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CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF THE ESSENTIAL OIL FROM *THYMUS VULGARIS* CULTIVATED IN MOROCCO (TAOUNATE) AGAINST TEN BACTERIA

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ABSTRACT

In order to valorize the medicinal and aromatic plants of Morocco, this study focuses on the chemical characterization and study of the antibacterial activity of the essential oil of *Thymus vulgaris*. In view to search for new bioactive natural products, this investigation was carried out against pathogenic bacteria which are frequent and cause problems in the medical field and food. Analysis by gas chromatography and Retention Index of the essential oil of *T. vulgaris* obtained by hydrodistillation of the aerial parts gave as major compounds, Thymol (40.0%), γ-Terpinene (12.0%), P-Cymene (12.0%) followed by Linalool (4.4%) and Carvacrol (3.1%).Thereafter minimal inhibitory concentrations of the essential oil added to Luria-Bertani agar medium in defining concentrations were determined by a screening test with the agar dilution method. The results indicated that MICs were ranged from 0.2% to 0.03% (v/v) for all trains, except *Pseudomonas aeruginosa*, which was least susceptible and inhibited by 0.4% (v/v).

KEYWORDS: *Thymus vulgaris*, essential oil; antibacterial activity, chemical composition, Thymol.

INTRODUCTION

Development of resistance for many bacteria to antibiotics and detergents used in various procedures, including, sterilization, disinfection equipment in food, medical and food preservation,[1] and nosocomial infections related to implants has become a major public health problem and is a global concern. [2] Moreover the situation is particularly concerning in hospitals and food due to the colonization of the implant surface and infections of prosthetic devices or central line-by bacteria contaminants *Staphylococcus aureus* and *Escherichia coli*.[3] As well as the growth and toxigenic power of several bacteria and fungi that causes food borne illness.[4, 5]

Therefore, nowadays increased interest has focused on naturally occurring molecules, in particular essential oils and the aromatic extracts containing phenolic compounds because of their wide acceptance by many consumers and their different functions, such as flavoring properties and radical-scavenging activity.[6, 7, 8] Moreover, the search for new products against pathogenic microorganisms with the active biomolecules naturally present in medicinal and aromatic plants can register as an ecological solution at lower cost.[9]

In recent years, the essential oils of many plants have become popular and their bioactive principles have recently used in several industry sectors.[10-13] However, despite their wide application spectrum insecticidal agents, antioxidant and antifungal agents, their use as antibacterial agents against microorganisms causing problems at the surfaces used in food and medicine is rarely studied.

Thymus (*Thymus vulgaris L.*), is an aromatic and medicinal plant which belong to the *Labiateae* family and which had increasing importance in horticulture. It is widely used in folk medicine for the treatment of a variety of diseases, including gastroenteric and broncho-pulmonary disorders and antispasmodic.[14]

Until now, the studies on the antifungal activity, antiaflatoxigenuc activity and antimycotoxigenic fungi of essential oil components have been reported by numerous investigators. [15, 16, 17] Also, many studies have focused an antimycotic, antioxidant activity and food preservative properties. [4, 18] In this present paper, the main goal, which is carried out for the first time for this plant grown in the garden of the National Institute of Medicinal and Aromatic Plants of Morocco Taounate, was to determine the component of *T. vulgaris L* essential oil and to evaluate in vitro its antibacterial properties against ten strains.

MATERIALS AND METHODS

MATERIAL

Plant Material

The aerial part (leaves and stems) of *Thymus vulgaris L. (Labiateae*) was freshly harvested and collected in March of 2011, in the National Institute of the Medicinal and Aromatic Plants. The plant was identified and deposited in the herbarium of the (NIMAP) Taounate-Morocco.

Bacterial strains

The bacterial species and strains used in this work were: *Pseudomonas aeruginosa* A22, *Staphylococcus aureus* ATCC 25922, *Staphylococcus aureus* CIP54354, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* AL52, *Escherichia coli* O128B12, *Escherichia coli* CIP5412, *Escherichia coli* HB101, *Bacillus subtilis*ILP142B and *Bacillus subtilis* ILP1428B. Strains were taken from frozen (-80 ° C) stock, rejuvenated on Luria-Bertani-Agar medium (LB) and sub-cultured as need.

METHODS

Preparation of plant oil

Extraction of essential oil was performed by a hydro-distillation method using Clevenger-type apparatus. [19] Two distillations of 200 g of fresh plant material were carried out in boiling for 120 minutes with 1 liter of water in a 21-ball surmounted by a column of 60 cm length connected to a condenser. The essential oil yield was determined relative to the dry matter estimated from two samples of 40 g dried for 24 hours in drying oven at 100 °C. The essential oils obtained were kept in dark at 4°C until further process.

Essential oil analysis

Chromatography analyzes were performed with a Thermo gas chromatograph equipped with a capillary apolar column type RTX-440 (30m × 0.32 mm × 0.25 microns in diameter, film thickness). The detection is provided by a flame ionization detector (FID) supplied with a gas mixture H2/air. The carrier gas used is nitrogen with a flow rate of 1 ml/min. The unit is equipped with an injector PVT (Programmed Temperature Vaporization) of split-split less. The injection mode was split (leakage: 1/50, flow rate: 1mL/min).

At the beginning of the injection, the temperature is at 50°C., the leak is closed. The temperature remains constant until removing the solvent accompanying the injected sample

and then rose to 250°C at a 4°C/min rate. The identification of the volatile compound was based on data obtained by chromatographic analyzes and using a retention index of Adams database.

Minimum inhibitory concentration of essential oil

The minimum inhibitory concentrations (MICs) of essential oil vis-a-vis the bacteria were determined according to the method reported by Remmal[20] and Satrani.[21] Due to the immiscibility of essential oils to the water and thus the culture medium, the emulsification was carried out with a solution agar in 0, 2%. It provides, in the middle of the homogeneous distribution of essential oil and to maximize the contact germ / compound. The dilutions were prepared at 10 %, 4%, 2 %, 1 %, 0, 5 %, 0, 33 % and 0, 2 % (v/v). Subsequently, in each test tube containing 13.5 ml of LB agar autoclaved (20 min at 121 °C) and cooled to 45 ° C, 1.5 ml of each dilution were added in order to obtain a final concentration of 1 %, 0,4 %, 0,2 %, 0,1 %, 0,05 %, 0,033 % and 0,02 % (v/ v). Then the tubes were shaken properly before pouring into Petri dishes.

Controls, containing the culture medium and the agar solution at 0.2% alone, are also prepared.

The inoculation of each isolate per plate is done by streaking using a calibrated platinum loop. The inoculums suspensions were adjusted to standard McFarland scale 0.5. The incubation temperature is 37°C. for 24 hours. Each test was repeated three times to minimize experimental error.

RESULTS AND DISCUSSION

Oil composition

The CPG analysis (Table 1) shows that 27 compounds are identified in oil essential of the *T. vulgaris,* representing 88.6% of the oil. The major constituents are Thymol (40.0%), γ-Terpinene (12.0%), P-Cymene (12.0%), Linalool (4.4%), Carvacrol (3.1%), beside other compounds with relatively low levels, including Thymol methyl ether (2.1%), Myrcene (2.1%), α-Thujene (2.1%), α-Terpinene (2.0%), α-Pinène (1.7%). This chemical composition of *T. vulgaris* of the National Institute of the Medicinal and Aromatic Plants of Taounate Morocco is broadly similar to that of *T. vulgaris* of Québec mainly composed by Thymol (18.1%), Carvacrol (8.9%), P-Cymene(20.8%), γ-Terpinene **(**0.2%), Linalool (13.3%), and significantly analogue to that of Spain that gave high levels of thymol (57.7%), P.Cymene

(18.7%) and Carvacrol (2.8%), [22] against only 40.0%, 12.0% and 3.1% respectively. The original essence of Tunisia dominated by carvacrol (62-83%) followed by p-cymene (5-17%), theγ-terpinene (2-14%) and theβ-caryophyllene (1-4%), depending on location and growing season [23] Other samples of the *T. vulgaris* from different countries such as Iran, Italy and Spain had given a similar profile as our *T. vulgaris*. However, quantitative variations in percentages of the components were noticed, [22, 24, 25] which may be at the origin of several factors such as species, origin, phenological stage, environmental influences and genetic heritage. [26, 27, 28]

Table 1: Chemical composition of the essential oil of *Thymus vulgaris*

RI	Compounds*	Percentage (%) **
931	α-Thujene	2.1
939	α-Pinene	1.7
980	β-Pinene	0.7
988	Octan-l-en-3-ol	0.1
991	Myrcene	2.1
1005	α-Phellandrene	0.5
1018	α-Terpinene	2.0
1026	P-Cymene	**12.0**
1031	Limonene	1.0
1033	1,8-cineole	0.8
1062	□-Terpinene	**12.0**
1088	Terpinolene	0.8
1098	Linalool	4.4
1177	Terpinene-4-ol	0.1
1235	Thymol methylether	2.1
1290	Thymol	40.0
1298	Carvacrol	3.1
1352	Terpinylacetate	0.4
1391	β-elemene	0.1
1401	MethylEugenol	0.4
1418	β-Caryophyllene	0.8
1430	β-copaene	0.1
1454	α-Humlene	0.3
1480	Germacrene D	0.3
1509	□-Bisabolene	0.3
1520	δ-cadinene	0.1
1581	Caryophyllene oxide	0.3
Total		88.6

RI: Retention index, *: constituents identified by GC-IK, **: Percentages of compounds provided by CPG

Antibacterial effect of essential oil from *T. vulgaris*

The antibacterial activity against various bacteria was evaluated by observing the inhibition of the growth of these stains tested in contact with the sample of the essential oil of *Thymus vulgaris* at different concentrations.

The results of the antibacterial activity of the essential oil are represented in Table 2. As can be seen in this table, the essential of *T. vulgaris* exercised an important inhibitory activity *vis-a-vis* all bacteria studied. Especially, *Staphylococcus aureus* ATCC 25922 and *Bacillus subtilis*ILP142B have shown a high sensitivity to the oil, they were inhibited from very low concentration of 0.033% (v/v). Also, the concentration of 0.05% (v/v) was sufficient to stop the growth of *S. aureus* CIP54354. *P. aeruginosa* ATCC 27853 and P. *aeruginosa* A22 was only inhibited by concentrations as high as 0.4% (v/v). It is also important to mention that Gram positive bacteria were generally found to be more sensitive than Gram negative (*Escherichia coli* O128B12; *Escherichia coli* CIP5412; *Escherichia coli* HB101 and *Escherichia coli* AL52); *P. aeruginosa* ATCC 27853 and *P. aeruginosa* A22 being least susceptible.

The essential oil of *T. vulgaris* proved very effective against the ten bacteria tested. This antibacterial activity is mainly attributed to its chemical composition which rich by monoterpenes hydrocarbon and oxygenated monoterpenes, and for its major compounds, including terpene alcohols (Thymol; Linalool and Carvacrol) and γ-Terpinène P-Cymen. Indeed, terpenols are known for their higher efficiency and broader spectrum of antimicrobial activity.[28, 29, 30] However, this great power antibacterial activity could also be attributed to the synergy between the various components of this oil.[32, 33] A correlation of the antimicrobial activity of the essential oil and its chemical composition suggests that this activity could be attributed to the presence of high concentrations of thymol and carvacrol. These compounds, which characterized by phenolic group, are indeed among the most efficient plant antibacterial agents known to date.[32, 34] Thus, the Thymol is able to alter the outer membrane and the Carvacrol can destabilize the cytoplasmic membrane and can act as a proton exchange, thereby reducing the pH gradient across the membrane. With conducted to cell death resulting from the collapse of the proton motive force and depletion of the ATP pool.[35] However, the major constituents are not necessarily responsible for the total antimicrobial activity; the contribution of less abundant components should also be considered.[35]

The more effective of the essential oil of *T. vulgaris* against Gram-positive bacteria than Gram-negative ones could be explained by the structure of the cell envelope. Gram-negative bacteria possess an additional membrane, termed outer membrane which composed primarily of lipopolysaccharid which provide protection against the effect of toxic agents and delineating the periplasmic space with the cytoplasmic membrane that restricts the diffusion of hydrophobic compounds.[36] Against, by the absence of the outer membrane in Gram-positive bacteria allowed the high permeability and external agents can disturb easily the cytoplasmic membrane.[32]

P. aeruginosa was less susceptible to the tested essential oil; this result is similar to that found with *T. maroccanus* and *T. broussoneti* essential oils from Essaouira region. [37] This high resistance could be due to its external membrane structure, particularly impermeable of biological molecules and the action of efflux mechanisms, which provide the protection to the bacteria against the action of essential oils.[38] Also, several studies have reported that *P. aeruginosa* is the least sensitive to essential oils.[32, 39]

This important antibacterial activity of essential oil of *T. vulgaris* against ten bacteria tested could be due to its strong proportion of Thymol 40.0%, which had a pronounced effect on the bacterial membrane[32, 35] and a broad specter antimicrobial.[14] In the limits of the present in vitro study, we conclude that *T. vulgaris* essential oil is an effective growth inhibitor and alternative agent against these bacteria which contribute to the formation of biofilm on the surface of wet food and medical.

Table 2: Antibacterial activity of *Thymus vulgaris* essential oil

Bacteria	Concentrations v/v							
	1% (v/v)	0.4% (v/v)	0.2% (v/v)	0.1% (v/v)	0.05% (v/v)	0.033% (v/v)	0.02% (v /v)	Control
Staphylococcus aureus CIP54354	_	_	_	_	_	+	+	+
Staphylococcus aureus ATCC 25922	_	_	_	_	_	_	+	+
Bacillus subtilis ILP142B	_	_	_	_	_	_	+	+
Bacillus subtilis ILP1428B	_	_	_	_	+	+	+	+
Escherichia coli AL52	_	_	_	_	+	+	+	+
Escherichia coli O128B12	_	_	_	+	+	+	+	+

Escherichia coli CIP5412	_	_	_	+	+	+	+	+
Escherichia coli HB101	_	_	_	+	+	+	+	+
Pseudomonas aeruginosa ATCC 27853	_	_	+	+	+	+	+	+
Pseudomonas aeruginosa A22	_	_	+	+	+	+	+	+

- : Inhibition ; + growth.

CONCLUSION

The development of resistance for many pathogenic bacteria to antibiotics, detergents and the nosocomial diseases related to implants has become a major public health concern. Thereby, the search and the development of new products and the formulations based on medicinal and aromatic plants were much needed.

Qualitative and quantitative analysis of essential oil of the *T. vulgaris* has allowed identifying 24 constituents representing 88.6% of the oil. The major constituents were Thymol (40.0%), γ-Terpinene (12.0%), P-Cymene (12.0%), Linalool (4.4%) and Carvacrol (3.1%).

The essential of *T. vulgaris* had showed an important inhibitory activity *vis-a-vis* all bacteria studied. This great power bioactive that observed in the essential oils mainly attributed to its high content of terpene phenols (Thymol, Carvacrol and Linalool). According to these results, we can assume that the essential oil of this kind of thyme deserves further study to exploit its antibacterial properties in medical and food and it can register as an ecological solution at lower cost.

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