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ORIGINAL PAPER

Composition and antimicrobial activity of *Cistus munbyi* essential oil: an endemic plant from Algeria

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Abstract The chemical composition and antimicrobial potential of *Cistus munbyi* essential oil were studied for the first time. GC and GC–MS analysis revealed 48 compounds representing 96.98% of the oil. Terpinen-4-ol (23.75%), *meta*-Cymene (17.30%), and Sabinene (12.38%) were the major constituents. Antimicrobial activity was evaluated against nine pathogens using the disc diffusion and broth micro-dilution methods. Results show that *C. munbyi* essential oil possesses strong antimicrobial activity against all strains, regardless if Gram-positive or Gram-negative bacteria, or yeast, with MICs values not exceeding 10 (mg/ml). In addition to its efficacy, *C. munbyi* essential oil has an unusual antimicrobial potency which is attributed to its specific chemical composition. Thus, findings presented here suggest that endemic *C. munbyi* contains a very interesting essential oil that may be valuable in several areas.

Keywords Antimicrobial activity · Chemical composition · *Cistus munbyi* · Essential oil

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Introduction

In recent years, considerable academic research has been focused on medicinal plants due to their biological potential, including antioxidant activities (Krishnaiah et al. 2011), biocidal effects against microbes (Silva and Fernandes Júnior 2010; Saleem et al. 2010), and numerous medicinal properties. The antimicrobial activities by molecules from natural sources is currently receiving the most attention as there is an increased need for new antimicrobial agents in several domains, notably for antibiotics (Saleem et al. 2010). This is of paramount importance, given the worldwide problems of healthcare associated infections (Högberg et al. 2010) and because of a lack of novel antibiotics currently available (Cheng et al. 2009).

Essential oils are interesting plant secondary metabolites that possess several biological proprieties (Kalemba et al. 2012). The antimicrobial potential of essential oils may address all classes of microorganisms as well as those volatile molecules is able to eradicate pathogens at their planktonic and biofilm states (Kavanaugh and Ribbeck 2012; Jadhav et al. 2013; Benbelaïd et al. 2014). Thus, antimicrobial potencies of essential oils may be valuable in human medicine and in various applications such as for food conservation (Benchaar et al. 2008) and oral health (Van Leeuwen et al. 2011).

Endemic plants, including aromatic ones, appear as a possible source for new substances that, perhaps, possess biological activities. This is the reason that interests scientists in researching the chemical composition and biological activities of unstudied botanical species, in order to discover new medicinal compounds that may have value in pharmacy and in other fields. To this end, essential oils are among plant extracts targeted in recent research because of their richness of molecules and their essence in several therapeutic impacts (Miguel 2010; Lang and Buchbauer 2012).

The genus *Cistus* L. (Cistaceae) consists of 21 species distributed primarily in the Mediterranean area. Most *Cistus* species are widespread, but a few are endemics with a small distribution (Guzmán and Vargas 2005). *Cistus munbyi* Pomel (*C. munbyi*) is one of these endemics species, known also as *Cistus sericeus* Munbyi and described as rare from Algeria's extreme northwest and north eastern Morocco (Quézel and Santa 1962). Known as "Chaboba elfediya", *C. munbyi* is a medicinal plant widely used by local populations against a variety of infectious diseases such pulmonary infections.

In the absence of any pharmacological studies that evaluate *C. munbyi*, this research aims to identify the chemical composition and assess the antimicrobial activity of the essential oils obtained from this species for the first time.

Fig. 1 *Cistus munbyi* Pomel at full inflorescence

Materials and methods

Plant material

Arial parts of *C. munbyi* (Fig. 1) were collected during June 2011 at full inflorescence in the coastal region of Honaine, located in northwest Algeria at 35°17′23″North and 1°66′36″West. A specimen sample was deposited at LAMAABE (Laboratoire de Microbiologie Appliquée à l'Agroalimentaire, au Biomédical et à l'Environnement) laboratory, Tlemcen University under code CS-BF070611. The plant material was identified in the Laboratory of Ecological Management of Natural Ecosystems at the same institution. Collected plant material (approximately three kilograms of leaves, flowers, and seeds) was dried by spreading in the open air and away from sunlight for 10 days.

Obtaining essential oil

The dried aerial parts were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus according to European pharmacopoeia (European-Pharmacopoeia 2005). The extraction yielded 0.8% of yellow oil that was collected and dried over anhydrous sodium sulphate, then stored at 4 °C until analysis.

GC and GC/MS condition

Gas chromatography (GC) analysis was performed using a Perkin Elmer Autosystem GC-type chromatograph equipped with two flame ionization detectors (FID) for the detection of volatile compounds, one injector/splitter and two polar (Rtx-Wax, polyethylene glycol) and nonpolar (Rtx-1, polydimethylsiloxane) columns (60 m × 0.22 mm inner diameter, film thickness 0.25 µm). Helium was the carrier gas (1 ml/min) with a column head pressure of 25 psi. The injector temperature was 250 °C and that of the detector was 280 °C. The temperature was programmed to increase from 60 to 230 °C at the rate of 2 °C/min, and then kept constant for 45 min at 230 °C. The injection was done by split mode with a split ratio of 1/50. The amount of essential oil injected was 0.2 µl. Quantification was made by the direct electronic integration of peak areas.

GC/MS (gas chromatography–mass spectrometry) analysis was similar to the preceding except that a Perkin Elmer Autosystem XL chromatograph was used along with a Perkin Elmer TurboMass mass detector. The carrier gas

was again helium (1 ml/min) with a column head pressure of 25 psi, injector temperature of 250 °C and programmed to rise from 60 to 230 °C at the rate of 2 °C/min, and then kept constant for 35 min at 230 °C. The injection was done by split mode but with a split ratio of 1/80. The amount of essential oil injected was 0.2 µl. Detection was carried out by a quadrupole analyser which consisted of an assembly of four parallel electrodes with a cylindrical section. The source temperature was 150 °C. The device functioned in electron impact and fragmentation was performed at an electric field of 70 eV.

Two methods were used to identify the essential oil constituents. Mass spectra databases were compared with those of computerized libraries (Adams 2007), and the Kovats index was used in which polar and nonpolar retention indices were calculated relative to retention times of a series of *n*-alkanes (Kovats 1958).

Antimicrobial activity determination

Microbial strains

Nine microbial strains have been used in this study, varying from a fungus strain to bacterial species representing various infection sources. The fungal strain was yeast, *Candida albicans* ATCC 10231 (*C. albicans*) while the bacterial strains included four Gram-negative ones: *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*); *Escherichia coli* ATCC 25922 (*E. coli*); *Salmonella enteritidis* ATCC 2453 (*S. enteritidis*); and, *Klebsiella pneumoniae* ATCC 70603 (*K. pneumoniae*), and four Gram-positive ones: *Enterococcus faecalis* ATCC 29212 (*E. faecalis*); *Staphylococcus aureus* ATCC 25923 (*S. aureus*); *Listeria monocytogenes* ATCC 19115 (*L. monocytogenes*); and, *Bacillus cereus* ATCC 11778 (*B. cereus*).

Inoculums preparation

The microbial strains were revivified and purified, then inoculated into tubes with 5 ml of Mueller-Hinton broth (Fluka®, India) for bacteria and Sabouraud broth (Fluka®, India) for yeast at 37 °C for 24 h. After incubation, suspensions were prepared through the standardization of cultures at an optical density of 0.08 to 0.13 at 625 nm wavelength, which corresponds to 0.5 McFarland (CLSI 2006). The inoculums final concentration was be 10^8 CFU/ml.

Paper disc diffusion

Kirby-Bauer's agar disc diffusion method (Bauer et al. 1966) was used for the initial overview of the antimicrobial potential of *C. munbyi* essential oil. Standardized suspensions were inoculated homogeneously using a swab on Petri dishes filled with Mueller-Hinton agar (Fluka®, India) for bacteria and Sabouraud agar (Fluka®, India) for yeast. Filter paper discs (6 mm diameter) were impregnated with 2 µL of the essential oil and placed on the inoculated Petri dishes. Discs of Gentamicin (10 µg per disc, Oxoid, England) and Amphotericin B (100 µg per disc, Sanofi diagnostics Pasteur, French) were also tested in the same way and served as positive controls for bacterial and fungal strains respectively. The results were analysed after 24 h of incubation at 37 °C according to the width of the inhibition zones around the discs, measured in mm by Vernier scale. All tests were performed in triplicate.

Minimum inhibitory concentration determination

The minimum inhibitory concentrations (MICs) of C. *munbyi* essential oil with the microbial strains were determined by the micro dilution method (Benbelaïd et al. 2014) modified from Wiegand et al. (2008). In the first step, 10 dilutions of essential oil were prepared in sterile laboratory tubes using Two-fold serial dilutions. Oil dilution was carried out in a mixture of culture media, Mueller-Hinton broth for bacteria and Sabouraud broth for yeast, with 1% of Twin 80 (Polysorbate 80). A 96-well microplate was prepared by distributing 90 µl of 5×10^5 CFU/ml inoculums (prepared by 1/200 dilution of 10^8 CFU/ml inoculums) with 10 µl of each concentration; the 10 final concentrations of essential oil in the wells ranged from 4.0 to 0.007% (v/v) and the final concentration of Twin 80 was 0.1% in each well. The MIC was defined as the lowest concentration of essential oil inhibiting visible growth. Experimental control was performed according to CLSI (2010) using Gentamicin and Amphotericin B (Calbiochem®, Germany) for bacterial and fungal strains respectively. All tests were performed in triplicate.

Results and discussion

Chemical composition of the essential oil

Qualitative and quantitative analytical results by GC and GC–MS are shown in Table 1. A total of 48 compounds (96.98%) were identified using two chromatographic techniques. *C. munbyi* essential oil is constituted principally by monoterpenes, especially hydrocarbons (more than half of the total oil volume), and oxygenates (more than a quarter of the oil). Other compounds in trace amounts include phenylpropanoids, hydrocarbon sesquiterpenes, and oxygenated sesquiterpenes. In general, there is no principal compound in essential oil but rather an equivalent mixture of Terpinen-4-ol (23.75%), *meta-*

Table 1 Chemical composition of *C. munbyi* essential oil

#	Components	IRa	IRp	%	Identification
1	α-Thujene	922	1023	5.22	RI, MS
2	α-Pinene	931	1022	1.86	RI, MS
3	2,4(10)-Thujadiene	942	1205	0.10	RI, MS
4	Camphene	943	1066	2.60	RI, MS
5	Sabinene	964	1120	12.38	RI, MS
6	*β*-Pinene	970	1110	0.81	RI, MS
7	Dehydro-1,8-cineole	979	1197	0.34	RI, MS
8	α-Phellandrene	997	1164	0.10	RI, MS
9	α-Terpinene	1008	1178	0.25	RI, MS
10	*meta*-Cymene	1010	1269	17.30	RI, MS
11	*para*-Cymene	1011	1268	0.25	RI, MS
12	Limonene	1020	1199	0.26	RI, MS
13	1,8-Cineole	1020	1209	0.23	RI, MS
14	*γ*-Terpinene	1047	1243	1.54	RI, MS
15	*trans*-Sabinene hydrate	1051	1451	5.62	RI, MS
16	Linalool oxide	1057	1435	0.10	RI, MS
17	Terpinolene	1078	1280	0.47	RI, MS
18	Linalool	1081	1544	0.15	RI, MS
19	*cis*-Sabinene hydrate	1083	1345	4.00	RI, MS
20	α-Thujone	1089	1396	0.14	RI, MS
21	*β*-Thujone	1103	1422	0.24	RI, MS
22	Camphor	1123	1517	1.65	RI, MS
23	(*E*)-Verbenol	1127	1655	1.51	RI, MS
24	(*Z*)-Verbenol	1129	1676	0.55	RI, MS
25	Borneol	1148	1698	0.86	RI, MS
26	Terpinen-4-ol	1161	1600	23.75	RI, MS
27	Myrtenol	1177	1789	0.38	RI, MS
28	α-Terpineol	1179	1700	1.02	RI, MS
29	(*E*)-Piperitol	1182	1457	0.25	RI, MS
30	γ-Terpineol	1183	1702	0.74	RI, MS
31	(*Z*)-Carveol	1196	1832	0.57	RI, MS
32	Cuminaldehyde	1217	1782	0.72	RI, MS
33	Linal1yl acetate	1240	1557	0.56	RI, MS
34	Lyratyl acetate	1256	1630	0.41	RI, MS
35	*p*-Cymen-7-ol	1266	1690	1.47	RI, MS
36	Bornyl acetate	1269	1515	0.40	RI, MS
37	*iso*-Bornyl acetate	1272	1581	0.41	RI, MS
38	Carvacrol	1278	2219	0.40	RI, MS
39	Terpinen-4-ol acetate	1282	2250	0.30	RI, MS
40	Perillyl alcohol	1284	2005	0.51	RI, MS
41	α-Terpinyl acetate	1334	1695	2.63	RI, MS
42	α-Ionone	1407	1848	0.13	RI, MS
43	*γ*-Himachalene	1479	1693	0.10	RI, MS
44	*cis*-Calamenene	1512	1816	1.01	RI, MS
45	α-Calocarene	1531	1895	0.11	RI, MS
46	Caryophyllene oxide	1576	1980	2.35	RI, MS
47	Viridiflorol	1591	2089	0.11	RI, MS
48	Cadalene	1654	2203	0.12	RI, MS
Total identified (%)				96.98	
Monoterpene hydrocarbons				44.79	

Table 1 continued

#	Components	IRa	IRp	%	Identification
	Oxygenated monoterpenes			47.54	
	Phenylpropanoids			0.72	
	Sesquiterpene hydrocarbons			1.47	
	Oxygenated sesquiterpenes			2.46	

Results are in percentage (%) of components. Percentages and elution order of individual components are provided in the nonpolar column. Retention indices nRI and pRI are shown respectively in the nonpolar (Rtx-1) and polar (Rtx-Wax) columns. ID: identification method by comparison of (RI) retention indices and (MS) mass spectra

Cymene (17.30%), and Sabinene (12.38%). There is a significant amount of *trans*-Sabinene hydrate (5.62%), *alpha*-Thujene (5.22%), and *cis*-Sabinene hydrate (4%), and to a lesser degree, *alpha*-Terpinyl acetate (2.63%), Camphene (2.60%), and Caryophyllene oxide (2.35%).

Antimicrobial activity

Diameters of inhibition zones and minimum inhibitory concentrations (MICs) are shown in Table 2. *C. munbyi* essential oil has demonstrated good antimicrobial activity against majority of the studied strains, and produced wide zones of inhibition ranging from 8 ± 0 mm up to 22 ± 1 mm. MICs values ranged between (02.10 ± 0.07/ 10.00 ± 0.00) mg/mL. With the disc diffusion method, *C. munbyi* essential oil produced the largest inhibition zones (>20 mm) against *B. cereus* and *S. aureus*, while *L. monocytogenes* and *P. aeruginosa* appeared less sensitive l with the smallest inhibition zones recorded. However, quantitative analyses (MICs determination) demonstrated that *C. munbyi* essential oil has almost equivalent activity against all studied strains, with an average of ≈7 mg/mL.

According to the literature, essential oils are more active on Gram-positive bacteria than on Gram-negative ones (Burt 2004). This is fundamentally due to the physiological difference between the outer membranes of Gram-negative bacteria, in which the walls are more impermeable to essential oil than the walls of Gram-positive ones. In contrast, we found that some Gram-negative species were more sensitive to *C. munbyi* oil than Gram-positive bacteria, for example, *E. coli* and *S. enteritidis* compared to *E. faecalis*. Another significant result recorded in this study was that the essential oil was active against *P. aeruginosa*. While this bacterium species is resistant to many, perhaps all known essential oils (Khadir et al. 2013; Kalemba et al. 2012). *P. aeruginosa* resistance against most of essential oils is due to its specific outer membrane (Mann et al. 2000).

The chemical analysis of *C. munbyi* essential oil may explain the reason for its antimicrobial activity. When

Table 2 Antimicrobial activity of *C. munbyi* essential oil

Strains	Disc diffusion methods (mm)			MIC determination		
	E. coil (2 µl)	Antibiotics		*E. coil* (mg ml^{-1})	Antibiotics (µg ml^{-1})	
		Gen[a] (10 µg)	AmB[b] (100 µg)		Gen[a]	AmB[b]
B. cereus	22 ± 1	21 ± 1	na	10.00 ± 0.00	4.00 ± 0.00	na
C. albicans	12 ± 1	Na	19 ± 1	10.00 ± 0.00	na	1.00 ± 0.00
E. faecalis	10 ± 1	20 ± 1	na	10.00 ± 0.00	8.00 ± 0.00	na
E. coli	12 ± 0	21 ± 1	na	02.50 ± 0.00	0.50 ± 0.00	na
K. pneumoniae	12 ± 1	20 ± 1	na	10.00 ± 0.00	2.00 ± 0.00	na
L. monocytogenes	08 ± 0	20 ± 1	na	05.00 ± 0.00	2.00 ± 0.00	na
P. aeruginosa	08 ± 0	18 ± 0	na	10.00 ± 0.00	1.33 ± 0.58	na
S. enteritidis	11 ± 1	20 ± 1	na	05.00 ± 0.00	2.00 ± 0.00	na
S. aureus	20 ± 1	21 ± 2	na	02.10 ± 0.07	0.25 ± 0.00	na

Results shown are mean ± standard deviation of three replicates

na not applicable

[a] Gentamicin

[b] Amphotericin B

evaluating its chemical composition, such activity may be due to its Terpinen-4-ol content, since this substance is responsible for the antimicrobial potency of several known plant essential oils, such as *Melaleuca alternifolia* (Carson et al. 2006; Hammer et al. 2012). However, the unusual antimicrobial activity of *C. munbyi* oil suggests there may be synergetic effects amongst the oil components. This may strengthens the theory that some essential oils in their entirety are more interesting than their component monoterpenes alone (Benbelaïd et al. 2014). Furthermore, the significant antimicrobial activity of *C. munbyi* oil on *P. aeruginosa* (MIC = 10 mg mL^{-1}) supports this proposition, since this bacterial species display an intrinsic resistance to a wide variety of essential oils (Kalemba et al. 2012).

Conclusion

This is the first academic study on the essential oil of *C. munbyi*, an endemic medicinal plant from Algeria. Extraction of the essential oil yielded 0.8% (w/w) and 48 compounds some 96.98% of the total extract. Overall results indicate that the essential oil shows not only a broad spectrum of antimicrobial activity against all referenced strains, but also a specific and interesting activity that characterizes this oil. According to the findings in this study, *C. munbyi* essential oil is a potential new source of natural antimicrobial agent.

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