active components [27].

Indeed, carvacrol as a major component of LMEO was proved to induce membrane damage by dissipate the potassium gradient in *S. aureus* and *E. coli* [28] which cause ultrastructural alterations and the loss of cell viability [29], proposed other mechanism of action for carvacrol based on its diffusion back and forth through the bacterial membrane, while exchanging the acidic proton for another cation on the cytosolic side of the membrane and the opposite cation exchange at the exterior side [29]. So this mechanism could play a role in the antimicrobial action of carvacrol [30]. Furthermore, many minor constituents of the EO have a significant antibacterial activity as it was reported previously by Dorman and Deans [31]. Therefore, the diversity of major and minor constituents in the essential oil and its synergistic or antagonistic effects should be taken into account for their biological activity [32].

In our investigation, we found that the activity of the essential oil depends on the strain of tested bacteria. The Gram positive bacteria were more susceptible to the antimicrobial properties of essential oil than Gram negative ones. This difference observed could be attributed in part to the structure or composition of membrane. Gram-positive bacteria have a

thick layer of peptidoglycan closely attached to the cell membrane compared to the complex double membrane of Gram-negative, it have a thinner cell wall, including an outer membrane and a periplasmic space which contains enzymes that protect the bacteria; make it less susceptible to antibiotics [33]. In this way, many researchers reported the relationship between the essential oil's chemical composition and the antibacterial activity [32]. According to several authors, Gram-negative bacteria appear to be the least sensitive to the action of many others essential oils [7-35-36]. *Ps. aeruginosa* is known to possess high intrinsic resistance and an exceptional capacity to develop even more resistance to conventional antibiotics. This resistance appears to be related to the nature of its membrane which is composed of lipopolysaccharide that forms an effective barrier against hydrophobic compounds [37] those are confirmed by our results.

In general, it was explained in many studies that the antimicrobial activities is through C10 and C15 terpenes with aromatic rings and phenolic hydroxyl groups whom with active sites of the target enzymes, are capable to form hydrogen bonds [38]. Thus, enantiomers of α-pinene, β-pinene have a considerable antibacterial activity. It has been demonstrated that they are able to destroy cellular integrity, and by this way, inhibit ion transport processes and cell respiration [39-40]. Finely synergistic effects of the major and minor of essential oils constituents should be taken into consideration to explain their biological activity [41].

Our results clearly indicate that the susceptibility of essential oil to growth inhibition of the tested bacterial stain do not necessarily depend on the size of inhibition zones. The largest inhibition zone from Gram-positive bacteria was of *S. aureus* ATCC (16.000 ± 2.828 mm). For Gram-negative bacteria the largest one was *P. mirabilis* with 12.500 ± 0.707 mm, however their sensitivity to essential oil was different, proven by the curve kinetics of bacterial growth which join our explanation mentioned before about the difference between Gram-positive and Gram-negative bacteria stains.

5 CONCLUSION

The results of the present study indicate a considerable natural antibacterial property of the *Lavandula multifida* L. essential oil collected from the Northwest of Morocco against ten bacterial stains. In summary, this essential oil may be used for pharmaceutical and natural therapies.

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