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Study of Antibacterial Activity of Essential Oils of Three Aromatic and Medicinal Plants

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Abstract **- The three botanical species: Mentha spicata, Thymus vulgaris, and Citrus limonum are a medicinal plants widely used in Morocco. The aromatic fractions of these plants offer new perspectives in herbal medicine through the development of new pharmaceutical preparations for therapeutic purposes. The essential oils (EOs) are extracted from dried plants, in the open air and away from light. The choice of these medicinal plants was made following an investigation and a statistical study conducted in various regions of Morocco. The results of physico-chemical analysis of the EOs oils are consistent with those of the AFNOR [1] standards. The analysis of their chemical composition was determined by gas chromatography-mass spectrometry (GC/MS). The antibacterial activity of these EOs was tested in two types of bacterial germs and the results for the in vitro activity show that these two germs has shown high sensitivity to these three EOs.**

Key words: Thymus vulgaris, Citrus limonum, Mentha spicata, **essential oil,** ***Staphylococcus aureus, Escherichia coli,*** **antibacterial actvity**

1. INTRODUCTION

Historically, human has used its environment and especially medicinal plants to treat against diseases. It is estimated that two thirds of current drugs have a natural origin, obtained by semisynthesis or by modification of a natural product and only a third of marketed drugs have a purely synthetic origin.

Our country has a plant biodiversity, with a very large number of plants used as herbs, for the therapeutic purposes. Many different natural substances have been identified and many of them are used in traditional medicine for the prophylaxis and for the treatment of diseases. Recent studies have shown that EOs and their constituents have significant potential as an antimicrobial agent and it's used in a many industrial and medical fields **[2 and 13].**

EOs of plants has found their place in aromatherapy, pharmacy, perfumery, cosmetics and food preservation. Their use is related to their broad spectrum of recognized biological activities **[24, 9, 10, 23, 19 and 8].**

The objective of our work is to study the antibacterial activity of the EOs of three Moroccan plants: *M. spicata*, *T. vulgaris* and *C. limonum* of two bacterial strains:

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*) and that by the use of agar diffusion test.

2. MATERIALS AND METHODS

a. Plant material

Medicinal plants tested were collected in various regions of Morocco. Samples of *T. vulgaris* were harvested from the Tafilelt province, *C. limonum* from the Agadir province and *M. spicata* from Settat province (Guisser). The choice of these medicinal plants, was made following an investigation and statistical study conducted in various regions of Morocco. Samples of the aerial part were used for extraction of EOs.

b. Biological model

The choice of bacterial strains, *Staphylococcus aureus* and *Escherichia coli*, as a model for our study is based on the fact that these bacteria are most frequently involved in superinfection of contact eczema.
The bacterial strains studied were made from the microbiology laboratory of the Pasteur Institute of Casablanca.

c. Bacterial culture media

To achieve our bacteriological study, we used two types of culture media:

- The ordinary nutrient agar

In order to isolate bacterial strains studied (*S. aureus* and *E. coli*), we used an ordinary nutrient agar, which represents a basic non-selective media for the isolation of bacteria.
To prepare the ordinary nutrient agar, 2 g of yeast extract was suspended with 1 g of meat extract, 5 g of peptone, 5 g of sodium chloride and 15 g of bacteriological agar, all these compounds are supplemented by distilled water up to 1 liter. The mixture was slowly heated, stirring and maintained for the necessary time to its dissolution. After adjusting of the pH to 7.4 ± 0.2, the medium was autoclaved at 120 °C for 20 minutes. Under laminar flow, the agar medium is poured into the plastic Petri dishes and was then sealed with parafilm **[22]**.

➢ The Mueller-Hinton agar

The Mueller-Hinton agar is recognized by all experts as the reference medium for the study of the sensitivity of bacteria to antibiotics.

To prepare the Mueller-Hinton medium: 17.5 g of casein acid hydrolyzate was suspended with 2 g of meat infusion, 1.5 g of soluble starch and 17 g of bacteriological agar, all these compounds are supplemented by distilled water up to 1 liter. The mixture was slowly heated, stirring and maintained for the necessary time to its dissolution. After adjusting of the pH to 7.3 ± 0.2, the medium was autoclaved at 120 °C for 20 minutes. Under laminar flow, the agar medium was poured into the plastic Petri dishes and was then sealed with parafilm **[21 and 6]**.

d. Extraction of essential oils

In our study, the aromatic species were treated after harvest. The extraction of EOs was performed by steam distillation using a Clevenger-type apparatus. The percent yields of EOs were determined in relative to the dry matter, estimated from dried samples for three days at room temperature. EOs was stored in a refrigerator prior to analysis.

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

Analysis of the chemical composition of EOs was carried out at the Unit Technical Analysis for Scientific Research (UATRS) National Center for Scientific and Technical Research (CNRST) Rabat by GC/MS. The EOs 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, 0.25 mm 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 was performed by peak area measurement.

f. Disc diffusion method

To evaluate the antibacterial activity of the three EOs, we adopted the disc diffusion method, which is based on a technique used in medical bacteriology.

This technique uses paper disks impregnated with EOs, and then deposited on the surface of agar uniformly seeded with a suspension of the bacteria studied. After incubation the colonies grow on the surface of the agar leaving blank zones around disks called an inhibition zone. More the diameter of the inhibition zone is large, the strain is sensitive to the EOs tested, if it's smaller the bacteria is resistant. The diameter of the zones of inhibition is proportional to the bacterial activity of the EOs on the bacteria tested **[15]**.

➢ Seeding of the suspension

One ml of the bacterial culture suspension with a concentration of 10^6 CFU / ml, was prepared from a stock culture of 18 hours, and then spread on the surface of Muller-Hinton medium.

➢ Deposit of the discs impregnated with essential oils

Using sterile forceps, sterile cellulose discs (diameter: 6 mm) are removed and soaked with EOs putting on contacting the end of the discs, and then deposited in Petri dishes which are placed in incubator chamber at 37°C for 24h. The experiment was carried out in triplicate. The diameter of inhibition zones were measured in millimeters **[26]**.

3. RESULTS AND DISCUSSION

a. *The yields of the three essential oils studied*

The Table 1 shows the yields of the EOs of the three Moroccan medicinal plants studied: *C. limonum*, *M. spicata* and *T. vulgaris.*

Table 1. Mean of the yields of the essential oils of the three plants studied

Medicinal plants	**Yields (%)**
Citrus limonum	0.75 ± 0.07
Mentha spicata	0.72 ± 0.03
Thymus vulgaris	0.65 ± 0.14

b. Chemical composition of three essential oils

➢ *Mentha spicata*

After achievement of the analysis by (GC / MS) we identify the major chemical compounds of the EOs of *M. spicata* (Table 2).

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

Compound	**Percentage**
α-pinene	0.322
Sabinene	0.327
β-pinene	0.607
Myrcene	0.380
Limonene	**9.140**
1,8 cineole	3.800
Linalol	0.212
α-terpineol	1.986
Cis carveol	1.176
Carvone	**57.00**
Piperitenone	0.147
β-bourbonene	2.796
β-caryophyllene	2.969
Germacrene-D	**8,120**

Delta cadinene	0.290
Oxyde de caryophyllene	0.650

The major chemical compounds are the carvone (57%), the limonene (9,140%) and the germacrene-D (8,120%). Previous study focused on the variation of the chemical composition of the EOs of *Mentha spicata* has revealed the existence of major components: carvone (59.40%), limonene (6.129%) and germacrene-D (4.665%) **[16]**. The difference in chemical composition observed in the EOs is likely to be investigated in relation to abiotic factors such as the specific climate of the regions of origin of the samples, geographical factors such as altitude, soil type and the picking season.

- *Citrus limonum*

On performing the analysis of the chemical composition of the EOs of the botanical species *C. limonum* by GC/MS, we identify the major chemical compounds of the EOs from *C. limonum* (Table 3).

Table 3: Chemical composition (%) of essential oils of *C. limonum*

Compound	Percentage
Alpha pinene +	2.66
Alpha thujene	2.66
β -pinene	**13.80**
Sabinene	2.17
Myrcene	1.59
Limonene	**66**
β-phellandrene	0.29
γ- terpinene	**9,10**
Para cymene	0.76
Citronellal	0.06
Linalol	0.15

The major chemical compounds are the limonene (66%), the β-pinene (13.80%) and the γ-terpinene (9, 10%). The previous work has revealed the existence of majority compounds as limonene (54.6%), γ-terpinene (19.1%) and β-pinene (14.5%) **[3]** There are many factors that can explain the difference in the chemical composition of the EOs of *C. limonum* between the result of our study and it's of the anterior work such as such as climate regions of origin of the samples, the genetics of the plant, altitude and the soil.

- ***Thymus vulgaris***

To determine the major chemical compounds of the EOs of *T. vulgaris* we used as technique the GC / MS, the results are shown in (Table 4).

Table 4: Chemical composition (%) of essential oils of *T. vulgaris*

Compound	Percentage
Borneol	5,1
Terpinene-4-ol	1.4
α-Terpineol	0.5
Thymol	**42**
Carvacrol	2.4
α-Pinene	1.2
Camphene	1.2
Sabinene	0.6
Myrcene	0.4
p-Cymene	**23.7**
γ-Terpinene	**15.5**

The major chemical compounds are the thymol (42%), the p-cymene (23.7%) and the γ-Terpinene (15.5%). In 2010, Benazzedine S. **[4]** has revealed the following major compounds: the carvacrol (85%), the p-cymene (45%) and the thymol (35%). The difference in chemical composition observed in the EOs is likely to be investigated in relation to abiotic factors such as the specific climate regions of origin of the samples, geographical factors such as altitude, age and the type of soil.

c. Antibacterial activity of the essential oils

To evaluate the antibacterial activity of three EOs, we adopted the technique of diffusion on agar medium.

Table 5: Diameter of inhibition (mm) of three essential oils

Strains	Inhibition zone diameter in (mm)		
	1	***2***	***3***
St.Aureus	21.50 ±0.81	22.00 ±1.29	29.25 ± 0.81
E. coli	18.00 ±1.29	22.50 ±0.81	20.00 ± 0.95

(1): *Thymus vulgaris*, (2): *Mentha spicata*, (3): *Citrus limonum*

The sensitivity of the germs to the three EOs is determined by the diameter of the halo of inhibition by the disc diffusion method**.** The results of the antibacterial activity obtained using the technique mentioned above, show that the strain of *Staphylococcus aureus* showed different sensitivities to these EOs.
The EOs of *T. vulgaris* showed the lowest antibacterial activity compared with those of *M. spicata* and *C. limonum*, with an average diameter of inhibition 21.50 ±

0.81. As to the EOs of *M. spicata* showed high antibacterial activity, the diameter of the zone of inhibition is 22.00 ± 1.29 mm. It should be noted that the highest antibacterial activity was recorded with the EOs of *C. limonum* with an average value of the halo of inhibition 29.25 ± 0.81 mm.
The *Escherichia coli* strain gave a variable sensitivity to these three EOs. The EOs of *T. vulgaris* showed the lowest antibacterial activity compared with those of *M. spicata* and *C. limonum*, with an average diameter of inhibition 18.00 ± 1.29 mm. The EOs of *C. limonum* showed high antibacterial activity with the diameter of the inhibition zone is located at 20.00 ± 0.95 mm. It should be noted that the highest antibacterial activity was recorded in EO of *M. spicata* with an average value of the halo of inhibition 22.50 ± 0.81 mm.

This sensitivity more marked of Gram positive (*S. aureus*) against EOs was also already observed by various authors **[12, 14, 5 and 18]**. On comparing the data obtained from different studies, most publications report a generalization of the antibacterial activity of EOs against Gram (+) and Gram (-). According with **[17]**, the sensitivity of microorganism to EOs depends on the properties of the EOs and the microorganism itself. It is well known that the Gram (+) are more sensitive to the EOs that Gram (-). Several studies testing the inhibitory activity of EOs confirm this phenomenon **[25, 8 and 5]**. According to **[25, 5, and 7]** the resistance of Gram (-) against EOs is partly due to the complexity of the cell envelope of these microorganisms which contains a double membrane, unlike the single membrane structure of Gram (+).
Conversely, **[11]** have pointed out that the action of the volatile EOs has little influence on the inhibition of the growth of Gram (-) and Gram (+). However, comparing the efficiency of the EOs through the various publications is difficult to achieve due to various external parameters uncontrollable as the chemical composition of EOs varies depending on environmental conditions of the plant.
So antibacterial activity of EOs can change by the chemical composition, the genotype, and the methods used to evaluate the antibacterial activity. The results obtained by different methods may be different; depending on the choice and physiological conditions of the microorganisms, the period of exposure of the microorganism to the EOs, the doses used of the EOs, the choice of the emulsifier to solubilize the EOs.
Theses all factors that may explain conflicting results from different studies.

4. CONCLUSION

In this work, we studied the chemical composition and antibacterial activity of EOs of three plants: *M. spicata*, *T. vulgaris* and *C. limonum*.
Measuring the average of the diameters of the zones of inhibition obtained by the disc diffusion method on agar show significant results:

- *T. vulgaris*: 18.00 ± 1.29 mm (*E. coli*) and 21.50 ± 0.81 (*S. aureus*)
- *M. spicata*: 22.50 ± 0.81 (*E. coli*) and 22.00 ± 1.29 (*S. aureus*)
- *C. limonum*: 20.00 ± 0.95 (*E. coli*) and 29.25 ± 0.81 (*S. aureus*)

Based on the above results, we can conclude that the three EOs tested have a remarkable antibacterial activity vis-à-vis the two pathogenic bacteria *S. aureus* and *E. coli*. New perspectives can be considered a further antibacterial activity not only for EOs used alone, but also in a mixture, allowing a possible synergy.
It would be interesting to continue this work on other bacteria to confirm the efficiency or not of EOs. The use of the antibacterial efficiency of these EOs may be envisaged in the domain of pharmaceutical industry.

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