Chemical Composition And Antibacterial Activity of Essential Oils of Two Aromatic Plants : *Mentha Spicata* and *Lippia Citriodora* Irrigated by Urban Wastewater

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Abstract

The objective of this study is the valorization of essential oils (EO) of two aromatic and medicinal plants: Mentha spicata and Lippia citriodora irrigated by urban wastewater from the city of Settat-Morocco. valorization consisite to test their antibacterial activity. Their chemical composition was studied by chromatography-mass spectrometry gas (GC/MS). These EOs were tested against three bacterial strains: Escherichia coli ATCC 25922, Streptococcus aureus ATCC 29213 and Pseudomonas aeruginosa ATCC 27853 ATCC. Our study showed that these essential oils from Menthe Spicata have significant antibacterial activity against the three species of bacteria studied (Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, and Pseudomonas aeruginosa ATCC 27853). Also, it is found that fresh leaves of Mentha spicata was the most active of all tested bacteria and the activity of this plant remains effective even when irrigated with wastewater, this is due to the presence of carvone (57,11%) and limonene (27,77%). On the other side, essential oils from Lippia citriodora, have not inhibitory activity against Pseudomonas aeruginosa, and fresh leaves of Lippia Citriodora have a more inhibitory activity when, it's irrigated with wastewater.Activity of fresh leaves of Lippia Citriodora irrigated by wastewater is due to the presence of neral (14,34%), geranial (14,75%) and limonene (25,86%).Generally, the irrigation by urban wastewater did not affect the MBC/MIC ratio, since this ratio values are the same for all of the essential oils value, indicating a bactericidal effect of the 5 oils tested under different conditions.

Key words : Mentha spicata, Lippia citriodora, essential oil, antibacterial activity, urbain wastewater.

1. Introduction

Medicinal plants have been traditionally used for pharmaceutical and dietary therapy in long history **[1]**. and pharmaceutical industries **[2]**. Essential oils are generally used in the cosmetic, medical and food industries. Antibiotic resistance has become a global concern **[3]**.Aromatic and medicinal plants had acquired particular attention in the field of intensive research on the natural antimicrobial com-pounds. They constitute a constant source of active reagents against pathogen germs **[4]**. Among these products, essential oils (EOs) produced by aromatic plants as secondary metabolites, have gained a net interest by many investigators **[5**, **6, 7 and 8]**
EOs are volatile natural complex compounds characterized by strong odour **[9]**, and represent very complex natural mixtures which may contain more than sixty individual components at quite different concentrations **[10]**. Major components can constitute up to 85% of the EOs, whereas other components are present only as trace **[6]**. It has been recognized that some EOs have antimicrobial, antifungal, anticancer and antioxidants properties [**11, 12, 13, 14, 15 and 16]**

Mentha spicata also known as spearmint belongs to the Lamiaceae (labiatae) family. The genus Mentha consists 25–30 species; and spearmint is the most common among them **[17]**. The plant is a glabrous perennial with creeping rhizomes **[18]**. Mint oil is of economic importance and is widely used in pharmaceutical, cosmetic, food, confectionary and beverage industries **[19]**. Spearmint oil contains monoterpenoids like carvone, limonene, dihydrocarveol and s-carvone. Some of them were found to possess high antioxidant activity than α-tocopherol **[20]**. The plant is also known for its ability to enhance memory **[21]**. Apart from being a stimulant and carminative, the mint plant is also known for its insecticidal, antimicrobial, antispasmodic and antiplatelet properties **[22, 23, 24 and 25]**.

The genus Lippia (Verbenaceae) includes approximately 200 species of herbs, shrubs and small trees **[26]**. Most of them are traditionally utilized as remedies for gastrointestinal and respiratory problems.Some species have shown antimalarial, antiviral and cytostatic properties. It is believed that their essential oils and phenolic compounds (flavonoids) are responsible for these properties **[27]**. It is cultivated mainly due to the lemon-like aroma emitted from its leaves that are utilized for the preparation of herbal tea, which is reputed to have antispasmodic, antipyretic, sedative and digestive properties **[28, 27, 29 and 30]**. Lemon verbena has a long history of folk uses in treating asthma, spasms, cold, fever, flatulence, colic, diarrhea, indigestion, insomnia and anxiety **[28 and 29]**. The essential oil from its leaves has been shown to exhibit antimicrobial activity **[31, 32, 33, 34 and 35]**..

2. Materials and methods

a. Plant material

Plantation was in May 2009 at experimental plots located in the Faculty of Science and Technics of Settat-Morocco (FSTS). The irrigations were made by urban wastewater purified by lagoon from Settat city (Morocco) and water well located in FSTS considered as a witness. Harvests for fresh leaves were made by hand. Water used for irrigation were characterized physico-chemical analyzes are determined previous work **[36]**.

b. Essential oil extraction

The chemical composition of essential oils was also determined using fresh and dried leaves of Lippia Citriodora and *Mentha spicata* harvested.
Distillation apparatus consisted of a heating cap, a 1.5 L extraction flask, a cooling system and a receiver for hydro distillate. Thirty grams of plant leaves and 800 mL of water were used and the distillation was carried out for 3 h after the mixture reached boiling at 100 °C. The essential oil obtained was dried under anhydrous sodium sulfate and stored at 4 °C in the dark. Each oil, after extraction has been divided into two parts, one was used for chemical analysis, while the second was used to study the antibacterial tests.
The following table presents the five essential oils used.

Table1: The essential oils used in this study.

Essential oil	Aromatic plant	Type leaves	Irrigation water
Mentha (1)	*Mentha spicata*	fresh leaves	well water
Mentha (2)	*Mentha spicata*	fresh leaves	wastewater
Lippia (1)	*Lippia Citriodora*	fresh leaves	well water
Lippia (2)	*Lippia Citriodora*	fresh leaves	wastewater
Lippia (3)	*Lippia Citriodora*	dried leaves	Wastewater

c. Gas chromatography-mass spectrometry analysis (GC/MS)

The essential oil was characterized using a gas chromatograph Trace GC Ultra equipped with an autoinjector (Triplus) directly interfaced with a mass spectrophotometer with a flame ionization detector (Pdains Q). Capillary column was DB-5 (5% of diphenyl and 95% of dimethypolysiloxane), 30m in length, 0.25mm thickness. Separation conditions were : 50°C for 2min, 50–200°C at 5°C/min. Temperature of the injector was 220°C. The volume injected was 0.1 µL. The carrier gas was helium with a flow rate of 1.4 ml.min−1. The oil constituents were identified by comparison of their retention indices and their mass spectra with those of authentic samples. Quantitative analysis (in percent) was performed by peak area measurement.

d. Bacterial strains

The essential oils were tested against the following bacteria: *Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, and Pseudomonas aeruginosa ATCC 27853*. All strains were obtained from Prof. Angeles Manresa Microbiology laboratory, Faculty of pharmacy, University of Barcelona. Spain.

e. Disc diffusion method

The agar disc diffusion method was employed for the determination of antibacterial activities of the tested EO as described previously **[7]** with some modifications.

The test was performed in sterile Petri plates containing Muller Hinton Agar. Sterile paper disk (6 mm in diameter) were impregnated with 10 µL of EO and were placed on the Petri plates previously inoculated with a microbial suspension adjusted to 10^6 CFU/ mL. Standard antibiotics amoxicillin (25 µg) were used as positive control. The Petri plates were incubated at 37° C for 24h. The diameters of inhibition zones were measured in millimeters. Tests were carried out in triplicate.

f. Determination of MIC and MBC

MIC (Minimal Inhibition Concentration) was determined by the method of micro-broth dilution **[37]**. A serial of dilution of essential oil ranging from 10 µL/mL to 0.15 µL/mL were prepared in test tubes containing Muller Hinton medium with 0.15% Agar **[38]**. Each tube was inoculated with a bacterial suspension adjusted to 10^6 CFU/mL. The MIC was the lowest concentration for which no growth was detected after 24h at 37° C **[39]**. While for the determination of MBC (Minimum Bactericidal Concentration), 10 µL of the tubes showing no growth were subcultured on nutrient agar plates and the Petri plates were incubated for 24h at 37° C. The MBC was the lowest concentration of the EO at which no growth occurred on the plates **[40]**. Each assay was repeated thrice

3. RESULTS AND DISCUSSION

A. Chemical composition of the essential oils

a. Essential oils of Mentha spicata

The Table 2 shows the major compounds of the two essential oils of *Mentha spicata*

Table 2. Chemical composition (%) of two essential oils of *Mentha spicata*.

Compounds	Retention time	*Mentha* (1)	*Mentha* (2)
α-Pinene	8.59	0,76	0,69
Camphene	9.06	0,20	Trace
3-Carene	10.04	1,33	1,01
β-Phellandrene	10.67	0,81	0,60
Limonene	12.01	31,13	27,77
1,8-Cineole	14.59	0,25	0,28
Cis-Ocimene	15.88	0,29	Trace
Borneol	16.85	0,56	0,84
Sabinene hydrate	17.28	Trace	0,39
Isocyclocitral	17.94	0,46	1,71
Trans-Carveol	18.87	3,90	1,79
Pulegone	19.42	0,33	0,30
Carvone	19.74	53,69	57,11
Bornyl acetate	21.63	0,26	0,27
iso-Limonene	22.79	0,22	trace
β-Bourbonene	24.32	0,68	0,71
Caryophyllene	25.12	0,40	Absent
Aromandrene	25.40	0,67	0,50
Germacrene D	27.31	0,87	0,65
Calamenene	27.78	0,22	0,22

Mentha spicata irrigated by well water contained carvone (53,69%), and limonene (31,13%) as the major compounds. The oils from *Mentha spicata* irrigated by wastewater are also characterized by a high percentage of carvone (57,11%), andlimonene (27,77%).

The chemical composition of two EOs of the *Mentha spicata* is qualitatively similar, although there are some differences in the concentrations of the compounds.
The biosynthesis of limonene and carvone in *Mentha spicata* are shown in previous work **[36]**.

The spearmint essential oil was characterized by the dominant presence of carvone (53.69%) from fresh leaves of *Mentha spicata* irrigated by well water , in agreement with other authors, such as **[41]**, who identified carvone occurring at 68.4% in samples collected in Greece **[42]**, who found it at 76.65% in samples collected in India, and **[43]**, who found it at 48.4% in samples collected in Turkey. Also, in essential oil from *M. spicata* collected in Montenegro (Europe), **[44]** reported carvone as the major constituent (49.52%),

b. Essential oils of Lippia Citriodora

The Table 3 shows the major compounds of the three essential oils of Lippia Citriodora

Table 3. Chemical composition (%) of three essential oils of *Lippia Citriodora* V(1), V(2) and V(3).

Compounds	Retention time	*Lippia* (1)	*Lippia* (2)	*Lippia* (3)
α-Pinene	8.62	0,94	-	1,06
Sabinene	10.00	2,22	2,33	2,42
Limonene	11.99	23,39	25,86	28,32
Trans- Ocimene	12.75	2,40	2,67	-
Nerol	17.77	1,64	1,50	1,40
Neral	19.55	15,29	14,34	13,85
Geranial	20.57	15,63	14,75	14,06
β-Caryophyllene	25.41	3,52	3,98	3,35
α-Curcumene	27.37	9,37	10,81	11,36
Neryl acetate	27.78	4,84	6,64	2,38
Copaene	28.28	2,60	3,42	2,38
α-Cadinene	28.59	1,52	-	-
Spathulenol	30.14	6,28	5,43	8,29
Caryophyllene oxide	30.29	6,37	4,23	7,07
ϒ-Cadinene	31.93	3,86	3,75	3,86

The main volatile components of fresh leaves *lippia Citriodora* irrigated by well water are limonene (23,39%), neral (15,29%) and geranial (15,63%), for EOs from fresh leaves of *Lippia citriodora* irrigated by wastewater , we found limonene (25,86%), neral (14,34%) and geranial (14,75%) were the main components. And EOs from dried leaves of *Lippia citriodora* irrigated by wastewater, we found limonene (28,32%), neral (13,85%) and geranial (14,06%) were the main components.

Also the chemical composition of three EOs of the *Lippia ciriodora* is qualitatively similar, although there are some differences in the concentrations of the compounds. These differences are due to irrigation with urban wastewater.

The chemical composition of the essential oil from the leaves of *Lippia Citriodora* has also been studied and reviewed **[45, 28, 46, 47, 48, 49, 33, 27, 29, 35, 26, 50, 51 and 52]**. The genus Lippia shows a rich genetic diversity, enabling it to synthesize a variety of essential oil constituents in plants grown in different parts of the world **[46 and 29]**. However, the composition of the essential oil obtained from the same plant stock remains constant under the same environmental conditions **[46 and 29]**.

For two waters used for irrigation, citral (neral and geranial) and limonene were the dominating compounds in the essential oil of *Lippia Citriodora* leaves, these results are in agreement with previous reports **[28, 48, 49, 33, 29, 35 and 53]**.

The results obtained by GC-MS analysis shows that each plant species has a specific quantitative and qualitative composition. The reasons of this variability can be due to different geographical sources, the genotype and the climate; all of this variability influences the chemical composition and the relative concentration of each constituent **[54, 55 and 56]**.

Also, the drying of leaves influence on composition of the essential oil of Lippia (3). Drying *Lippia Citriodora* had led to similar results [**57**] who noted qualitative and quantitative changes occurring in the essential oil of basil during drying in air at 25 ° C. Indeed, after drying for three days, he was a quantitative reduction of the following volatile compounds: 1ol-Octene-3, trans-ocimene, geraniol, geranial, eugenol, neryl acetate, humulene and Y-muurolene and compounds which increased quantitatively are: α-pinene, β-pinene, α-and β-copaene elemen.

B. Antibacterial activity of the essential oils

The results of antibacterial activity of the five EOs against The three species of microorganisms are summarized in **Table 4** for disk diffusion test**,** and **table 5** for the results of **MIC and MBC.**

Table 4: Diameter of inhibition zones in (mm)

Microorganism species	**Inhibition zone diameter in (mm)**					
	Mentha (1)	*Mentha* (2)	*Lippia* (1)	*Lippia* (2)	*Lippia* (3)	Positive control
St. Aureus ATCC 29213	19 ± 1.73	15 ± 0	14.5 ± 0.7	18.66 ± 1.52	14 ± 0	9 ± 0
E. coli ATCC 25922	13.66 ± 1.1	12.33 ± 0.57	15.33 ± 0.57	22 ± 2.64	15 ± 0	19 ± 0
Ps. aeruginosa ATCC 27853	9.5 ± 0.70	9 ± 1.41	0 ± 0	0 ± 0	0 ± 0	0 ± 0

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Table 5: MIC and MBC values

EOs	*St. aureus* ATCC 29213			*E. coli* ATCC 25922			*Ps. Aeruginosa* ATCC 27853		
	MIC (µl/ml)	**MBC (µl/ml)**	**MBC/MIC**	**MIC (µl/ml)**	**MBC (µl/ml)**	**MBC/MIC**	**MIC (µl/ml)**	**MBC (µl/ml)**	**MBC/MIC**
Mentha (1)	1.25	1.25	1	1.25	2.5	2	>10	>10	
Mentha (2)	1.25	1.25	1	5	5	1	>10	>10	
Lippia (1)	0.625	0.625	1	5	5	1	–	–	
Lippia (2)	0.625	1.25	2	5	5	1	–	–	
Lippia (3)	1.25	1.25	1	5	5	1	–	–	

By analyzing Table 4, it is seen that the EO *Mentha spicata* inhibit the growth of three species with a maximum inhibition on *St. aureus* ATCC 29213, against by the EO of *Lippia citriodora*, have no effect on *Ps. Aeruginosa* ATCC 27853, and maximum inhibition for *E. coli* ATCC 25922. The resistance of *Pseudomonas aeruginosa* may be due to hydrophobic lipopolysaccharide in the outer membrane which provides protection against different agents **[58]**.
By a comparison of EO from fresh leaves of plants irrigated by well water (*Mentha* (1) and *Lippia* (1)) and those by wastewater (*Mentha* (2) and *Lippia* (2)), we see that for *Mentha spicata* the antibacterial activity decreases when the plant irrigated by wastewater. At the *Lippia citriodora*, we see the opposite effect, the activity is higher when the plant irrigated by wastewater, however when the EO is extracted from dried leaves, irrigated by wastewater (*Lippia* (3)), activity that is similar irrigated with well water.

In Table 5, there is shown the MIC and MBC of 5 EO, it was found that, in most cases, the MIC value was equivalent to the MBC value, indicating a bactericidal effect of the oils tested.
Generally, the irrigation by urban wastewater did not affect the MBC/MIC ratio, since this ratio values are the same for all of the essential oils.
The MBC / MIC ratio is used to classify antibiotics according to their characters as bactericides (close to 1) or bacteriostatic (greater than 4) **[59]**. Our results showed that EO tested has bactericidal activity against E. coli ATCC 25922 and st. aureus ATCC 29213.
The antibacterial activity of EO tested would be result of the presence of citral (neral and geranial), carvone and limonene, known for their antiseptic activity **[60]**. There is evidence in the literature that the essential oils of some plants of the Lamiaceae family have a moderate to good antibacterial activity **[43 and 44].**

4. Conclusion

In this work, we studied the chemical composition and antibacterial activity of two plants: *Mentha spicata* and *Lippia Citriodora* irrigated by well water or wastewater.

Our study showed that the essential oils from *Mentha spicata* inhibits the growth of 3 bacterial species with a maximum inhibition for *Staphylococcus aureus* ATCC 29213, while the EO of *Lippia citriodora*, has no inhibitory action on *Pseudomonas aeruginosa* ATCC 27853, and their maximum of inhibition is for *Escherichia coli* ATCC 25922.

It is found that fresh leaves of *Mentha spicata* was the most active of all tested bacteria and the activity of this plant remains effective even when irrigated with wastewater, this is due to the presence of carvone (57,11%) and limonene (27,77%). On the other side, fresh leaves of *Lippia Citriodora* have a more inhibitory activity when, it's irrigated with wastewater.

Generally, the irrigation by urban wastewater did not affect the MBC/MIC ratio, since this ratio values are the same for all of the essential oils value, indicating a bactericidal effect of the 5oils tested under different conditions.

This study allowed us to prove the efficacy of OE from these plants against these bacteria. This shows that even these Moroccan flora irrigated by wastewater can be an important subject of interesting plant species, including active principles can be used in several areas such as the food and pharmaceutical industries

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