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Antimicrobial activity and chemical composition of essential oil and hydrosol extract of *Nepeta nepetella* subsp. amethystina (Poir.) Briq. from Algeria

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ABSTRACT

The essential oil and hydrosol extract of *Nepeta nepetella* subsp. amethystina, obtained by hydrodistillation and analysed by GC, GC–MS, 1H and 13C NMR, were evaluated for their antimicrobial activity using disks diffusion method and MICs against thirteen pathogenic microorganisms. The chemical analysis of essential oil and hydrosol extract allowed the identification of 34 and 12 constituents representing 96.77% and 80.07% of total oil and hydrosol extract, respectively. The two isomers 4aα,7α,7aβ-nepetalactone and 4aα, 7α, 7aα-nepetalactone were found to be the major components in *N. amethystina* essential oil and hydrosol extract, α, 7α-nepetalactone and 4aβ,7aα,7αrespectively. The two isomers 4a -nepetalactone were found to be the major components in N.α7a amethystina essential oil and hydrosol extract. *Candida albicans* strains were the most sensitive microorganisms towards *N. amethystina* essential oil and hydrosol extract, which have the largest inhibition zones (>30mm) and the lower MICs values (<1.25%). The anti-mold activity, carried out by the diffusion method, showed that *N. amethystina* essential oil has a very interesting activity against *Aspergillus flavus* and *Cladosporium herbarium*.

INTRODUCTION

The genus *Nepeta* which belonged to Lamiaceae family is represented by more than 250 species distributed mainly in Africa, Europe and Asia (Mabberly, 1997). 7% of total Nepeta species growth wild in the Iberian Peninsula and North Africa, in which only five species exist in Algeria (Quézel and Santa, 1962; Greuter *et al.*, 1986). According to taxonomic revision of Ubera & Valdes (Castrillón and Jiménez, 1983), *N. amethystina* is a subspecies of *N. nepetella*. This medicinal plant is found in Saharan Atlas in dry stony places. Locally, *N. amehtystina* is named Gouzia and it's mostly used as a medicinal plant for its febrifuge, antirheumatic, antispasmodic and diuretic effects. The essential oils of some Nepeta species have been extensively studied, which are mainly characterized by nepetalactone (Dabiri and Sefidkon, 2003; Sefidkon *et al.*, 2006). The compounds 1,8-cineole and D-germacrene were also identified as the main constituent of the essential oils of *N. ispahanica* and *N. ucrainica* (Rustaiyan and Nadji, 1999; Javidnia *et al.*, 2005), respectively. Essential oil obtained from *N. amethystina* subsp. *amethystina* of Spanish origin showed that 4aα,7α,7aα-nepetalactone was the main constituents (41.29%) followed by 4aα, 7α, 7aβ-nepetalactone (29.42%) and an aldehyde not identified (5.30%) (Velasco–Negueruela *et al.*, 1989). To our knowledge there are no reports on the antimicrobial properties of *N. amethystina*. This study aimed the determination of chemical composition and antimicrobial activity of *N. amethystina* essential oil and hydrosol extract.

MATERIALS AND METHODS

Plant material

The aerial parts of *N. amethystina* were collected during flowering stage in June 2011 from Aïn-Safra region located in western Algeria. The plant material was identified by Dr. Boumediene Medjahdi according to (Quézel and Santa, 1962).

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A voucher specimen has been deposited in the Herbarium of the Laboratory of Botany, Department of Biology, Tlemcen University, Algeria.

Preparation of essential oil (EO) and hydrosol extract (HY)

EO from air-dried aerial parts (250g) was obtained by hydrodistillation for 5 h using a Clevenger type apparatus according to the (European-Pharmacopoeia, 2005). The oil was separated from hydrosol and dried over anhydrous sodium sulfate and stored in sealed vials at 4 C°. The 500 ml first liters of water hydro-distillation (hydrosol) were extracted three times with 100 ml of diethyl ether (Et_2O) by liquid-liquid extraction. The organic layer was dried over Na_2SO_4, evaporated at room temperature until obtained pure extract and stored in sealed vials at low temperature.

EO and HY analysis procedure

GC analyses were carried out using a Perkin Elmer Clarus 600 apparatus equipped with a dual flame ionisation detection system and two fused-silica capillary columns (60 m x 0.22 mm i.d., film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethyleneglycol). GC conditions used were: programmed heating from 60 °C to 230 °C at 2°C/min, followed by 35 min under isothermal conditions. The injector and the detector were maintained at 280 °C. Helium was the carrier gas at 1 ml/min; 0.2 µL of EO was injected in the split mode (1:50). EO and HY was analysed with a Perkin Elmer Turbo Mass detector, directly coupled to a Perkin Elmer Autosystem XL equipped with fused-silica capillary columns (60 m x 0.22 mm i.d., film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethyleneglycol). GC-MS (EI) conditions: Ion source temperature: 150°C; energy ionization: 70 eV; electron ionisation mass spectra were acquired oven the mass range 35-350 Da. Split: 1/80. Identification of the components was based i) on the comparison of their GC retention indices (RI) on non-polar and polar columns, determined relative to the retention time of a series of n-alkanes C9-C24 with linear interpolation, with those of authentic compounds or literature data; and ii) on computer matching with commercial mass spectral libraries (Hochmunth, 2001; Köning *et al.*, 2001) and comparison of spectra with those of our personal library "Aromes". Relative amount of individual components were performed on the basis of their GC peak areas on the two capillary Rtx-1 and Rtx-Wax columns, without FID response factor correction.

Isolation of nepetalactones and NMR analysis

The dichloromethane extract of aerial part of *N. amethystine* was subjected to flash chromatography (silica gel 200–500µm), eluting with different solvents of increasing polarity. We obtained several fractions with the highest was subjected to fine chromatography (silica gel 63–200µm).

Thus, we recovered 8 fractions. The sixth fraction (0.02 g) obtained with petrol ether/dichloromethane (75/25) was analyzed by NMR. The NMR spectra were recorded on a Bruker DPX 300 instrument in deuterated chloroform.

Antimicrobial activity

Microbial strains

N. amethystina EO and HY were evaluated against eight bacterial reference strains, which are *Escherichia coli* (*E. coli*) ATCC 25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, *Klebsiella pneumonia* (*K. pneumoniae*) ATCC 700603, *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Bacillus cereus* (*B. cereus*) ATCC 11778, *Bacillus subtilis* (*B. subtilis*) ATCC 6633, *Listeria monocytogenes* (*L. monocytogenes*) ATCC 19115 and against three different strains of *Candida albicans* (*C. albicans*) ATCC 10231, *C. albicans* ATCC 26790 and *C. albicans* IPP 444 (**I**nstitut **P**asteur **P**aris). The anti-mold activity was tested against two mold strains obtained from **N**ational **M**useum of **N**atural **H**istory of Paris (MNHN), which are *Aspergillus flavus* (*A. flavus*) MNHN 994294 and *Cladosporium herbarum* (*C. herbarum*) MNHN 3369.

Inoculums preparation

The inoculums of the bacterial and yeasts strains were prepared from overnight broth cultures. The suspensions were set to 0.5 McFarland or an optical density from 0.08 to 0.13 at 625 nm wavelength, which corresponds to 10^8 cfu/mL (CLSI, 2006).

Disc diffusion assay

EO and HY of *N. amethystina* were tested for their antimicrobial activity using the diffusion technique on solid media (Benbelaïd *et al.*, 2014). Discs of sterile Whatman paper (∅6mm) were impregnated with 4µl of extracts EO and HY, and then placed on dish plates of Mueller-Hinton Agar (Pronadisa™, Spain), which had been inoculated with an inoculum of 10^8 cfu/mL. The plates were then incubated for 24 h at 37°C for bacteria and 30°C for yeast. The results were recorded by measuring the growth inhibition zones surrounding the discs. All tests were performed in triplicate.

Determination of minimum inhibitory concentration (MIC)

The MIC was established by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2006).

DMSO was used to dissolve the EO and HY. A serial doubling dilution of the oil was prepared in a 96 well microliter plate over the range of 40.00-0.08 mg/mL inoculated in Mueller-Hinton broth. The MIC was defined as the lowest concentration of EO and HY that inhibiting visible growth. All tests were performed in triplicate.

Anti-mold activity (Agar dilution method)

The anti-mold activity of *N. amethystina* EO and HY was determinate using the agar dilution method (Hili *et al.*, 1997). Briefly, 6 mm agar discs of each mold cultures were deposited in the centre of Petri plate (90mm) containing 20 ml of Potato Dextrose Agar (Merck, France), with various concentrations of EO

and HY. The testing dishes were incubated at 25°C for 7 days, the anti-mold indices were calculated as follows:

$$AI\ (\%) = [(DC - DE) / DC] \times 100$$

DE: the diameter of growth zone in the experimental dish (mm);
DC: the diameter of growth zone in the control dish (mm)

Statistical analysis

Statistical analyses were performed withthe GraphPad Prism 5 software. Statistical comparisons were made with two-way ANOVA followed by Bonferroni's test. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

The plant extractions provide yellow pale oil by hydrodistillation which yield 0.60%, whereas hydrosol extract gave dark yellow oil yielded 0.39%. The chemical composition of EO and HY from *N. amethystina* was listed in table 1.

A total of thirty-four compounds were identified in *N. amethystina* EO and HY obtained from the aerial parts of *N. amethystina*, representing 96.77 and 80.07 % respectively. All components were identified by comparison of their EI–MS and GC-retention indices and mass spectral with those of the "Arômes" library. Oxygenated monoterpenes compounds were the major groups in EO and HY, while hydrocarbon monoterpenes and hydrocarbon sesquiterpenes were absent in HY. The major components were found to be 4aα, 7α, 7aβ-nepetalactone (72.40%, 49.21%) and 4aα, 7α, 7aα-nepetalactone (16.25%, 4.71%) of EO and HY respectively. This result contrasts with that obtained by Velasco–Negueruela et al. (1989). These authors have found cis-trans- nepetalactone (41.90%) as the main compound followed by the isomer trans-cis- nepetalactone (39.40%) of EO from *N. amethystina* of Spanish origin. The isomers nepetalactone finding in our work looks like the species of *N. argolic*, *N. nepetella* subsp. *aragonensis* and *N. nuda* (De Pooter *et al.*, 1987; Velasco–Negueruela *et al.*, 1998; Tzakou *et al.*, 2000). We note also the presence of myrtenal, carvacrol, β-farnesene, β-ionone, α muurolene and γ-cadinene which were reported for the first time in *N. amethystina* from Algeria. The antibacterial and anti-fungal activities of *N. amethystina* EO and HY were assessed by disc diffusion and MIC methods.

Table 1: Chemical composition of essential oil (EO) and hydrosol extract (HY) of Algerian *N. amethystina*.

Sl. No.	Components	RI[a]	RI[b]	EO	HY	Identification
1	(z)-Hex-2-en-1-ol	826	1405	Tr	-	RI, MS
2	α-Pinene	931	1022	0.20	-	RI, MS
3	Camphene	945	1066	Tr	-	RI, MS
4	Octan-3-one	959	1337	0.10	-	RI, MS
5	Sabinene	966	1120	Tr	-	RI, MS
6	β-Pinene	971	1110	0.41	-	RI, MS
7	p-Cymene	1014	1268	0.23	-	RI, MS
8	1,8-Cineole	1022	1209	0.45	2.25	RI, MS
9	(Z)-β-Ocimene	1024	1230	0.10	-	RI, MS
10	γ-Terpinene	1050	1243	Tr	-	RI, MS
11	Linalool	1086	1544	0.12	1.70	RI, MS
12	trans-p-Menth-2-en-1-ol	1123	1625	0.10	1.40	RI, MS
13	Camphor	1124	1517	0.21	1.31	RI, MS
14	Terpinene 4-ol	1166	1600	0.32	6.11	RI, MS
15	α-Terpineol	1181	1700	0.41	2.80	RI, MS
16	Myrtenal	1182	1498	0.21	0.11	RI, MS
17	trans-Piperitol	1189	1738	0.40	1.50	RI, MS
18	Linalyl acetate	1247	1565	Tr	-	RI, MS
19	**Thymol**	**1277**	**2189**	**2.30**	1.51	RI, MS
20	Bornyl acetate	1280	1575	0.50	-	RI, MS
21	**Carvacrol**	**1283**	**2219**	**0.35**	0.23	RI, MS
22	**4aα, 7α, 7aα-nepetalactone**	**1333**	**1953**	**16.25**	**4.71**	RI, MS, ^{1}H and ^{13}C NMR
23	**4aα, 7α, 7aβ-nepetalactone**	**1373**	**2019**	**72.40**	**49.21**	RI, MS, ^{1}H and ^{13}C NMR
24	(E)-β-Caryophyllene	1420	1591	0,60	0.31	RI, MS
25	α-Humulene	1453	1665	0.10	-	RI, MS
26	(E)-β-Farnesene	1452	1665	0.21	-	RI, MS
27	β-Ionone	1462	1923	Tr	-	RI, MS
28	α-Muurolene	1494	1716	0.10	-	RI, MS
29	γ-Cadinene	1509	1752	0.20	-	RI, MS
30	Elemol	1536	2076	Tr	2.21	RI, MS
31	Spathulenol	1560	2119	Tr	1.20	RI, MS
32	Caryophyllene oxide	1572	1980	0.40	1.31	RI, MS
33	τ-Cadinol	1631	2163	0.10	1.50	RI, MS
34	α-Cadinol	1643	2227	Tr	0.70	RI, MS
35	Total			96.77	80.07	
36	Yields % (w/w)			0.60	0.39	
37	Hydrocarbon monoterpenes			0,84	-	
38	Oxygenated monoterpenes			94,72	73.15	
39	Hydrocarbon sesquiterpenes			0,61	-	
40	Oxygenated sesquiterpenes			0,50	6,92	

Results are in percentage (%) of components of *N. amethystina* EO and HY. Percentages and elution order of individual components are given on no polar column. Retention indices nRI and pRI are given relative to C6 - C24 n-alkanes on no polar (Rtx-1) and polar (Rtx-Wax) columns. ID: identification method by comparison of (RI) retention indices and (MS) mass spectra. ^{1}H and ^{13}C NMR: proton and carbon nuclear magnetic resonance.

Table 2: Antibacterial and anti-yeast activities of *N. amethystina* essential oil (EO) and hydrosol extract (HY).

The microbial stumps	IZ		MIC	
	EO	**HY**	**EO**	**HY**
E. coli	11±1	9±1	20±0.000^{g}	40±0.000^{g}
P. aeruginosa	8±0	7±1	40±0.000	40±0.000
K. pneumoniae	8±1	7±1	40±0.000	40±0.000
E. faecalis	14±1	12±1	10±0.000^{h}	20±0.000^{h}
S. aureus	**22**±1^{a}	18±1^{a}	1.25±0.000	1.25±0.000
B. cereus	24±2^{b}	18±1^{b}	1.25±0.000^{i}	2.5±0.000^{i}
B. subtilis	20±1^{c}	16±1^{c}	1.25±0.000^{j}	2.5±0.000^{j}
L. monocytogenes	12±1	10±1	10±0.000^{k}	20±0.000^{k}
C. albicans IPP 444	37±2^{d}	32±1^{d}	0.520±0.180^{l}	1.25±0.000^{l}
C. albicans ATTC 10231	45±2^{e}	37±2^{e}	0.416±0.180	0.520±0.180
C. albicans ATTC 26790	40±1^{f}	35±2^{f}	0.625±0.000^{m}	1.25±0.000^{m}

All results shown in this Table are: Mean ± Standard Deviation (SD) of three repeats. The data were analysed by two-way ANOVA followed by Bonferroni's test. Values in the same row followed by the same letter are significantly different (P<0.001). **IZ**: inhibition zones in millimetre. **MIC**: minimal inhibitory concentration in mg/ml.

Table 3: Results of inhibiting effect of *N. amethystina* essential oil (EO) and hydrosol extract (HY) on mycelia growth.

Mold strain	Volume added	(AI) of EO%	(AI) of HY %
A. flavus MNHN 994294	10	4.75	12.5
	50	40.47	37.5
	100	**100**	**66.66**
C. Herbarum MNHN 3369	10	27.71	2.4
	50	68.67	45.78
	100	**79.51**	**61.44**

AI: anti-mould indice.

The EO and HY showed strong antimicrobial activity against microbial species (Table 2), especially against yeast. In most, the *Gram positive* bacteria are more sensitive to EO and HY than Gram negative ones. *B. cereus* was the most sensitive bacterial species, with inhibition zones larger than 18 mm. However, *P. aeruginosa* and *K. pneumoniae* appears resistant to *N. amethystina* EO and HY. While *C. Albicans* was very sensitive to both EO and HY with inhibition zones larger than 30 mm. The MICs of the studied oil and HY ranged between 0.416 and 40 mg/mL (Table 02). The lowest MICs were observed against *C. albicans* ATCC10231 with an MIC of 0.416 mg/ml for oil and 0.520mg/ml for HY. Against molds, EO and HY of *N. amethystina* have showed a good activity (Table 03). The EO exhibited more activity against A. *flavus* than *C. herbarum*. The antimicrobial activity of *N. amethystina* EO and HY may be related to their major monoterpenoid component i.e. nepetalactone, since it's known by its antimicrobial potency (Farag *et al.,* 1989). The antimicrobial activity of EOs is also due to minor components might contribute into antimicrobial activity (Kobaisy *et al.,* 2005). Indeed, the minor constituents like 1,8 cineol, terpinene 4-ol, α-terpineol, and thymol may involve in the antimicrobial activity of *N. amethystina* EO and HY (Tao *et al.,* 2014; Zhou *et al.,* 2014). Many previous studies have shown the relationship between the antimicrobial activity of EOs and their chemical composition (Deans and Svoboda, 1989; Farag *et al.,* 1989) and the role of synergy in the antimicrobial activity of EOs (Benbelaïd *et al.,* 2014). We also find that the EO is more active on microorganisms than the HY. This result is contrary to that of the literature that reports that the hydrosol rich in hydrophilic oxygen molecules exerts more activity than the oil rich in lipophilic compounds (Rose, 1999; Rao *et al.,* 2002). In this study, we have evaluated the antimicrobial activities of the EO and HY of *N. amethystina* harvested in Algeria. *N. amethystina* EO and HY were effective in growth inhibition of all tested strains. According to the extracts, EO was more active than the HY, but according to strains, *C. albicans* and *B. cereus* were shown to be more sensitive to both extracts. As a consequence this oil can be used as possible alternatives or complementary therapeutic agent against candidiasis.

Also, the oil can be useful for developing alternative compounds to preserved food contaminations caused by *B. cereus* and inhibits mold growths.

CONCLUSION

In conclusion, we show once again the interest of natural products in the control of microbial growth and especially the use of oil and its by-product of steam distillation hydrolat as antimicrobial agent's preservatives in agro- foods or as antiseptic agents in therapies.

Conflict of interest statement

We declare that we have no conflict of interest.

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