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Full Length Research Paper

The essentials oils and antimicrobial activity of four nepeta species from Morocco

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The chemical composition of essential oils (EO) isolated from four Nepeta species (*Nepeta atlantica* Ball, *Nepeta tuberosa* L. subsp reticulata (Desf.) Maire, *Nepeta cataria* L., *Nepeta granatensis* Boiss) were characterized and the antibacterial activity tested. Chemical analyses indicated that the major component of these EO was the sterioisomere 4a-α, 7-α, 7a-β-nepetalactone which represented more than 70% of oils from *N. atlantica*, *N. tuberosa*, *N. cataria* and 39.4% of oils from *N. granatensis.* The EO from *N. atlantica*, *N. tuberosa*, and *N. cataria* presented comparable antibacterial activity against tested bacteria. Statistical analysis indicated that *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) strains were the most sensitive to the EO (MICs ranged from 4.37 to 16.25 µL/mL), these strains were more sensitive than clinically isolated *E. coli*, *S. aureus* and *S. enteritidis* and *P. aeruginosa* (ATCC 27853) strains. The EO from *N. granatensis* contained low nepetalactone concentrations and showed relatively poor antibacterial activity against tested bacteria (MICs ranged from 22.50 to 80.00 µL/mL). These results indicate that the antibacterial activity of EO from the Nepeta genus depends on their chemical composition and suggests that nepetalactone plays an important role in antibacterial activity against *E. coli* and *S. aureus* strains, two microorganisms which cause morbidity and mortality worldwide.

Key words: *Nepeta atlantica, Nepeta tuberosa, Nepeta granatensis, Nepeta cataria* , Lamiaceae, essential oil composition, 4a-α, 7-α,7a-β-nepetalactone, antibacterial activity.

INTRODUCTION

Nepeta is a genus of annual or perennial herbs; it belongs to the Lamiaceae family, which includes approxi-mately 250 species. These plants are localized to central and southern Europe, Asia, the Middle East, northern Africa, and to tropical mountains in Africa (Ghannadi et al., 2003; Gkinis et al., 2003).

Nepeta species are used in the traditional medicine of many countries and have a large ethnobotanical effect: diuretic, diaphoretic, vulnerary, antitussive, antispasmo-dic, antiasthmatic, tonic, febrifuge, emmenagogue and carminative (Ghannadi et al., 2003; Nostro et al., 2001). However, neither the antimicrobial activity of EO of Ne-peta species growing spontaneously in Morocco nor the composition of the essential oils has been published. In fact antibacterial properties of Lamiaceae oils compounds such as borneol and pinene has been reported (Dorman and Deans, 2000; Tabanca et al., 2001; Vardar-Unlü et al., 2003). It has also been reported that plant oils rich in phellandrene and limonene show high antimicrobial acti-vity (Gonzalez et al., 2004). In addition, plants rich in car-yophyllene and sesquiterpenoid hydrocarbons exhibited a broad spectrum of antibacterial activities against Gram-positive and Gram-negative bacteria (Oyedeji & Afolayani, 2005). In this context we were interested in analyzing the composition and the antibacterial activity of EO from Nepeta species. In this report we studied the composition of the oils from four Nepeta species growing in the wild in Morocco (Fennane and Ibn Tattou, 1998) i.e. *N. tuberosa* L. subsp *reticulata* (Desf.) Maire, *N. cataria* L., *N. granatensis* Boiss, and *N. atlantica* Ball and we evaluated the antibacterial activity of their EO against Gram-positive and Gram-negative bacteria.

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MATERIALS AND METHODS

Plant material

Whole plants of *N. cataria*, *N. granatensis*, *N. tuberosa* and *N. atlantica* were collected from Moroccan regions with agreement from the authorities and respecting the United Nations Convention on Biodiversity. *N. cataria* was collected in the region of Kenitra, *N. granatensis* and *N. tuberosa* were collected in the region of Ifran, and *N. atlantica* was collected in Boumia-Tounfit.

Essential oil extraction

Plant material (100 g) was submitted to steam distillation and the distillate was subject to successive ethyl acetate extractions (3 x 100 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The yield of extraction (percentage of weight of oil/weight of dry plant) was: 0.96% for *N. granatensis*, 1.04% for *N. atlantica*, 1.02% for *N. cataria*, and 1.2% for *N. tuberosa.*

Analytical techniques

GC-MS analysis of the EO was performed on a TRACE GC ULTRA equipped with non-polar VB5 (5% phenyl; 95% methylpolisyloxane) capillary column (30 m x 0.25 mm x 0.25 m film thickness), directly coupled to a mass spectrometer (Polaris Q) (EI 70 eV). The temperature of the injector and detector was set at 250°C and 300°C, respectively. The oven temperature was programmed from 60°C to 200°C at 2°C/min, and then from 200°C at 300°C at 20°C/min. The components of the oil were identified by comparison of their mass spectra with those in the Wiley-NIST 7^{th} edition library of mass spectral data. The percentage composition of the oil sample was calculated from GC-MS peak areas.

Microorganisms and inoculum preparation

The bacteria *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Salmonella enteritidis* (*S. enteritidis*) isolated from patients were obtained from the culture collection of the microbiology department (Microthec Unity) at the National Institute of Health (Rabat, Morocco). Bacteria were identified based on the colony morphology, gram stain, API enterobacteria and biochemical gallery. Reference strains were obtained from the American Type Culture Collection: *Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922), *Staphylococcus aureus* ATCC 25923 (*S. aureus* ATCC 25923), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa* ATCC 27853).

Each isolate was inoculated into Muller Hinton Agar with a scraping from a glycerol stock frozen at –20°C and incubated during 18 - 20 h at 37°C. For the inoculum, colonies were selected from 18 - 20 h old cultures. Turbidity was adjusted to that of a 0.5 MacFarland standard (1.5 x 10^8 CFU/mL) using sterile NaCl solution (0.9%).

Determination of minimum inhibitory concentration (MIC)

The viability indicator MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) and the quick microplate method (Eloff, 1998) were used for the determination of the minimum inhibitory concentration (MIC) as we described previously (Oumzil et al., 2002). A range of doubling dilutions of Nepeta EO was prepared in Muller Hinton Broth (BMH) in a 96-well microplate. Tween-80 was included in all assays at a final concentration of 0.001% (v/v) to enhance oil solubility. Each well was inoculated with 25 µl of a bacterial suspension. The covered microplates were incubated overnight at 37°C.

The bacterial suspension changed to blue when bacterial growth occurred. To verify that there was no abiotic reduction of MTT, tested products were incubated directly with the viability indicator without any bacterial suspension (negative control). The MIC is defined as the lowest concentration of antibiotic or extract at which there is no visible growth. The stock solution and the dilutions of antibiotic were prepared as described (Andrews, 2001; EUCAST, 2003). As an internal control, we determined the MIC for chloramphenicol against *E. coli* ATCC 25922; the value obtained was in agreement with the NCCLS standards (NCCLS, 1990).

Statistical analysis

Statistical analysis was performed using a regression qualitative model, ANOVA and least differences analysis was carried out with J.M.P. version 5.1: SAS Institute. Inc, 2003.

RESULTS

The chemical composition of EO from tested Nepeta species was determined by GC -MS analysis. Table 1 shows that the EO of each plant species has a specific quantitative and qualitative composition. The major component was neptalactone (the sterioisomere 4a-α, 7-α, 7a-β-nepetalactone and the dihydronepetalactone) which represents about 74% for *N. atlantica*, 82% for both *N. tuberosa* and *N. cataria* but only 42% in *N. granatensis*. This result is comparable to that obtained for others Nepeta species (Ghannadi et al., 2003; Gkinis et al., 2003) . Of note *N. atlantica* is characterized by its relatively high - caryophyllene (8.2%) and farnesol (2.5%) content while *N. granatensis* was relatively rich in other components (eucalyptol 24%, -pinene 6.3 %, -phellandrene 5.8%). In addition, *N. cataria* is characterized by its relative content in terpinene (4.2%) and limonene (4.1%).

The screening for antibacterial activity indicates that the oils from *N. atlantica*, *N. tuberosa*, and *N. cataria* present comparable activity against all strains of tested bacteria (MICs ranged from 4.37 to 53.33µL/mL) (Table 2). The oils from *N. granatensis*, was found to possess a relatively low antibacterial activity (MICs ranged from 22.5 to 80µL/mL). For example the MICs of EO from *N. tuberosa*, *N. atlantica*, and *N. cataria* against *S. aureus (*ATCC 25923) were 4.37, 7.50 and 5.00 µL/mL respectively, while the MIC of EO from *N. granatensis* was of 22.50µL/mL (Table 2).

Of interest, *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were more sensitive to the oils of *N. atlantica*, *N. tuberosa*, and *N. cataria* (MICs ranged from 4.37 to 16.25 µL/mL) . For isolated *E. coli* and *S. aureus* the MICs ranged from 7.96 to 30 µL/mL. *S. enteritidis* and *P. aeruginosa* (ATCC 27853) were less sensitive to EO, with MICs below 53.33 µL/mL (Table 2). Interestingly, the oil of *N. granatensis,* which is poor in nepetalactone, has a relatively low antibacterial activity against tested microorganisms (MICs ranged from 22.5 to 80 µL/mL).

Table 1. Chemical composition (%) of four oils of Nepeta species

Compounds *	*N. atlantica*	*N. tuberosa*	*N. cataria*	*N. granatensis*
- pinene	1.1	1.3	0.4	6.3
- pinene	1.1	0.9	0.9	2.3
camphene	1.1	0.3	0.9	1.1
eucalyptol	1.1	1.2	0.5	24.0
– phellandrene	1.1	0.6	0.2	5.8
terpinene	1.1	0.9	4.2	0.5
sabinene	0.2	0.6	0.2	0.5
-Cymene	0.3	0.9	0.2	3.8
limonene	0.2	0.5	4.1	0.3
menthone	0.2	0.4	0.2	0.7
thujone	0.1	0.4	0.4	0.6
borneol	0.1	0.6	0.3	0.6
linalyl acetate	0.5	0.4	0.2	1.4
menthol	0.6	1.6	0.2	0.4
linalool	0.6	0.5	0.4	1.4
pulegone	0.4	0.9	0.2	1.6
thymol	0.8	0.6	1.3	0.9
-terpineol	0.6	0.5	0.3	0.5
citronellol	0.8	0.6	0.2	0.6
4a-α, 7-α, 7a-β-nepetalactone	71.4	76.8	77.4	39.4
dihydronepetalactone	3.1	5.9	5.0	2.8
methyleugenol	0.9	0.8	0.1	0.9
methylisoeugenol	0.5	0.6	0.4	0.6
-caryophyllene	8.2	0.7	0.2	1.5
– curcumene	1.3	0.7	0.6	0.9
farnesol	2.5	0.8	0.9	0.5

*Compounds are listed in order of their elution from a VB-5 column.

Table 2. Minimal inhibitory concentration (MIC) of Nepeta species oils against different bacteria.

Strains	MIC ± Std Err Means			
	N. tuberosa	*N. atlantica*	*N. cataria*	*N. granatensis*
E. coli ATCC 25922	6.25 ± 1.25	13.13 ± 4.25	16.25 ± 3.75	35.00 ± 15.00
E. coli	30.0 ± 5.77	7.96 ± 4.07	16.60 ± 3.33	70.00 ± 10.00
P. aeruginosa ATCC 27853	40.0 ± 0.00	22.50 ± 6.29	37.50 ± 15.47	40.00 ± 0.00
S. aureus ATCC 25923	4.37 ± 0.62	7.50 ± 1.44	5.00 ± 0.00	22.50 ± 6.29
S. aureus	23.33 ± 8.81	18.43 ± 7.92	10.00 ± 3.53	68.00 ± 12.00
S. enteritidis	20.00 ± 0.00	20.00 ± 0.00	53.33 ± 13.33	80.00 ± 0.00

Data represent the MICs (µL/mL) ± standard error of the means for 4 experiments.

In summary, our results indicate that *E. coli* . and *S. aureus* are more sensitive than *P. aeruginosa* to EO from the Nepeta genus ($p < 0.001$).

DISCUSSION

The results suggest that the antibacterial activity of the oils of Nepeta species may be related to their major monoterpenoid component *i.e.* nepetalactone, however this does not exclude the possibility that the other monoterpenes may account for the antibacterial property of the oils. This may be supported at least in part by the differrences observed in the sensitivity of tested bacteria to EO from Nepeta species that have similar levels of nepetalactone (Table 1). In fact we found that nepetalactone represents 42% and eucalyptol represents 24% of the oil from *N. granatensis* and other reports showed that eucalyptol has antibacterial activity towards *S. vaureus*

(Karlovic et al., 2000). Thus, one may take into consideration that the antibacterial effect of the oils could result from a synergistic or antagonistic action of different oils components.

The antibacterial effect of the oils could be explained by disturbance of the permeability barrier of the bacterial membrane structure (Cowan, 1999). Indeed, recent findings revealed that tea tree oil damages the cell membrane structure of *E. coli*, *S. aureus* and *Candida albicans* (Cox et al., 2000). Such a phenomenon is due to the penetration of monoterpenes through the cell wall and cell membrane. In fact, monoterpenes are lipophilic, and may induce the expansion of cell membranes, increases fluidity, destroy the membrane structure and inhibit membrane embedded enzymes (Cox et al., 2000; Cox et al., 2001). In the case of *E. coli*, tea tree oil also caused a leakage of cellular potassium and inhibits glucose dependent respiration (Cox et al., 1998). Thus, further investgations should be made to explore the mechanisms of action of the oils of Nepeta species showing high activity against bacteria.

In summary, our data indicate that the EO from the Nepeta genus efficaciously inhibit *E. coli* and *S. aureus* strains, two resistant microorganisms which make serious sanitary problems worldwide. This indicates that this plant may be useful for developing alternative compounds to treat infections caused by these antibiotic resistant pathogens. As suggested in a recent report (Betoni et al., 2006), these compounds of plant origin may be used together with known drugs in the development of pharmacological agents against pathogens.

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