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Chemical composition and antibacterial activity of the essential oil of *Thymus ciliatus* growing wild in North Eastern Algeria

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ABSTRACT

The essential oil extracted from the aerial parts of *Thymus ciliatus* harvested in the region of Tawra (North-East of Algeria) gave a yield of 2.5%. Its analysis by GC / MS has allowed the identification of twenty- four elements, mainly phenols and terpenes. The main components were: Thymol (67.78 %), p- cymene (12.25%), pseudo-limonene (5.10 %), and γ-terpinène (4.42 %). Antimicrobial activity was evaluated on two pathogenic foodborne bacterial strains: *Bacillus cereus* and *Listeria monocytogenes*. These pathogens showed high sensitivity with respect to the essential oil with respective inhibition diameters of 28.6and 40mm of relatively low MICs of 0.18mg / ml. Due to these results, we may propose the use of this natural substance as a substitute for additives used in food protection.

INTRODUCTION

Bacillus cereus, a gram-positive rod has a reputation as a foodborne pathogen responsible for poisoning causing diarrheal or emetic syndromes (Tossa *et al.,* 2009). It is an opportunistic associated with certain infection wich might endanger the life of immune compromised individuals, such endophthalmitis, wounds, bacteremia, septicemia, meningitis, pneumonia, endocarditis, pericarditis. It is a ubiquitous saprophyte of the environment, especially the soil, it can be met at all stages of the food production chain. The selection by the heat retaining endospores, is an important factor in the frequency of its isolation as a contaminant. The spore causes deterioration of food after its subsequent germination (Delmas *et al.,* 2010). In Algeria, *B. cereus* is the 4th cause of foodborne outbreaks (INVS, 2004). *Listeria monocytogenes*, saprophytic ubiquitous (soil, plants, water) is an intracellular opportunistic pathogen agent.

Its ability to multiply at 4 °C allows it to reach high concentrations in foods stored in the refrigerator (Viuda *et al.,* 2011). *Listeria* has gained increasing attention as a causative agent of listeriosis, with a mortality rate of 24% (Datta *et al.,* 2003). It is also responsible for, bacteremia, meningitis, encephalitis, meningoencephalitis, miscarriage, premature births, stillbirths' human births etc. Infection occurs mainly due to the occasional contamination of food products, especially meat (Bubonja *et al.,* 2011; Lauchlin *et al.,* 2004). Many efforts have been made to guard against contamination by such microorganisms by the use of various types of preservatives.

However, capacity of resistance acquisition of these bacteria, forced into a continual search for new antimicrobials. In addition to tall the side effects that can generate these chemical conservative substances of synthetic origin. The plant world, presents an inexhaustible and renewable source whose traditional and medical use has been known since a long time. Among the aromatic plant species, the genus of Thymus offers a wide variety of natural substances with antimicrobial effects. The aim of this work is to evaluate the effectiveness of essential oil of *Thymus ciliatus,* an abundant endemic plant in the North -east of Algeria on both bacteria *Bacillus cereus - Listeria monocytogenes.*

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This species of Thyme produces an essential oil that has a very broad spectrum of action since it inhibits the growth of bacteria. This species produces an essential oil rich in phenolic compounds: Thymol and carvacrol, the most active antimicrobial molecules described to date(Kempf *et al.,* 2011). The development of these herbs has affected several areas of the food industry (Ozkan *et al.*, 2010). Where they can be applied as an effective alternative to chemical preservatives controlling pathogens in food products such as *Bacillus cereus* and *Listeria monocytogenes*. These strains are implicated in the contamination of food products stored at room temperature and the deterioration of refrigerated foods; also they are responsible for food poisoning.

MATERIALS AND METHODS

Bacterial Strains

We chose two strains which are implicated, one in the contamination of food products stored at room temperature and the other in the deterioration of refrigerated foods. *Bacillus cereus* originally isolated from a meat dish, preserved and identified by API system (Bio- Mérieux). *Listeria monocytogenes* is part of ATCC, kindly made available to us by the team at the Pasteur Institute in Algiers; were used as the test object.

Essential oil

Plant material

The aerial parts (stems, leaves and flowers) of *Thymus ciliatus* of Tawra in the province of Souk Ahras (Northeastern Algeria) were harvested in full bloom period (May-June 2012). The samples thus collected were dried in the open air and in the shade.

Extraction of essential oil

The essential oil was obtained by steam distillation using a LINKENS- NICKERSON type apparatus. The distillation was carried out by boiling 100 g of dry matter in 1 liter of water in a 2 liter flask surmounted by a column of 60 cm length connected to a condenser (Bruneton, 1999). After an extraction of 2 hours, the oil is recovered in small opaque bottle and stored at 4 °C.

Chemical analysis of the essential oil

Chromatographic analysis of the extracted essential oil was performed on a gas chromatograph coupled to a type of mass spectrometer Shimadzu QP 2010. Column used is SE- 30 type 25 m in length, 0.25m in diameter internally, the film thickness is 0.25 microns. The injection mode is split. Helium was used as carrier gas at a constant pressure of 25.6KPa. The temperatures of the injector and transfer line were brought to 250 °C. The oven temperature was programmed according to the following conditions:

Initial column temperature was 60 °C, increasing the temperature of 3 °C / min to 120 °C, and is kept isothermal for 5 minutes then increased to 10 °C / min to 180 °C. The injected volume was 1µl.

The analysis was performed in mode electron impact (EI)ionization with ionization energy of 70 EV using in scan mode (45-450µ). After obtaining the chromatogram of the essential oil, identification of chromatograms was done by querying the database NIST (National Institute of Standards and Technology). The internal normalization method was used for determining the amount of each component.

Study of the Antibacterial Activity

Aromatogram

The antibacterial activity of the essential oil of *Thymus ciliates* on studied strains was determined by the solid medium diffusion method using sterile filter paper discs. The principle of this method is inspired of Antibiogramas recommended by the Clinical Laboratory Standards Institute (CLSI) and those of the Committee of French Society for Microbiology (CA-SFM ; EUCAST ; 2014).

This test is performed by depositing two sterile discs : one is impregnated with 20µl of the crude essential oil, the other is a witness disk devoid of any substance, on medium Muller-Hinton (MH) previously inoculated by a bacterial suspension concentration equivalent to 0.5 McFarland [10^6 – 10^8CFU / ml] , (CFU= Colony- Forming Unit).

These prepared dishes were incubated at 37 ° C for 24h. The antibacterial activity, when present, is estimated by measuring the diameters of zones of inhibition around the discs.

Determination of minimum inhibitory concentrations (MIC)

MICs of the oil extracted on the studied bacterial strains were determined by the medium agar incorporation method as recommended by CLSI (CA-SFM ; EUCAST ; 2014).

A range of dilutions of the essential oil in DMSO (dimethylsulfoxide) were prepared so as to obtain Final concentrations of 1%, 0.5 %, 0.4 % , 0.3% , 0.25 % , 0.2% , 0.15 %, 0.1% , 0.075% , 0.05% , 0.025 % and 0.012 % , essential oil per milliliter of culture medium (Djahoudi *et al.,* 2011; Amrouni *et al.*, 2014).

These dilutions are added to MH agar melted and cooled in a water bath at 45 °C. S pots of 2 µl standardized inoculum to 0.5 McFarland are deposited on agar plates using a multi-applicator; witness boxes containing only MH were used as negative control.

All dishes are incubated in an incubator at 37 °C for 24 h. The MIC is defined as the lowest concentration in the presence of which no bacterial growth is visible to the naked eye similar to the growth of the strain on the control box (CA-SFM ; EUCAST ; 2014).

RESULTS AND DISCUSSION

Yield of essential oil extracted

The average yield of essential oil of *Thymus ciliatus* was 2.5 %. It is more important than that obtained from *Thymus ciliatus* of Morocco, which is 1.2 % (Amarti *et al.*, 2010). Thus, *Thymus vulgaris* essential oil of North America, obtained by steam distillation, gave a lower yield of about 1.8 % (Yaouba *et al.*, 2011). While essential oil *Thymus piperella* of Island of Great Comoro revealed an average yield of 1.31 % (Satrani *et al.*, 2010).

According to the study (Faleiro *et al.*, 2003)on Portuguese Thyme:essential oil of *Thymus mastichina*, established a rather low yield of about 0.5%.

Indeed, these variations in performance can be attributed, not only to the portion of the extracted plant, but also to climatic and environmental factors, the intensity of the metabolism of plants, species, age, the period of gathering and the specific geographical location of this species.

Chemical composition of essential oil extracted

Chemical analysis of the volatile essential oil components *Thymus ciliatus* GC-MS has allowed the identification of twenty four elements representing 99.87 % of total oil (Table-1). It is composed mainly of phenolic monoterpenes represented by Thymol (67.78 %) and carvacrol (2.70 %); followed by hydrogenated monoterpenes , p- cymene (12.25 %) associated to other components at relatively low levels, such as the pseudo-limonene (5.10 %) , the γ-terpinene (4.42 %).

Our results are similar to those of Cherchar *et al.*, (2014)which showed that the essential oil of *T. ciliatus* species from Tlemcen (West Algeria) consists essentially of Thymol (60.52 %) followed by p-cymene (17.2%) and γ-terpinene (8.03%), while carvacrol is (0.2%).

Indeed, the same results were obtained for the essential oil of *T. vulgaris* collected in the city of Ifran located in the Middle Atlas of Morocco which is Thymol chemotype (41.39 %),γ - terpinene (22.25 %) (El ouali *et al* ., 2013).

Thus, essential oil of *T. serpyllum* of western Himalayas (Kumaon region), has the same major compounds but with different percentages dominated by Thymol (60.1 %), followed by γ-terpinene (13.8 %) and of p-cymene (10.4 %) (El ouali *et al.*, 2013).

By cons, another Tunisian variety of *T. capitata* (Zaghouan region) has a completely different composition from that of *T. ciliatus* with comparable percentages;carvacrol (88.98 %), p- cymene (1.14 %), Thymol (0.51 %) and γ - terpinene (0.40 %) (El Abed *et al.*, 2014).

Table 1: Chemical composition of essential oil of *Thymus ciliatus*.

Compound Name	***RT***	***Content %***
α-Thugène	6.38	0.23%
1R-α-pinène	6.56	0.93%
1-octène-3-ol	7.80	0.78%
α-pinène	8.12	0.45%
β- thugène	8.79	0.01%
α- terpinolène	8.99	1.00%
p-cymène	**9.30**	**12.25%**
Limonène	9.41	0.51%
γ-terpinène	10.51	4.42%
3-carène	10.87	0.28%
Pseudo-Limonène	12.14	5.10%
5-isopropyl-2-methylbicyclo[3.1]hexan-2-ol	15.33	0.23%
Thymol methyl ether	17.74	0.93%
Thymoquinone	18.45	0.60%
Thymol	**20.54**	**67.78%**
Carvacrol	20.82	2.70%
α-Cubebène	23.73	0.007%
β-bourbonène	24.11	0.02%
Caryophylène	25.55	0.92%
5-Muurolène	27.87	0.06%
Δ-Cadinène	29.74	0.13%
Epi-Bcyclosesquiphellandrène	29.37	0.03%
2,5-Dimethoxyethylbenzène	31.26	0.43%
Total:		**99.87%**

RT: retention time.

Antibacterial activity of essential oil

The diameters of the zones of inhibition around the discs loaded with essential oil are very important and so, class our strains, in the category of extremely sensitive microorganisms. Indeed inhibition diameters were 28.6 mm for *L. monocytogenes* and 40 mm for *B. cereus* and MICs of about 0.18mg / ml (Table-2).

Table 2: Diameter of inhibition and MIC EO of *Thymus ciliates.*

Bacteria tested	EO of *T. ciliatus* (S. Ahras)	
	Ø (mm)	MIC (mg/ml)
L. monocytogenes ATCC 19118	28.6	0.18
B. cereus	40	0.18

Other studies have shown that the essential oil of *Thymus capitata* harvested in Tunisia (El Abed *et al.*, 2014), has had a more importance activity on *L. monocytogenes* ATCC 19118and *B. cereus* with an inhibition diameter of 70 to 80mm and MIC least low of 2.5 and 1.25mg/ml for the two respective strains. For this, another essential oil of France *Thymus vulgaris* (Cristani *et al.*, 2007) exerted an inhibitory effect on stem with low MIC 0,45mg / ml.

These values show excellent sensitivity of Gram-positive strains, tested, towards this essential oil. This would be attributed to the particular structure of their less complex membrane consisting of a thin layer of peptidoglycan in which proteins are related. Allowing hydrophobic molecules easily penetrate and act on the cell wall and into the cytoplasm (Burt *et al.*, 2004; Cristani *et al.*, 2007). The antibacterial action of essential oil (EO) of *T. ciliatus* is also related to its high content of phenolic derivatives (Thymol and carvacrol). These may destabilize the cytoplasmic membrane and interfere with energy metabolism and cellular integrity leading to cell death (Ultee *et al.*, 2002; Nguefack *et al.*, 2004; Cristani *et al.*, 2007).

It should be noted that Thymol, main component of our EO, is known for its broad spectrum of antibacterial activity (Dorman *et al.*, 2000). Indeed, Thymol binds to membrane proteins and increased membrane permeability of the bacterial cell.

Other studies suggest that the volatile compound is responsible for the inactivation of enzymes, including those involved in the production of energy and synthesis of Structural components (Trombetta *et al.*, 2005).

On the other hand, hydrogenated monoterpenes such as γ-terpinene and p-cymene are both precursors in the biosynthesis of carvacrol. Present in sufficient quantity (12.25 %), p- cymene facilitates intracellular penetration of carvacrol and potentiating its action (Ultee *et al.*, 2002); it shows a high affinity for cell membranes and may disturb and affect them (Rassouli *et al.*, 2006). Logically, its activity is the first link in the destabilization of cellular integrity process. This antibacterial effect is mainly provided by carvacrol. Thus, it has ATPase inhibitory activity (Gill and Holley, 2006) inducing dissipation proton- motive force by inhibiting other enzymes in the periplasmic space after the treatment of the bacterial cytoplasmic membrane (Ultee *et al.*, 2002; Benarfa *et al.*,2006 ; Cristani *et al.*,2007 ; Xu *et al.*, 2008).

While other minority compounds of our EO such as terpene hydrocarbons, pseudo-limonene known for its low antibacterial activity (Vardar Vnlu *et al*., 2003; Nejad Ebrahimi *et al.*, 2008) and α-terpinolene, can exert synergistic interactions. Their target and their mode of action remain to be determined.

CONCLUSION

Listeria monocytogenes and *Bacillus cereus* are two pathogenic bacteria responsible for food poisoning can be fatal. Many efforts have been made to protect food from contamination by this type of microorganisms by the use of various types of preservatives. However, the ability of these bacteria to develop resistance forced a continual search for new antimicrobials.

In this perspective, we were interested in the EO of *Thymus ciliatus* harvested in the region of Tawra (northeastern Algeria), and the GC / MS unveiled its chemotype “Thymol”. This oil showed a very good antibacterial activity against pathogenic strains of *Listeria monocytogenes* and *Bacillus cereus*.

This result suggests the use of such molecules in food as a substitute for conventional chemical preservatives. Knowing that in terms of taste, Thyme is much appreciated and constitutes an ingredient in several recipes, this opens the prospect of its use for the prevention and fight against deterioration of food stored at room temperature or 4 °C.

In addition to its economic impact, this EO would contribute to the fight against food poisoning.

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