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Essential Oil Composition and Antimicrobial Activity of *Ballota nigra* L. ssp *foetida*

Daniele Fraternale, Anahi Bucchini, Laura Giamperi and Donata Ricci*

Istituto di Botanica e Orto Botanico "P. Scaramella", Facoltà di Farmacia,
Università degli Studi di Urbino "Carlo Bo", Urbino (PU) Italy

donata.ricci@uniurb.it

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The chemical composition of the essential oil of *Ballota nigra* L. ssp *foetida* obtained from the flowering aerial parts was analyzed by GC/MS. From the 37 identified constituents of the oil, β-caryophyllene (20.0%), germacrene D (18.0%) and caryophyllene oxide (15.0%) were the major components. The oil was active against both Gram-negative and Gram-positive bacteria as well as against three *Candida* species.

Keywords: *Ballota nigra* L., essential oil, caryophyllene oxide, antimicrobial activity.

The genus *Ballota* (Lamiaceae) comprises seven species which are widespread in Europe [1a]. *B. nigra* L. ssp *foetida*, commonly known as black horehound, is a small perennial herb, distributed in areas with mild climate; the flowering aerial parts are used for medicinal purposes [1b]. *Ballota* species have been traditionally used to cure nausea, vomiting and nervous dyspepsia, as well as a sedative, mild astringent and antibacterial agent [2a]. *B. nigra* ssp *foetida* is traditionally used internally as an infusion, either alone or mixed with other herbs due to its unpleasant smell, in the treatment of upset stomach, nausea and vomiting, in the symptomatic treatment of mild sleeping disorders, and for the symptomatic treatment of coughs. Externally, *B. nigra* is used in cases of gout [1b] and as a trophic protective and antimicrobial in case of wounds and sprains [2b]. Previous reported components from the aerial parts of the plant include flavonoids [3a,3b], phenylpropanoids [3b-3d], and volatiles [3e,3f].

We have studied the composition of the essential oil of *B. nigra* ssp *foetida* grown in northern Marche (central Italy) and its antimicrobial activity. Thirty-seven compounds were identified from the oil, representing 88.3 % of the total (Table 1). The main components were: β-caryophyllene (20.0%), germacrene D (18.0%), and caryophyllene oxide (15.0%). The essential oil of *B. nigra* ssp *foetida* was poor in monoterpenes, both hydrocarbons and oxygenated derivatives (about 3.8%). The percentage of sesquiterpenes was higher (68.7%), made up of several hydrocarbons (53.0%) and two oxygenated compounds, *trans*-nerolidol (0.7%) and caryophyllene oxide (15.0%). The only two previously reported studies regarding *B. nigra* ssp *foetida* essential oil are from plants grown in Iran [3f] and Pisa (Italy) [3e]. The same components in slightly different percentages were found in our oil in comparison with the Pisa grown plants. The Iranian oil was less similar, but the major components were sesquiterpenes (89.9%), although some of the major compounds recorded were lacking in the Italian oils [*epi*-α.-muurolol (6.6%), α-cadinol (6.3%), γ-amorphene (4.3%), aromadendrene (3.4%)].The main compound of the oil from Iran, caryophyllene oxide (7.9%), was also a substantial component of our oil (15.0%), although less so in the oil from Pisa (4.2%).

The diameters of the zones of inhibition (DI_s), minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) of *B. nigra* essential oil for the microorganisms tested are shown in Table 2. The essential oil showed inhibition against all the microorganisms tested; larger DI_s values are correlated with lower MIC_s. This correlation is more evident for Gram-positive bacteria and less so for fungi, whose MIC_s are more uniform.

Table 1: GC-MS analysis of *Ballota nigra* ssp *foetida* essential oil.

Compounds	*RI DB-5*	*RI HP-WAX*	*%*
(*E*)-2- Hexenal	856	1173	6.1
α -Tujene	932	1038	t
α –Pinene	940	1040	0.6
Benzaldehyde	963	1521	t
Sabinene	977	1132	t
1-Octen-3-ol	980	1396	6.8
3-Octanone	988	1242	t
3-Octanol	995	1395	t
p-Cimene	1028	1272	t
Limonene	1032	1207	t
(*z*)-Ocimene	1040	1250	0.2
Phenylacetaldehyde	1045	1574	0.5
γ-Terpinene	1063	1252	t
Linalool	1099	1541	1.5
Nonanal	1103	1354	1.5
cis-Verbenol	1143	-	t
(*E*)-2-Nonenal	1165	1440	0.5
4-Terpineol	1179	1603	0.5
α-Terpineol	1190	1668	0.7
Decanal	1205	1480	0.4
β-Cyclocitral	1223	1601	0.3
Eugenol	1357	2165	t
α-Copaene	1377	1491	2.5
β-Bourbonene	1385	1520	3.2
β-Cubebene	1390	1548	0.5
β-Elemene	1393	1592	0.5
α-Gurjunene	1409	1595	t
β-Caryophyllene	1419	1598	20.0
β-Gurjunene	1432	1660	t
α-Humulene	1456	1676	4.5
β-Chamigrene	1475	1740	0.8
Germacrene-D	1481	1699	18.0
Bicyclogermacrene	1495	1721	t
α-Selinene	1498	1742	1.5
δ-Cadinene	1523	1756	1.5
trans-Nerolidol	1564	2045	0.7
Caryophyllene oxide	1582	1966	15.0

t: traces (< 0.1%)
RI: Retention indexes, relative to the *n*-alkanes series, DB-5 column.

The essential oil of *B. nigra* exhibited significant antimicrobial activity against the organisms tested, particularly against Gram-positive bacteria. This was confirmed by MIC$_s$, MBC$_s$ and MFC$_s$. The data indicated that *S. aureus* was the most sensitive microorganism tested and *P. fluorescens* the least. The results of MIC indicated that *S. aureus* and *S. epidermidis* had the lowest MIC: 1.4 and 1.5 mg/mL respectively, whereas the highest was 7.5 mg/mL for *E. cloacae*. The lowest MBC was 1.5 mg/mL for *S. aureus*. *E. cloacae* and *P. aeruginosa* showed the highest MBC$_s$ of 12.0 mg/mL. MFC$_s$ for the three species of *C. albicans* tested were 15.0 mg/mL of the oil. In Table 3 is reported the antimicrobial activity of two positive controls, levofloxacin for bacteria and fluconazole for fungi.

The antimicrobial properties of the oil of *B. nigra* ssp. *foetida* may be associated with the presence of caryophyllene oxide (15.0%), which is known to possess antimicrobial activity [4a,4b]. In addition, components present in lower concentrations, such as *p*-cymene, linalool and eugenol, could also contribute

Table 2: Antimicrobial activity of the essential oil of *Ballota nigra* ssp. *Foetida*.

Microorganism ATCC	DI[a] mm	MIC mg/mL	MIC mg/mL
Bacteria			
Gram-negative			
Escherichia coli 25922	18 ± 1.1	3.3 ± 0.3	3.3 ± 0.2
Enterobacter cloacae 43560	14 ± 0.9	7.5 ± 0.9	12.0 ± 0.9
Pseudomonas aeruginosa 27583	15 ± 1.0	5.1 ± 0.5	12.0 ± 1.1
P. fluorescens 17400	9 ± 0.7	6.2 ± 0.6	10.0 ± 0.9
Gram-positive			
Staphylococcus aureus 25923	21 ± 1.7	1.4 ± 0.1	1.5 ± 0.1
S. epidermidis 35983	16 ± 1.3	1.5 ± 0.1	3.2 ± 0.2
Fungi			MFC mg/mL
Candida albicans 10231	15 ± 1.0	5.5 ± 0.4	15.0 ± 1.0
C. glabrata 15126	18 ± 1.1	5.5 ± 0.3	15.0 ± 1.1
C. tropicalis 20336	13 ± 0.9	5.5 ± 0.3	15.0 ± 0.9

DI: diameter of zone of inhibition, including disc diameter of 6 mm
a: Tested at a concentration of 3 mg/disc

Table 3: Antimicrobial activity of controls: levofloxacin for bacteria and fluconazole for fungi.

Microorganism ATCC	DI[c]mm	MIC μg/mL	MBC μg/mL
Bacteria			
Gram-negative			
Escherichia coli 25922	30 ± 2.6	0.6 ± 0.04	0.6 ± 0.05
Enterobacter cloacae 43560	17 ± 1.2	9.5 ± 0.07	20.2 ± 1.9
Pseudomonas aeruginosa 27583	25 ± 1.9	0.3 ± 0.02	0.6 ± 0.04
P. fluorescens 17400	13 ± 0.8	6.5 ± 0.3	20.6 ± 1.9
Gram-positive			
Staphylococcus aureus 25923	26 ± 1.9	0.4 ± 0.02	0.4 ± 0.02
S. epidermidis 35983	23 ± 1.2	0.6 ± 0.03	1.3 ± 0.08
Fungi			MFC μg/mL
Candida albicans 10231	21 ± 1.1	9.8 ± 0.7	18.5 ± 1.3
C. glabrata 15126	23 ± 1.3	9.8 ± 0.8	18.5 ± 1.2
C. tropicalis 20336	20 ± 1.7	9.8 ± 0.7	18.5 ± 1.3

c: tested at a concentration of 5 μg/disc

to the antimicrobial activity of the oils [5a,5b]. It is also possible that the components present in lower amounts might be involved in some type of synergism with the other active compounds [6].

Tables 4 and 5 show the results obtained from testing caryophyllene oxide alone and as a mixture with two of the minor compounds of the oil in the same percentages as those in the essential oil. Table 4 shows the antimicrobial activity of a 15% caryophyllene oxide solution in DMSO against all the microbial strains tested. The values reported show an antimicrobial activity that, although lower than that of *B. nigra* essential oil, is comparable with it. The addition of 1.5% linalool and 1.0% eugenol (minor compounds in our oil) to the 15% solution of caryophyllene oxide increased the antimicrobial activity of the solution (Table 5).

In conclusion, *B.nigra* essential oil possessed activity against a series of pathogens. This activity, although weaker in comparison to the tested standards (levofloxacin and fluconazole), might account for the use of this herbal drug for the treatment of various

Table 4: Antimicrobial activity of caryophyllene oxide (15.0% solution).

Microorganism ATCC	DI[a]mm	MIC mg/mL	MBC mg/mL
Bacteria			
Gram-negative			
Escherichia coli 25922	13 ±1.0	4.5 ± 0.3	4.8 ± 0.3
Enterobacter cloacae 43560	10 ± 0.1	8.1 ± 0.7	13.2 ± 1.0
Pseudomonas aeruginosa 27583	10 ± 0.9	6.7 ± 0.5	13.7 ± 1.0
P. fluorescens 17400	5 ± 0.4	8.2 ± 0.7	12.5 ± 1.0
Gram-positive			
Staphylococcus aureus 25923	16 ± 1.0	3.4 ± 0.2	4.6 ± 0.4
S. epidermidis 35983	10 ± 0.9	2.8 ± 0.2	5.5 ± 0.45
Fungi			MFC mg/mL
Candida albicans 10231	11 ± 1.1	6.5 ± 0.5	17.0 ± 1.4
C. glabrata 15126	13 ± 1.0	6.8 ± 0.5	17.0 ± 1.4
C. tropicalis 20336	11 ± 1.0	6.8 ± 0.5	17.0 ± 1.3

DI: diameter of zone of inhibition including disc diameter of 6 mm
a: Tested at a concentration of 3 mg/disc

Table 5: Antimicrobial activity of the mixture: caryophyllene oxide 15.0%, linalool 1.0% and eugenol 1.0%.

Microorganism ATCC	DI[a]mm	MIC mg/mL	MBC mg/mL
Bacteria			
Gram-negative			
Escherichia coli 25922	20 ± 2.0	2.9 ± 1.9	3.0 ± 0.3
Enterobacter cloacae 43560	15 ± 1.0	7.1 ± 0.6	11.9 ±1.0
Pseudomonas aeruginosa 27583	17 ± 1.1	4.8 ± 0.3	11.2 ±1.0
P. fluorescens 17400	11 ± 0.9	5.9 ± 0.4	10.0 ± 1.0
Gram-positive			
Staphylococcus aureus 25923	24 ± 1.9	1.5 ± 0.09	1.5 ± 0.09
S.epidermidis 35983	19 ± 1.4	1.0 ± 0.08	2.9 ± 0.2
Fungi			MFC mg/mL
Candida albicans 10231	23 ± 1.8	4.7 ± 0.3	13.7 ± 1.0
C. glabrata 15126	25 ± 1.9	5.1 ± 0.5	13.2 ± 1.0
C. tropicalis 20336	23 ± 1.7	4.7 ± 0.4	13.9 ± 1.0

DI: diameter of zone of inhibition including disc diameter of 6 mm.
a: Tested at a concentration of 3 mg/disc.

types of external infectious diseases. The popular use of this species as an anti-infective agent may be justified also by the presence of other non-volatile constituents, such as phenylpropanoid glycosides [3d], which possess moderate antimicrobial activity.

Experimental

Plant material: Aerial parts of *B. nigra* ssp *foetida* were collected during the flowering stage in July 2007 on the “San Cipriano” mountain, near Urbino (Italy), 750 m above sea level. The plant was identified by D. Ricci, Botany Professor, Urbino University. A voucher specimen (BN-63) is kept at the Herbarium of the Botany Institute.

Chemical analysis: A sample (400 g) of fresh plant material was coarsely ground and hydro-distilled for 2 h using a Clevenger-type apparatus (yield 0.06 % w/w). The essential oil was dried over anhydrous sodium sulfate. GC analyses were performed with a HP-5890 instrument equipped with a HP-WAX capillary column (30 m x 0.25 mm, 0.25 µm film thickness), working with the following temperature program: 60°C for 10 min, rising at 5°C/min to 220°C; injector and detector temperatures, 250°C; carrier gas, nitrogen (2 mL/min); detector, dual FID; split ratio, 1:30; injection (0.5 µL). The identification of the components was performed by comparison of their retention times with those of pure authentic samples and by means of their linear retention indices relative to a series of *n*-hydrocarbons. The relative proportions of the constituents were percentages obtained by FID peak-area normalization, all relative response factors being taken as one.

GC-EIMS analyses were performed with a HP 6890 gas chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm, coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures, 220 and 240°C, respectively; oven temperature programmed from 60°C to 240°C at 3°C/min; carrier gas, helium at 1 mL/min; injection 0.2 µL (10% *n*-hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, by comparison of calculated retention indices with those reported in the literature and by comparison with mass spectra from the Nist98 Mass Spectral Database. [3e,7a,7b].

Antimicrobial activity: *In vitro* antibacterial activity of the oil was carried out according to the disc agar diffusion method [8a,8b]. Antibacterial activity was tested against four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 43560), *Pseudomonas aeruginosa* (ATCC 27583) and *P. fluorescens* (ATCC 17400); and two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *S. epidermidis* (ATCC 35983). These microorganisms were grown in nutrient broth for 24 h and were used as inoculum.

The bacterial inoculum (2 x 10^8 CFU/mL) was streaked over the surface of Mueller-Hinton solidified agar medium into the sterile plates 90 mm in diameter. Filter paper discs (6 mm diameter Schleicher & Schuell) were individually impregnated with 3 mg of the oil, or 3 mg of the 15.0% caryophyllene oxide solution in DMSO, or 3 mg of a mixture of 15.0% caryophyllene oxide, 1.5% linalool and 1.0% eugenol in DMSO. (15 µL of the diluted oil or 15 µL of the diluted caryophyllene oxide solution or 15 µL of the diluted mixture aliquots from 200 mg/mL in DMSO). The discs were then aseptically applied to the surface of the agar plates at well-

spaced intervals. The plates were incubated at 37°C for 24 h and inhibition zones, including the diameter of the dishes, were measured. Control discs impregnated with aqueous solution of levofloxacin (5 μg/disc), were used as reference for bacteria.

The antifungal activity of the oil was carried out as above. The pathogenic fungi: *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 15126) and *C. tropicalis* (ATCC 20336) were cultured in modified Sabouraud's agar. The oil was tested as above and fluconazole (5 μg/disc) was used as reference. Both the assays were performed in triplicate and the results are the mean values ± standard deviations.

***Determination of MIC, MBC and MFC*:** The determinations of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), were performed by the broth microdilution method [9,10]. For bacteria, all tests were performed in Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/v); for fungi in Sabouraud's broth under the same conditions. Serial doubling dilutions of the oil prepared in a 96-well microtiter plate ranged from 0.05 to 200.00 mg/mL of the oil, or the caryophyllene oxide solution or the mixture. The final concentration of each microorganism was adjusted to 4 X 10^4 CFU/mL. Plates were incubated at 37°C for 24 h. The MIC was determined as the concentration of essential oil with no visible growth. MBC was determined taking broth from each well and incubating in Mueller Hinton Agar or Sabouraud's Agar at 37°C for 24 h. The MBC and the MFC were calculated as the lowest concentrations of oil which inhibited the recovery of bacteria or fungus. All the assays were performed in triplicate and the results are the mean values ± standard deviations.

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