Research Article

Sensibility of Methicillin Resistant *Staphylococcus aureus* to Essential Oil of *Thymus ciliatus*

[1]Heni Sonia, [2]Abdesselam Amira, [3]Kermiche Fella, [4]Bennadja Salima, [5]Djahoudi Abdelghani*
[1]Department of Biochemistry, Faculty of Sciences, University Badji Mokhtar, Annaba, Algeria.
[2]Department of Agronomy, Faculty of Sciences, University Chadli Ben Djedid, El Tarf, Algeria.
[3]Laboratory of Cellular Toxicology, Department of Biology, Faculty of Science, University Badji Mokhtar, Annaba, Algeria.
[4]Laboratory of Biochemistry and Environmental Toxicology, Faculty of Medicine, University Badji Mokhtar, Annaba, Algeria.
[5]*Laboratory of Microbiology, Department of Pharmacy, Faculty of Medicine, University Badji Mokhtar, Annaba, Algeria.
***Corresponding author's E-mail:** adjahoudi@yahoo.fr

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ABSTRACT

The essential oil extracted from the aerial parts of *Thymus ciliatus,* harvested in the region of Tawra (North-East Algeria), gave an excellent yield of 2.5%. Its analysis by GC/MS allowed identification of twenty-four elements, mainly phenols and terpens. The main components are: thymol (67.78%), p-cymen (12.25%), pseudo-limonen (5.10%), and γ -terpinen (4.42%). The main aim of this study is to evaluate the antibacterial activity of this essential oil on a bacterial population consisted of forty-four strains of *Staphylococcus aureus* resistant to methicillin (MRSA), isolated from different food products. For that, we used the agar diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing [CASFM- EUCAST; 2014- V2]. The calculation of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by the medium agar incorporation method as recommended by [CASFM- EUCAST; 2014- V2]. MRSA strains tested, showed a high sensitivity in respect of the essential oil with inhibition diameters ranging from 20mm to 50mm and relatively low MICs values ranging from 180µg/ml and 720µg/ml, and CMBs values ranging from 180µg/ml and 2160µg /ml. The essential oil of *T. ciliatus* showed a bactericidal effect on all isolated MRSA with a very important report CMB / CMI = 1. The bactericidal power of this oil suggests its use in foods as a substitute for conventional chemical preservatives.

Keywords: Essential oil - *Thymus ciliatus* - Antibacterial activity - CMI – CMB - MRSA.

INTRODUCTION

Staphylococcus aureus is a Gram-positive cocci bacteria, ubiquitous, no sporulating[1,2]. Opportunistic pathogen, it is common commensal of the skin and mucous membranes of humans and animals. It is a principal agent of contamination and food spoilage[3], carried by dust, water and the contact (hands especially) with food[4].

During the recent years, the emergence of *S. aureus* resistant to methicillin (MRSA), enterotoxin producers in fresh and preserved food products has become a major cosmopolitan public health problem[5]. Every year, it is estimated that 241,000 of food-borne diseases are caused by MRSA[6]. They are resistant not only to β-lactams but also to many other antibiotics[7,8].

Both in the field of health and agribusiness, many efforts have been made. However, this bacterium shows great adaptability and develops resistance against synthesized chemical antibacterial products[9]. In addition, the preservatives used in the food industry are not free of side effects. This requires continual search for new and more effective antimicrobial and is a great difficulty since the bacterial targets have summers exhausted[10].

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 test the anti-staphylococcal activity of the essential oil extracted from thyme, *Thymus ciliatus*: endemic and abundant plant which characterizes the plant environment of North-east Algeria (Tawra, wilaya of Souk Ahras). Furthermore, it is highly integrated in the culinary habits of several regions. This species produces an essential oil rich in phenolic compounds: Thymol and carvacrol, the most active antimicrobial molecules described to date[11,12]. The development of these herbs has affected several areas of the food industry[13]. Where they can be applied as an effective alternative to chemical preservatives controlling pathogens in food products such as methicillin-resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

Strains of *S. aureus*

Origin

In total 44 strains of *S. aureus* resistant to methicillin (MRSA) isolated from food products have been adopted in this work.

Identification

The identification of strains considered is based on conventional methods [API Staph (Bio-Merieux)].

Antibiogram

Antibiotic resistance was determined by the medium agar diffusion method in Muller-Hinton based on the recommendations by the (CASFM; CLSI; EU-CAST., 2014)[14].

Screening - Test oxacillin for *S. aureus*

In 10 ml of sterile distilled water, we dissolved 6 mg of oxacillin and then we achieved a ten-fold dilution in sterile distilled water[14].

From dilution previously prepared, 2 ml are placed in a sterile Petri dish of 90 mm diameter, to which 18 ml of Mueller-Hinton agar was added supplemented with 4% NaCl, melted and cooled to 45 °C. The whole is gently homogenized by rotary motion shaped of "8".

After solidification, the Petri dish is divided into four areas: one is inoculated with the strain to be tested, both by reference strains: *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 43300 (MRSA). The fourth quadrant is unseeded.

Plates are then incubated at 33 ° C for 24 hours. The presence of more than one colony is enough to indicate a resistance to oxacillin, involving resistance to all beta-lactams[14].

Chemical composition of essential oil *T. ciliatus*

The essential oil of *T. ciliatus* was obtained by steam distillation using a LINKENS- NICKERSON type device, identified by gas chromatography coupled with mass spectrometry and described (chemical composition and classes) in a previous work (in press). [Tab.1 and tab.2]

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

Compound name	Content (%)	Compound name	Content (%)
α-thugen	0.23%	5-isopropyl-2 methylbicyclo [3.1]hexan-2-ol	0.23%
1R-α-pinen	0.93%	thymol methyl ether	0.93%
1-octene-3-ol	0.78%	thymoquinone	0.60%
α-pinen	0.45%	**thymol**	**67.78%**
α- phellandren	0.08%	carvacrol	2.70%
β- thugen	0.01%	α-cubeben	0.007%
α- terpinolen	1.00%	β-bourbonen	0.02%
***p* – cymen**	**12.25%**	caryophylen	0.92%
limonen	0.51%	5-Muurolen	0.06%
γ-terpinen	4.42%	Δ-cadinen	0.13%
3-caren	0.28%	epi-Bcyclosesquiphellandren	0.03%
pseudo-limonen	5.10%	2,5-dimethoxyethylbenzen	0.43%
Total		**99.87 %**	

Table 2: Biochemical classes identified in the essential oil of *T. ciliatus*

Biochemical classes	Percentage (%)
Monoterpene phenols	70.48
Monoterpene hydrocarbons	26.50
Sesquiterpene hydrocarbons	1.15
Monoterpene Ketones	0.60
Monoterpen alcohols	0.78
Ether	1.59
Total	**99.87 %**

Table 3: Diameters of inhibition zones, MIC and MBC of EO towards tested strains

Strain n°	Ø (mm)	MIC (µg/m)	MBC (µg/ml)	MBC/MIC	Strain n°	Ø (mm)	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC
1	22	540	540	1	**24**	47	720	720	1
2	27	540	540	1	**25**	44	720	720	1
3	28	600	600	1	**26**	42	720	600	0,83
4	25.9	540	720	1,33	**27**	32,5	600	600	1
5	24.6	540	720	1,33	**28**	28	180	720	4
6	20	600	2160	3,6	**29**	45	540	1140	2,66
7	27	540	540	1	**30**	32	540	540	1
8	40	360	360	1	**31**	33	540	600	1,11
9	49	360	540	1,5	**32**	47	540	600	1,11
10	29	540	540	1	**33**	34	720	720	1
11	39	540	540	1	**34**	31,7	720	720	1
12	26	600	600	1	**35**	40	600	600	1
13	43	720	600	0,83	**36**	37,5	600	600	1
14	33	540	540	1	**37**	36	360	180	0,5
15	33	720	720	1	**38**	41	600	600	1
16	34,1	600	600	1	**39**	40,8	360	180	0,5
17	38	540	540	1	**40**	33,2	600	600	1
18	36	720	720	1	**41**	36,5	600	600	1
19	45	600	600	1	**42**	36	540	540	1
20	41	720	720	1	**43**	50	540	540	1
21	29	600	600	1	**44**	38,5	600	600	1
22	35	540	540	1	***S.aureus* ATCC 29213**	21	1010	1010	1
23	38	720	720	1	***S.aureus* ATCC 43300**	45	180	180	1
Modal values						**33**	**540**	**600**	**1**

Aromatogram

The antibacterial activity of the essential oil of *T. ciliatus* on the strains studied was determined by the solid medium diffusion method using sterile filter paper discs.

Method comparable to antibiogram as recommended by the Clinical Laboratory Standards Institute (CLSI) and complies with the recommendations of Committee of the antibiogram of French Society of Microbiology[14].

This test is performed by depositing a disk impregnated with 20µl of the crude essential oil on medium Muller-Hinton (MH) previously inoculated from a bacterial suspension of concentration equivalent to 0.5 McFarland (106-108 CFU).

The prepared plates are incubated at 37 °C for 24h. The antibacterial activity, when it exists, is estimated by measuring diameters of inhibition zones around the discs.

Determination of minimum inhibitory concentrations (MIC)

MICs of the essential oil were determined by the medium agar incorporation method as recommended by the CLSI[14].

A dilution range of the oil tested was performed in DMSO (Dimethyl sulphoxide). Once obtained, the dilutions are added to MH agar, melted in a water bath and cooled to 45 °C, so as to obtain 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[11,15].

2µl of spots of a standardized inoculum to 0.5 McFarland are deposited on the surface of MH medium with a micropipette.

All dishes are incubated in an oven and at 37 ° C for 24h.

Cultivation dishes witnesses, without thyme oil, are seeded and incubated in the same conditions.

MIC is defined as the lowest concentration, in the presence of which no bacterial growth is visible to the naked eye and similar to the growth of the strain on the control plate[14].

Determination of minimum bactericidal concentrations (MBC)

MBC essential oil towards the bacterial strains is determined by subcultures streaked on nutrient agar without oil from traces of spots without apparent growth corresponding to the MIC. The report MBC/MIC is then determined.

RESULTS AND DISCUSSION

Antibacterial activity of the essential oil tested

The diameters of the inhibition zones for *S. aureus* strains resistant to methicillin tested vary between 20mm and 50mm [tab 03]. This classifies these strains in the category of extremely sensitive bacteria by the criteria of Ponce and colleagues[16]. The activity is inversely proportional to the concentration; therefore MICs are very low and range from 180µg/ml to 720µg/ml. This is a major asset deviating seriously, the risk of toxicity and broadening the spectrum of fields of use of the substance. In a previous study, the same oil harvested in the Djebel Edough area in the wilaya of Annaba, has shown activity against *Pseudomonas aeruginosa* resistant to imipenem by producing VIM-2 carbapenemases[11]. The absolute values of MIC ranging from 1370 µg/ml to 2280 µg/ml attest a better sensitivity of gram-negative bacteria.

The values of the MBC are relatively low and vary between 180µg/ml and 216µg/ml, reflecting a bactericidal effect compared with a MBC/MIC ≤ 4[17].

This bactericidal effect is an asset when the prospect of using this substance as a disinfectant especially for surfaces, utensils, medical or surgical tools. It could operate in improving environments and disinfection during fumigation of hospital rooms. However, care should be taken in this bactericidal effect if used for food preservation.

While its business is exclusively bactericidal against MRSA strains tested. The oil of thyme is sometimes bacteriostatic and sometimes bactericidal on MSSA strains considered. MIC are included between 750µg/ml and 900µg/ml, and MBC are included between 760µg/ml and 900µg/ml. This could allow us to assume that the occurrence of mutation in the PLP does not affect the permeability of strains to this oil, or, this oil takes a different path and even, it acts differently. We might think, also that the emergence of resistance, makes Staphylococcus more susceptible, to volatile extracts of aromatic plants.

The bactericidal power of the HE on Gram-positive cocci, is assigned to the particular structure of their less complex membrane consisting of a thin layer of peptidoglycan in which proteins are embedded. This allow to hydrophobic molecules, to penetrate easily and act on the cell wall and into the cytoplasm[18-20].

Monoterpen phenols, with more than 70%, dominate the chemical composition of our oil. They are probably the most effective bactericidal known for their broad spectrum of antibacterial activity[21]. These are remarkable fungicide, virucidal and skin and intestinal antiparasitic, immune boosters and help decongest the airways[20]. Also, they are powerful atmospheric disinfectants. Thymol is the major component (67.78%), binds to membrane proteins and increased permeability, destabilizing cellular integrity. It also interferes with the synthesis of structural components[22] and energy metabolism leading to cell death[23,18,20,22] carvacrol, present in relatively small amounts accentuates this effect by inhibiting action on the ATPase activity[24].

P-cymene (hydrogenated monoterpen) is also present in sufficient quantity (12.25%), and is a precursor of the biosynthesis of carvacrol, facilitating its intracellular penetration and thus potentiating its action[23]. It shows a high affinity for cell membranes and can disrupt and assign them by causing them to swell to a greater extent than carvacrol[19,24].

Other minority compounds such as terpen hydrocarbons such: pseudo-limonen and α- terpinolen, can exert synergistic interactions, their targets and modes of action to be determined. It is recognized that the antimicrobial activity is dependent on chemical composition of the essential oil, functional groups of the major compounds (alcohols, phenols, terpen and ketone compounds) and also on minor compounds that act in a synergistic manner[25,26].

CONCLUSION

MRSA are pathogenic bacteria producing enterotoxins responsible for food poisoning and very intense and disabling transient acute morbidity. Moreover, this bacterium has a great ability to acquire resistance requiring continual search for new antimicrobials.

The main aim of this study was to evaluate the antibacterial activity of this essential oil on a bacterial population consisted of forty-four strains of *Staphylococcus aureus* resistant to methicillin (MRSA), isolated from different food products.

GC/MS revealed that HE *T. ciliatus* harvested in the region of Tawra (northeastern Algeria) is chemotype "Thymol" containing among other, p-cymen and low content of Carvacrol. The study showed a bactericidal activity of this oil on all MRSA strains tested since it generated inhibition diameters between 20mm and 50mm, with MICs and MBCs relatively low and the ratio of MBC/MIC equal to 1.

The MSSA strains generated variable behavior towards the extract (bactericidal and bacteriostatic) and *S. aureus* ATCC 43300 resistant to antibiotics. This one has proved more sensitive to oil than *S. aureus* ATCC 29213 antibiotic sensitive. Antibiotic resistance seems to make strains more susceptible with essential oils.

This result suggests considering the use of such molecules in food as a substitute for conventional chemical preservatives. It is known that the essential oil of Thyme is tasteful and highly appreciated since it enters several recipes.

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