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 GC/MS allowed the identification of 13 components, principally phenois 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 multiple-antibiotic resistance. This resistance responds to several defense mechanisms like the impermeability of the bacterial membrane to antibiotics2-4 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.7,8

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.4 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 plasmids9,10,11 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 resistance,6 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 buildjju.

**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 Hadef 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, Hadef 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 authors¹⁹⁻²⁰, 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 Hadef 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 precursor²¹,²². 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 constant. Works of Saidj et al., 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

<table>
<thead>
<tr>
<th>Components</th>
<th>T. numidicus 2011</th>
<th>T. numidicus 2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-phellandrene</td>
<td>0.2 %</td>
<td>0.4 %</td>
</tr>
<tr>
<td>α-pinène</td>
<td>1.01 %</td>
<td>1.8 %</td>
</tr>
<tr>
<td>δ-3-carène</td>
<td>0.18 %</td>
<td>/</td>
</tr>
<tr>
<td>α-terpinène</td>
<td>0.40 %</td>
<td>2.3 %</td>
</tr>
<tr>
<td>p-cymène</td>
<td>10.10 %</td>
<td>10.1%</td>
</tr>
<tr>
<td>γ-Terpénène</td>
<td>6.38 %</td>
<td>11.7%</td>
</tr>
<tr>
<td>β-pinène</td>
<td>3.16 %</td>
<td>2.5 %</td>
</tr>
<tr>
<td>Bornéol</td>
<td>0.2 %</td>
<td>/</td>
</tr>
<tr>
<td>Thymol</td>
<td>77.5 %</td>
<td>49.9%</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>0.17 %</td>
<td>2.5 %</td>
</tr>
<tr>
<td>Guai a 3,9 diène</td>
<td>0.2 %</td>
<td>/</td>
</tr>
<tr>
<td>γ -Cadénène</td>
<td>0.08 %</td>
<td>/</td>
</tr>
<tr>
<td>δ-cadinène</td>
<td>0.4 %</td>
<td>0.7%</td>
</tr>
<tr>
<td>Linalool</td>
<td>/</td>
<td>10%</td>
</tr>
</tbody>
</table>

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*

<table>
<thead>
<tr>
<th>Strains</th>
<th>D (mm)</th>
<th>MIC (mg/ml)</th>
<th>MIB (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S ATCC27853</td>
<td>38.5</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>S 139</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 36</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 67</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 71</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 72</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 74</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 87</td>
<td>43.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 218</td>
<td>17.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D: inhibition diameters; MIC: minimal inhibitory concentration, MIB: minimal bactericidal concentration

Despite the resistance of gram-negative bacteria to essential oils, 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. 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., binds to membrane proteins and increases the permeability of the bacterial cell membrane. Dorman et al., 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.

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., in a study on the sage. This was also highlighted by Lambert et al., 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., 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 permeability. 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


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