



Antipseudomonal Activity of the Essential Oil of *Thymus numidicus* Poiret

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ABSTRACT

The essential oil extracted from the leaves of *Thymus numidicus* Poiret harvested in the region of Berrahel (Annaba) gave a yield of 1.92%. Its analysis by CG/SM allowed the identification of 13 components, principally phenols and terpenes. The main constituents are thymol (77,5 %), P-cymene (10,1 %) and γ -terpinene (6,37 %). The antibiotic activity of this essential oil was assessed on 9 strains of *Pseudomonas aeruginosa*, using the method of diffusion in a solid medium. MIC was determined by the method of integration in an agar medium. The nine strains showed high sensitivity towards the essential oil with inhibition diameters ranging from 17.5 mm to 55 mm and a MIC of 0.4 mg/ml. The essential oil of *Thymus numidicus* proved to be endowed with bactericidal properties against *Pseudomonas aeruginosa* strains.

Keywords: *Pseudomonas aeruginosa*, *Thymus numidicus* Poiret, Essential oil, GC/MS, antibacterial activity.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative ubiquitous bacterium, very common in hospitals. It is resistant to numerous antibiotics such as penicillin, cephalosporin of 1st and 2nd generations, carbapenem, aminoglycosides and fluoroquinolones.¹

In fact, this germ fits quickly in medicinal attacks and poses a major health problem because of its multi-antibiotic resistance. This resistance responds to several defense mechanisms like the impermeability of the bacterial membrane to antibiotics¹⁻⁴ and the expression of several efflux systems, enabling these strains to expel antibiotics in the outside environment like a pump, using the energy of the electrochemical gradient of the cytoplasmic membrane.^{5,6}

The acquisition of plasmids carrying resistance genes to certain antibiotics is also a serious problem favoring the dissemination of the resistance factor.

Strains of *Pseudomonas aeruginosa* are also able to modify the constitution of their own cytoplasmic membrane and to create chromosomal mutations in the target of some antibiotics, reducing the affinity of the antibiotic for its target and making its fitting impossible.⁷⁻⁹

The aim of this study is to test the antibacterial activity of the essential oil of Thyme of Numidia on *P. aeruginosa* strains resistant to imipenem, which is the last beta-lactam antibiotic, it has a wide bacterial spectrum and a high stability against beta-lactamases. Its main action mode is the inhibition of the bacterial wall synthesis by fixing to the binding proteins.⁶ The strains of *Pseudomonas aeruginosa* are of wild-type sensitive to imipenem. They are however endowed with a high plasticity and a remarkable adaptation power involving

the appearance of resistant strains for which the rate never stops increasing. The acquisition of this resistance may be enzymatic, making use of metallo- β -lactamase. This mechanism leads us straight to a therapeutic impasse because it is transferable. The genes coding for the production of these enzymes are located in mobile platforms, transmission is realized by integrons situated in associated plasmids^{10,11} which causes high spread of resistant strains. The other mechanism of resistance is the loss of the porin Opr D2 in these strains reducing imipenem permeability.¹²

To address this well-established resistance⁶, aromatherapy could be an alternative to fight against this problem. Thymes are part of those miraculous plants that have many therapeutic properties and a recognized antibacterial activity.

This genus comprises more than 300 species, *Thymus numidicus* Poiret, which is typically endemic to north eastern Algeria and Tunisia¹³ is a bushy plant with erect stems, leaves are lanceolate, 2-5 times longer than wide, the floral leaves are distinctly broader while the flowers are pink, sessile or nearly so. Although it is a major element of Algerian traditional medicine, this species remains to date little known and has been the subject of few investigations. Its study is therefore well-founded.

MATERIALS AND METHODS

Materials

Essential oil of *Thymus numidicus*

The extraction of the essential oil was performed by hydrodistillation method (Likens Nickerson apparatus) during 1h30 min on dry leaves, harvested in April 2012 before flowering in Berrahel (Annaba) area. The oil

obtained was dried and then stored at 4° C in a dry place inaccessible to light and moisture.

Bacterial strains

The test strains were isolated from hospitals (University Hospitals, health centers) and private clinics of Annaba (Algeria). The tests were performed on nine strains of *Pseudomonas aeruginosa*: ATCC 27853, S36, S67, S71, S72, S74, S87, S139 and S218. All these strains are resistant to imipenem.

Methods

Chromatographic Analysis of the essential oil

The chemical composition of the oil was determined by the technique of gas chromatography coupled with mass spectrometry (GC/MS). The apparatus used is a gas chromatograph (GC UltraTrace) with a VB-5 column combined with a mass spectrometer (MS Polarisation trap). The analysis required ethyl acetate as solvent and XEI ionization type (70 eV) with a temperature of 200°C at the source. The carrier gas is helium, the injection mode is the split mode, the injection temperature is 220°C, while the interface temperature is 300°C. The column temperature follows an increase of 40 to 300°C at a rate of 4°C min⁻¹, and the injected volume is 1µl.

The identification of the chemical constituents of the essential oil was performed with reference to the following databases: NIST/ EPA /NIH Mass Spectral Library Version 2.0, January 2002 buildju.

Microbiological study

Aromatogram

The aromatogram has the same principle as the antibiogram technique. On Mueller Hinton medium we performed seeding tested strains as recommended by the Comity of the Antibiogram of French Society of Microbiology (CASFM)¹⁴. On the surface of the agar we introduced two sterile discs: one is impregnated with pure Thyme oil, the other is a witness disk devoid of any substance. After an incubation of 24 hours at 37° C, we proceeded to the reading of the results by measuring the diameter of the inhibition zones formed around the disc.

Determination of MIC (minimal inhibition concentration)

MIC is the lowest concentration of essential oil to which no bacterial drive is observed. The calculation of the MIC was conducted by the method of incorporation agar following the recommendations of the Clinical Laboratory Standards Institute (CLSI)¹⁵, the principle is to prepare dilutions of the essential oil in Dimethylsulfoxid (DMSO) from a stock solution; each dilution is incorporated into a fixed volume of medium Mueller Hinton then poured into a Petri dish. After the drying of the medium, we have placed on the surface of each dish spots, each one representing a tested strain. After an incubation of 24 hours, we distinguished susceptible strains from resistant strains for each concentration.

Determination of MBC (minimal bactericidal concentration)

The MBC is the lowest concentration of essential oil which destroys 99.9% of the bacterial inoculum, which is a bacterial count lower than an interval between 104 and 102 CFU/ml after 24 hours of incubation (the initial inoculum is between 106 and 108). Using a platinum loop, we collected a sample from each dish that showed no bacterial growth. Then these samples were plated on a nutritive agar and incubated at 37 °C for 24 hours. The minimum bactericidal concentration is the lowest concentration of the essential oil for which no growth was observed.

RESULTS AND DISCUSSION

Essential oil yield

Thyme leaves gave 1.92 % of essential oil to a test sample of 100 g of dried plant material. This result can be considered important when compared to the yield of 1.1% reported by Hadeff et al.,¹⁶ on the same species, growing in the same environment, harvested at the flowering stage (June). The difference would be due to the climatic conditions corresponding to the two years in question. It is important to mention the relationship between the yield and the distillation quality.

Chemical composition of the essential oil of *Thymus numidicus* Poiret

Analysis of the essential oil by GC/MS revealed the presence of 13 components, thymol is the major element with 77.52%. The other important components are respectively P-cymene (10.10 %), γ-terpinene (6.38%) and β-pinene (3.16). Carvacrol is in trace level (0.17%), but we note the presence of p-cymene which is its precursor. In 2007, Hadeff et al.,¹⁷ found in the same species within the same region 11 components (Table 1) whose thymol is also the major component with only 49.4%.

According to several authors¹⁸⁻²⁰, variations in the chemical composition of essential oils from the qualitative and quantitative stand point are due to genetic factors, environmental factors, the part of the plant used, its age and finally the period of the vegetative cycle during which the harvest has been made. The differences between our results and those of Hadeff can be attributed to this last factor. *Thymus numidicus* of 2011 was harvested in April thus before flowering; we note nevertheless a high concentration of thymol (77.5 %). Besides the absence of some components, we note the presence of their precursors; we can deduce their subsequent development during the vegetative cycle. We note particularly, the presence of carvacrol at a very low concentration level and also the presence of p-cymene of which it is the precursor^{21,22}. We remark also the absence of linalool in the essential oil of 2011. Climate variations can be implicated because they have a significant impact on the chemical composition of essential oils, especially in species with superficial storing histological structures such the secretory bristles in Lamiaceae, for when the

location of the essential oil is deeper quality is much more constant²³. Works of Saidj et al.,²⁴, which focused on *Thymus numidicus* in the area of Yakouren (Tizi Ouzou) harvested in April 2004, showed a predominance of oxygenated compounds which are thymol (51%) and carvacrol (9.4%) but also a small proportion of p-cymene (0.5%). Comparison between these results allows us to conclude that Thymol always prevails in this species.

Table 1: Chemical composition of the essential oil of *T. numidicus* in 2007 and 2011

Components	<i>T. numidicus</i> 2011	<i>T. numidicus</i> 2007
α -phellandrène	0.2 %	0.4%
α- pinène	1.01 %	1.8%
δ- 3- carène	0.18 %	/
α -terpinène	0.40 %	2.3%
p- cymène	10.10 %	10.1%
γ- Terpinène	6.38 %	11.7%
β-pinène	3.16 %	2.5%
Bornéol	0.2%	/
Thymol	77.5 %	49,9%
Carvacrol	0.17 %	2.5%
Guaia 3,9 diène	0,2 %	/
γ -Cadinène	0.08 %	/
δ-cadinène	0.4 %	0.7%
Linalool	/	10%

T. numidicus: *Thymus numidicus*

Results of antimicrobial study

Table 2 shows the inhibition diameters, MIC and MBC of *Pseudomonas aeruginosa* strains.

Table 2: Activity of the essential oil of *T.numidicus* on *Pseudomonas aeruginosa*

Strains	D (mm)	MIC (mg/ml)	MIB (mg/ml)
S ATCC27853	38.5	0,4	0,4
S 139	21		
S 36	55		
S 67	22		
S 71	30		
S 72	46		
S 74	34		
S 87	43.5		
S 218	17.5		

D ; inhibition diameters, MIC ; minimal inhibitrice concentration, MIB ; minimal bactericidal concentration

Despite the resistance of gram-negative bacteria to essential oils²¹, strains of *Pseudomonas aeruginosa* showed an interesting sensitivity against the essential oil of *Thymus numidicus*, with inhibition zones ranging from 17.5 mm in strain S 218 to 55 mm in S 36. MIC obtained

for all strains investigated (0,4 mg/ml) is very interesting and indicates a high activity of this oil on this bacterial species. This efficiency can be explained in terms of the high prevalence of phenolic derivatives that are the source of the antibacterial effect of essential oils according to several authors.²⁵⁻²⁷ The antibacterial activity of the essential oil of *T. numidicus* can be partly attributed to its high content level of thymol which, according to Lambert et al.,²⁸ binds to membrane proteins and increases the permeability of the bacterial cell membrane. Dorman et al.,²⁹ demonstrated that thymol is the compound that has the widest spectrum of antibacterial activity and that against 25 types of bacteria tested. Other studies suggest that volatile compounds are responsible of the inactivation of enzymes, including those involved in energy production and synthesis of structural components^{26,29}.

Minor components are not of lesser importance since they produce synergies with others and potentiate their effects; this is what was discovered by Marino et al.,³⁰ in a study on the sage. This was also highlighted by Lambert et al.,²⁸ who have tested the activity of thymol and carvacrol on strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Despite the low level of carvacrol in our essential oil we note the presence of its biological precursor P-cymene at high concentrations. It would appear that the P-cymene is a very good antibacterial agent. Ultee et al.,³¹ reported that P-cymene could cause swelling of the cytoplasmic membrane of *Bacillus cereus* and disturbances in its structure. Due to their hydrophobic nature, essential oils and their constituents are able to integrate with the bacterial membrane lipids, disrupt its structure and increase its permeability³². Therefore, it is an option tool in the fight against bacterial resistance.

CONCLUSION

The essential oil of *Thymus numidicus* Poiret harvested in the region of Berrahal (Eastern Algeria) is characterized by the presence of 13 components; the most important are thymol, P-cymene and γ-terpinene. The essential oil was tested over nine strains of *Pseudomonas aeruginosa* ATCC 27853, S36, S67, S71, S72, S74, S87, S139 and S218. All these strains have a profile of resistance to imipenem. The tests showed a strong antibacterial activity of essential oil of *Thymus numidicus* towards all the strains tested. This strength is mostly attributed to the high concentration of terpenes and phenolic compounds in this essential oil including thymol which is the major component. The minor components are not of lesser importance.

REFERENCES

- Brain Y, Hadda BD, Bonniun M, *Pseudomonas aeruginosa* et antibiotiques, Bulletin De L'institut Pasteur, Ed : Elsevier, 87, Paris, France, 1989, 125-170.
- Biendo M, Yala F, Kounkou R, Boudjoumba, Sensibilité à 9 antibiotiques de 46 souches de *Pseudomonas aeruginosa* isolées au CHU de Brazzaville, Médecine et maladies infectieuses, 21, 1991, 668-670.
- Barbier F et Wolff M, Multi-drug resistant *Pseudomonas aeruginosa*: towards a therapeutic dead end? MS. Médecine sciences, 26(11), 2010, 960-968.
- Kal S, Achour W, Abdelhamid A, Benjaoui M, Ben Hassen A, *Pseudomonas aeruginosa* isolées de patients immunodéprimés: résistance aux antibiotiques, sérotypage et typage moléculaire, Médecine et maladies infectieuses, 35, 2005, 530-535.
- Bert F, Lambert-Zechovzki N, Problèmes microbiologiques d'actualité = Current concepts on antibiotic resistance and therapeutic problems raised by *Pseudomonas aeruginosa*: Today's challenges in microbiology, La Presse médicale, 28(8), 1999, 451-458.
- Mesaros M, Van Bambeke F, Glupczynski Y, Vanhoof R, Tulkens PM, L'efflux des antibiotiques: Un mécanisme ubiquitaire conduisant à la résistance. Etat de la question et implications microbiologiques et cliniques, Louvain Médical, 124(8), 308-320.
- Avril JL, Dabernat H, Denis F, Monteil H, Bactériologie clinique, 2^{ème} édition, Edition Marketing, 1992, Pp -268-276.
- Xian-Zhi Li, Quinolone resistance in bacteria: emphasis on plasmid-mediated mechanisms, International Journal of Antimicrobial Agent, 25, 2005, 453-463.
- Honoré S, Lascols C, Malin D, Targaouchi R, Cattoir V, Legrand P, Soussy CJ, Cambau E, Emergence et diffusion chez les enterobactéries du nouveau mécanisme de résistance plasmidique aux quinolones Qnr (résultat de l'Hôpital Henri Mondor 2002-2005), Pathologie Biologie. Ed: Elsevier, France, 54, 2006, 270-279.
- Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo J D, Nordmann P, Characterization of Vim-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid-and integron-borne gene from a *pseudomonas aeruginosa* clinical isolate in France, *Antimicrob Agents Chemother*, 44, 2000, 891-897.
- Gral N, Andreumont A, Armand lefèvre L, Résistance aux carbapénèmes: vers une nouvelle impasse? Journal des Anti-infectieux, ANTINF-16: N° 16. Doi.10.1016/ J. antinf, 2011.03.005.
- Chun-Xin Wang and Zu-Huang Mi, Imipenem-Resistant *Pseudomonas aeruginosa* producing IMP 1 metallo B-lactamases and lacking the outer membrane protein Opr D, JMM: Journal of Medical Microbiology, 55(3), 2006, 353-354.
- Quezel P et Santa S, Nouvelle flore de l'Algérie et des régions désertiques méridionales, Edition Centre national de la recherche scientifique, Tome 2, 1963, 805.
- Comité de l'Antibiogramme de la Société Française de Microbiologie. Communiqué 2012, Paris, France, 2012, 5-7.
- Benslimani A, Techniques. In: Réseau Algérien de surveillance de la résistance aux antibiotiques des bactéries (Eds) Standardisation de l'antibiogramme à l'échelle nationale, médecine humaine et vétérinaire, 6^{ème} édition, 2011, 23-37.
- Hadef Y, Kaloustian J, Giordan R, Regli P, Chefrou A, Abou L, Mikail C et Portugal H, Composition chimique et activité antifongique des huiles essentielles de *Thymus vulgaris* L. et *Thymus numidicus* Poiret d'Algérie, 6^{ème} symposium international d'aromathérapie scientifique et plantes médicinales, Grasse, France, 2004, pp 5.
- Hadef Y, Kaloustian J, Chefrou A, Mikail C, Abou L, Giodani R, Nicolay L and Portugal H, Chemical composition and variability of the essential oil of *Thymus numidicus* Poir. from Algeria, *Acta Bot. Gallica*, 154(2), 2007, 265-274.
- Karousou R, Koureas DN & Kokkini S, Essential oil composition is related to the natural habitats: *Corido thymus capitatus* and *Satureja thymbrina* NATURA 2000 sites of Crete, *Phytochemistry*, 66(22), 2005, 2668-2673.
- Thompson JD, Chalchat JC, Michet A, Linhart YB, Ehlers B, Qualitative and quantitative variation in monoterpene co-occurrence and composition in the essential oil of *Thymus vulgaris* chemotype, *J. Chem. Ecol*, 29(4), 2003, 859-880.
- Benjilali B, Hammouni M, Richard H, Chemical polymorphism of Moroccan thyme essential oils: compounds characterization, *Sci. Aliments*, 7, 1987, 77-91.
- Burt S, Van der Zee R, Koets AP, De Graaff AM, Knapen F, Gastra W, Aagsman HP and Veldhuizen EJA, Carvacrol Induces Heat Shock Protein 60 and Inhibits Synthesis of Flagellin in *Escherichia coli* O157:H7, *Applied and Environmental Microbiology*, 73(14), 2007, 4484-4490.
- Simon JA, The total synthesis of natural products, Vol (2), A Wiley-interscience publication, John Wiley & sons, Toronto, 1973, Pp: 1-197.
- Bruneton J, Pharmacognosie, Phytochimie, Plantes médicinales, 2^{ème} édition TEC&DOC-Lavoisier, Paris, 1993, Pp: 406-417.
- Saidj F, Rezzoug SA, Bentahar F, Boutekedjiret C, Chemical Composition and insecticidal Properties of *Thymus numidicus* Poiret Essential Oil from Algeria, *Journal of essential oil-bearing plants*, 11(4), 2008, 397-405.
- Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E, Palmas F, In vitro antimicrobial activity and



- chemical composition of Sardinian Thymus essential oils, Letters in Applied Microbiology, 29, 1999, 130– 135.
26. Trombetta D, Saija A, Bisignano G, Arena S, Caruso S, Mazzanti G, Uccella N Castelli, Study on the mechanisms of the antibacterial action of some plant alpha, beta-unsaturated aldehydes, *Lett. Appl. Microbiol*, 35(4), 2002, 285-290.
27. Amarti F, Satrani B, Ghanmi M, Farah A, Aafi A, Aarab L, El Ajjouri M et Chaouch A, Composition chimique et activité antimicrobienne des huiles essentielles de *Thymus algeriensis* Boiss. & Reut. Et *Thymus ciliatus* (Desf.) Benth du Maroc, *Biotechnol. Agron. Soc. Environ*, 14 (1), 2010, 141-148.
28. Lambert RJW, Skandamis PN, Coote P, Nychas GJE, A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol, *Journal of Applied Microbiology*, 91, 2001, 453–462.
29. Dorman HJD & Deans SG, Antimicrobial agents from plants: antimicrobial activity of plant volatile oils, *J. Appl. Microbiol*, 88, 2000, 308-316.
30. Marino M, Bersani C, Comi G, Impedance measurements to study the antimicrobial activity of essential oils from Lamiacea and Compositae, *International Journal of Food Microbiology*, 67, 2001, 187– 195.
31. Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ, Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol, *Archives of Microbiology*, 174(4), 2000, 233– 238.
32. Knobloch K, Weigand H, Weis N, Schwarm HM, Vigneschow H, Action of terpenoids on energy metabolism, In: Brunke, E.J. (Ed.), *Progress in Essential Oil Research: 16th International Symposium on Essential Oils*, De Gruyter, Berlin, 1986, 429– 445.

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