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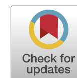
Original article

Antimicrobial activity and chemical composition of essential oil from *Helichrysum microphyllum* Cambess. subsp. *tyrrhenicum* Bacch., Brullo & Giusso collected in South-West Sardinia



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ABSTRACT

The aim of this study was to evaluate the chemical composition and the antimicrobial activity of essential oils of *Helichrysum microphyllum* subsp. *tyrrhenicum* collected in four different stations in South-Western Sardinia.

The composition of the essential oils was determined by gas chromatography and gas chromatography/mass spectrometry. The oil samples showed different chromatographic profiles. The oil of the station 4 revealed the presence of significant amount of neryl acetate (33.6%); in oils from stations 1 and 2 we found γ-curcumene (28%) and in station 3 γ-curcumene (12%) and linalool (11%), while there was no trace of neryl acetate. Standard microbiological assays demonstrated that essential oils obtained by plants collected in station 1 and 2, very rich in curcumene, showed an interesting anticandidal activity, dose- and time-dependent, which is enhanced by sub-inhibitory concentrations of chitosan.

Our results suggest that the essential oil of *Helichrysum microphyllum* subsp. *tyrrhenicum,* associated with chitosan in innovative formulations, could be considered as a therapeutic alternative in the treatment of *Candida* opportunistic infections. The results of this study shows that the chemotypization of the species examined could lead to their targeted clinical use, in a concept of a rational scientific aromatherapy.

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1. Introduction

The genus *Helichrysum* Mill. is currently widely distributed in Africa, Madagascar, the Mediterranean basin, Macaronesia, central Asia and India, and, according to different authors, it includes ca 500 to ca. 600 species (Hilliard, 1983; Anderberg, 1991). The Mediterranean, European, Western and Central Asia *Helichrysum* species have been classified into two main groups: *Helichrysum* sect. *Helichrysum,* mainly equivalent to de Cadolle's unranked group *Stoechadina*, and *Helichrysum* sect. *Virginea* (DC) Gren & Gord. All species belonging to the *H.* sect. *Helichrysum* are shrubs or herbaceous perennials with capitula in terminal corymbose yellow inflorescences, arachnoid to densely tomentose. A taxonomic revision of *Helichrysum* sect. *Stoechadina* has been proposed (Galbany-Casals et al., 2006). Each one of them possesses several vegetative stems that can be erect, ascendant, or decumbent, and leafy throughout their length.

Helichrysum microphyllum Cambess. subsp. *tyrrhenicum* Bacch., Brullo & Giusso (Angiolini et al., 2005), synonym of *Helichrysum italicum* subsp. *microphyllum*, and *H. italicum* subsp. *siculum* nearly always bear axillary leaf fascicles on the vegetative stems. On the other hand, intermediate specimens between subsp. *italicum* and *H. microphyllum* subsp. *tyrrenicum* (as regards the height of the plant, the length of its leaf and the presence of axillary leaf-fascicles on vegetative steams) are very frequent in areas where they co-occur. Particularly in Southern Corsica, we can easily find specimens exhibiting all the degrees of morphological variation

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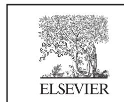

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between these two subspecies. *H. microphyllum* subsp. *tyrrenicum* is wide spread, occupying diverse open habitats, including shrubby formations in the mountain regions, road banks and path margins, maritime rocks and sand dunes. With an altitudinal range 0–1950 m. *H. microphyllum* subsp. *tyrrenicum* presents a disjoint geographical distribution among the Mediterranean islands of Majorca, Corsica, Sardinia, Crete and Cyprus (Galbany-Casals et al., 2006).

In Sardinia, *H. microphyllum* subsp. *tyrrenicum* was abundantly used as a dermo-protective product and in the formulation of tonic baths. It is an extraordinary component with multiple health benefits due to its numerous properties, such as strengthening hair, fighting hair loss, burn relief, choleretic, diuretic, expectorant, analgesic, antirheumatic and used against headaches (Atzei, 2003). It is to remember that *H. microphyllum* subsp. *tyrrenicum* is a synonimum of *H. italicum* subsp. *mycrophyllum;* for this reason we can referred to previous papers on this specie. Scientific studies on this plant also demonstrated its anti-oxidant (Rosa et al., 2007), anti-inflammatory (Guinoiseau et al., 2013), antiviral (Nostro et al., 2003) and anti-HIV activity (Appendino et al., 2007). Most of the species have been studied for their content of essential oils (Maoddi and Usai, 1997; Usai et al., 2000; Chessa et al., 2000; Angioni et al., 2003; Bianchini et al., 2003; Paolini et al., 2006; Perrini et al., 2009; Usai et al., 2010; Ornano et al., 2015).

Currently, many researches have reported the antimicrobial activity of other species of *Helichrysum* genus, but limited data are available on different extracts of *Helichrysum italicum,* documenting an effect against Gram + bacteria and mycetes (Nostro et al., 2000; 2001; 2004; Mastelic et al., 2005; Tundis et al., 2005; Rossi et al., 2007; Stupar et al., 2014).

Therefore, the main aim of our study was to estimate the antimicrobial potential of different samples of essential oils obtained from *H. microphyllum* subsp. *tyrrhenicum* collected in different South-West Sardinian locations; we particularly focused on the anticandidal activity of these oils, since *Candida* is now recognized as an increasingly important human pathogen responsible for severe infections, especially in immunocompromised hosts (Deng et al., 2010; Pakfetrat et al., 2015). Moreover, we investigated an eventual synergistic interaction between *H. microphyllum* subsp. *tyrrhenicum* essential oils and chitosan, a biopolymer obtained from partial deacetylation of chitin that possesses, among others, antifungal and antioxidant properties (Gavhane Yogeshkumar et al., 2013) and is able to enhance the anticandidal activity of Tea Tree essential oil (Juliano et al., 2008). Afterwards, the chemical composition of the essential oils we tested was determined by GC and GC/MS in the attempt to relate differences in antimicrobial activity to the presence of specific components.

2. Materials and methods

2.1. Plant species collection and identification

H. microphyllum subsp. *tyrrhenicum* was collected at flowering stage (June 2014) in South-Western Sardinia by Giorgio Congiu. Voucher specimens were deposited at the Herbarium S.A.S.S.A. (identified by M. Usai; cumulative identification number: 729) of the Department of Chemistry and Pharmacy, University of Sassari. The four stations are all located in South Western Sardinia and include an area particularly rich in essences. Station 1 is located 4 km far from Iglesias and 135 m above sea level; its soil is characterized by the presence of landfilled mining materials containing lead mixed with zinc. Station 2 is located 10 km far from Carbonia and 90 m above sea level; the soil is rich in argentiferous lead ores. Station 3 (Portoscuso area) is located 18 km far from Carbonia and around 100 m above sea level; its subsoil was exploited for the presence of coal. Station 4 is located 19 km far from Oristano and its subsoil is mostly alluvial.

2.2. Oil distillation and yield

For every station, we submitted an amount of 10 kg of plant material for 3 h to hydrodistillation using a crafted extractor; the reached yields were between 0.17 and 0.19% (w/w). The oils were separated from the water by decantation and stored at −20 °C until analysis. To confirm the composition and yields of the essential oils, for every station we also extracted a sample of plant material, following the indications of the Italian Pharmacopeia (FUI) (Commissione permanente per la revisione e la pubblicazione della Farmacopea ufficiale, 2008). An amount of 300 g of plant material was submitted for 4 h to hydrodistillation using a Clevenger-type apparatus; the reached yields were between 0.19 and 0.20% (w/w). The oils were dried over anhydrous sodium sulphate and stored at −20 °C until analysis.

2.3. Oil analyses

2.3.1. Gas chromatography

Three replicates of each sample were analyzed by using a Hewlett-Packard Model 5890A GC, equipped with a flame ionization detector and fitted with a 60 m × 0.25 mm (I.D.), thickness 0.25 μm ZB-5 fused silica capillary column (Phenomenex). Injection port and detector temperatures were maintained at 280 °C. The column temperature was programmed from 50 °C to 135 °C at 5 °C/min (1 min), 5 °C/min up 225 °C (5 min), 5 °C/min up 260 °C and then held for 10 min. The samples (0.1 μL each), generally analyzed without dilution (using 2,6-dimethylphenol as internal standard), were injected using a split/splitless automatic injector HP 7673 and using helium as the carrier gas. The analytical data reported in Table 1 represent the average results of three GC injections. The quantization of each compound was expressed as absolute weight percentage using internal standard and response factors. The detector response factors (RFs) were determined for key components relative to 2,6-dimethylphenol and assigned to other components on the basis of functional group and/or structural similarity.

2.3.2. Gas chromatography/mass spectrometry

MS analyses were carried out with an Agilent Technologies model 7820A connected with a MS detector 5977E MSD (Agilent), and using the same conditions and column described above. The column was connected with the ion source of the mass spectrometer. Mass units were monitored from 10 to 900 at 70 eV. The identification of constituents was based on comparison of the R_t values and mass spectra with those obtained from authentic samples and/or the NIST and Wiley library spectra (INational Institute of Standards and Technology, 1999); Adams, 2007), or on the interpretation of the EI-fragmentation of the molecules.

2.3.3. Statistical analysis

Prior to chemometric analysis, setting the total integral areas to 100 normalized the data and the generated ASCII file was imported into Microsoft EXCEL for the addition of labels. The matrix was imported into SIMCA-P software version 12.0, (Umetrics AB, Umeå, Sweden) for statistical analysis. A data matrix for subsequent analysis was set. Data were submitted to multivariate statistical evaluation using the Simca-p software package (Umetrics Umeå, Sweden). Especially, the Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were applied. In order to avoid model overfitting, a 300-time permutation test validated the supervised models.

Table 1
Essential oils composition (%) of *H. microphyllum* subsp. *tyrrhenicum*.

RI		[c]Class of compounds	Compounds	Samples				[a]ID	References
				Station 1 (Iglesias)	Station 2 (Carbonia)	Station 3 (Portoscuso)	Station 4 (Oristano)		
				[b]Yield %					
				0.38	0.41	0.35	0.42		
Exp.	Lit.								
940	939	MH	α-pinene	0.25 ± 0.01	0.33 ± 0.02	0.42 ± 0.04	nd	Std	
950	953	MH	α-fenchene	0.04 ± 0.03	0.35 ± 0.01	0.16 ± 0.02	nd	Std	
956	954	MH	camphene	nd	nd	0.05 ± 0.01	nd	Std	
991	991	MH	β-myrcene	nd	nd	0.13 ± 0.02	0.44 ± 0.02	Std	
1031	1029	MH	(+)-limonene	5.28 ± 0.01	7.33 ± 0.05	4.14 ± 0.03	1.19 ± 0.01	Std	
1039	1042	MH	α-ocimene	nd	0.99 ± 0.02	0.39 ± 0.02	nd	RI, MS	Tzakou et al. (1997)
1075	1089	MH	α-terpinolene	0.80 ± 0.04	nd	0.22 ± 0.01	0.29 ± 0.03	Std	
1093	1090	No TCC	2-nonanone	0.54 ± 0.02	0.65 ± 0.01	0.33 ± 0.02	nd	RI, MS	
1095	1097	OM	linalool	14.21 ± 0.03	11.66 ± 0.01	11.47 ± 0.02	3.16 ± 0.02	Std	
1102	1100	AE	isopentyl-2-methylbutanoate	0.71 ± 0.01	0.07 ± 0.02	0.44 ± 0.02	nd	RI, MS	
1142	1144	No TCC	(*E*)-tagetone	4.48 ± 0.04	3.28 ± 0.02	1.72 ± 0.01	0.84 ± 0.01	RI, MS	
1152	1152	No TCC	(*Z*)-tagetone	1.49 ± 0.02	2.46 ± 0.02	1.57 ± 0.01	nd	RI, MS	
1162	1166	OM	δ-terpineol	1.65 ± 0.02	nd	nd	0.79 ± 0.03	RI, MS	
1180	1160	No TCC	4,6-dimethyloctan-3,5-dione	1.62 ± 0.02	0.94 ± 0.01	1.59 ± 0.01	0.92 ± 0.01	RI, MS	Bianchini et al. (2003)
1190	1189	OM	α-terpineol	0.55 ± 0.02	0.48 ± 0.02	0.36 ± 0.01	0.22 ± 0.01	Std	
1301	1298	OM	geranyl formate	nd	nd	nd	4.93 ± 0.05	RI, MS	
1353	1351	SH	α-cubebene	nd	nd	1.31 ± 0.02	nd	Std	
1366	1362	OM	neryl acetate	nd	nd	nd	33.60 ± 0.04	Std	
1405	1405	SH	di-*epi*-α-cedrene	1.54 ± 0.01	6.08 ± 0.02	3.35 ± 0.03	1.37 ± 0.01	RI, MS	Juteau et al. (2003)
1409	1412	SH	α-cedrene	4.17 ± 0.02	0.29 ± 0.02	0.14 ± 0.02	3.52 ± 0.02	Std	
1415	1413	SH	*cis*-α-bergamotene	2.10 ± 0.01	2.79 ± 0.03	0.77 ± 0.01	1.41 ± 0.02	Std	
1421	1419	SH	(*E*)-β-caryophyllene	2.01 ± 0.01	1.64 ± 0.01	2.98 ± 0.03	0.80 ± 0.01	Std	
1435	1435	SH	*trans*-α-bergamotene	1.60 ± 0.02	1.75 ± 0.02	0.65 ± 0.01	1.08 ± 0.02	Std	
1439	1440	SH	α-guaiene	nd	nd	0.26 ± 0.04	nd	RI, MS	
1443	1441	SH	aromadendrene	nd	nd	0.50 ± 0.02	nd	RI, MS	
1454	1455	SH	α-humulene	nd	nd	0.40 ± 0.05	nd	Std	
1459	1457	SH	(*E*)-β-farnesene	0.54 ± 0.02	0.85 ± 0.01	0.41 ± 0.01	7.58 ± 0.03	Std	
1460	1460	SH	alloaromadendrene	1.38 ± 0.02	1.08 ± 0.04	2.25 ± 0.02	1.21 ± 0.03	RI, MS	
1465	1463	SH	dehydroaromadendrene	0.50 ± 0.03	0.53 ± 0.03	0.39 ± 0.01	nd	RI, MS	
1480	1483	SH	γ-curcumene	28.94 ± 0.02	28.22 ± 0.02	12.22 ± 0.03	11.08 ± 0.01	RI, MS	
1483	1486	SH	ar-curcumene	0.53 ± 0.01	4.69 ± 0.01	3.12 ± 0.03	nd	RI, MS	
1490	1495	ArE	phenethyl isovalerate	1.27 ± 0.03	1.38 ± 0.01	0.75 ± 0.02	nd	RI, MS	Petrakis et al. (2001)
1495		No TCC	tricyclo[M,N,O,Pxy]undec-2-en-4-one	1.03 ± 0.02	0.81 ± 0.03	0.63 ± 0.01	nd	MS	
1497	1498	SH	β-selinene	nd	1.42 ± 0.01	1.39 ± 0.03	nd	Std	
1500	1500	SH	α-muurolene	1.50 ± 0.05	1.78 ± 0.02	2.44 ± 0.02	0.96 ± 0.02	Std	
1508	1506	SH	β-bisabolene	0.57 ± 0.03	0.63 ± 0.02	nd	0.48 ± 0.02	Std	
1514	1522	SH	7-*epi*-α-selinene	1.83 ± 0.02	1.50 ± 0.02	1.40 ± 0.01	1.61 ± 0.03	RI, MS	
1514	1514	SH	γ-cadinene	0.95 ± 0.01	0.70 ± 0.01	3.29 ± 0.01	nd	Std	
1522	1523	SH	δ-cadinene	1.65 ± 0.03	1.19 ± 0.02	4.45 ± 0.02	0.51 ± 0.01	Std	
1523		SH	7-*diepi*-α-selinene	nd	nd	0.55 ± 0.02	nd	MS	
1542	1546	SH	α-calacorene	0.50 ± 0.02	0.81 ± 0.03	0.74 ± 0.02	0.67 ± 0.02	RI, MS	
1547		SH	4,5,9,10-dehydro-isolongifolene	nd	nd	1.96 ± 0.01	nd	MS	
1549	1547	SH	selina-3,7(11)diene	nd	nd	1.01 ± 0.03	nd	RI, MS	
1563	1567	OM	*trans*-nerolidol	0.45 ± 0.01	0.46 ± 0.03	5.07 ± 0.02	0.27 ± 0.02	Std	Zeng et al. (2007)
1590	1585	OS	globulol	nd	nd	0.29 ± 0.03	nd	RI, MS	

(*continued on next page*)

Table 1 (*continued*)

RI		[c]Class of compounds	Compounds	Samples				[a]ID	References
				Station 1 (Iglesias)	Station 2 (Carbonia)	Station 3 (Portoscuso)	Station 4 (Oristano)		
				[b]Yield %					
				0.38	0.41	0.35	0.42		
Exp.	Lit.								
1597	1601	OS	guaiol	1.88 ± 0.02	1.42 ± 0.02	2.08 ± 0.01	1.33 ± 0.02	Std	
1618	1619	OS	1,10-di-epi-cubenol	0.38 ± 0.01	0.55 ± 0.01	0.47 ± 0.02	0.37 ± 0.01	RI, MS	
1621		OS	5-eudesmen-11-ol	9.81 ± 0.04	7.39 ± 0.02	10.35 ± 0.02	4.34 ± 0.01	MS	
1624	1624	OS	10-epi-γ-eudesmol	1.05 ± 0.01	0.75 ± 0.01	0.39 ± 0.03	1.52 ± 0.01	RI, MS	
1647	1651	OS	β-eudesmol	0.56 ± 0.05	0.36 ± 0.03	1.06 ± 0.01	1.64 ± 0.01	Std	
1654	1654	OS	α-eudesmol	0.90 ± 0.02	0.51 ± 0.01	0.98 ± 0.03	0.32 ± 0.02	Std	
1662	1658	OS	patchouli alcohol	0.47 ± 0.02	0.32 ± 0.01	0.58 ± 0.01	0.40 ± 0.06	Std	
			Total	99.73	98.44	91.62	88.97		

Data are the mean of three replicates ± SD. Not detected compouns were indicated as nd.
RI by comparison of retention index with those reported in literature.
Std by comparison of the retention time and mass spectrum of available authentic standards.
[a] Identification methods: MS by comparison of the Mass spectrum with those of the computer mass libraries Adams, Nist 11 and by interpretation of the mass spectra fragmentations.
[b] Yield of essential oils calculated on fresh material.
[c] MH = Monoterpene Hydrocarbon; No TCC = No Terpenic Carmonil Compound; OM = Oxygenated Monoterpene; AE = Aliphatic Ester; SH = Sesquiterpene Hydrocarbon; OS = Oxygenate sesquiterpene; ArE = Aromatic Ester.

2.4. Antimicrobial activity

2.4.1. Materials

Stock solutions of the essential oils were prepared by dissolving them in PEG-200 in order to obtain a concentration of 100 mg mL^{-1} (10% w/vol); solutions were then sterilized by filtration using sterile membrane filters (Sartorius, pore size 0.22 µm) and stored at −20 °C until use. Chitosan (molecular weight 190–310 kDa; deacetylation degree 75–85%; viscosity Brookfield, 1% solution in acetic acid 200–800 cps; manufacturer values) was supplied by Aldrich (Milwaukee, WI). Its aqueous solution was prepared by dissolving chitosan in hydrochloric acid 0.1 mol l^{-1} at 1% w/vol and by evaporating the resulting solution to dryness in a Rotavapor R110 (Buchi, Switzerland) at 70 °C under vacuum; the residual was then redissolved in 100 ml of Milli-Q water and sterilised by filtration through 0.2 µm Sartorious filters. The pH of this solution, evaluated with a pH-meter Hanna 8417, was 2.35 ± 0.04.

2.4.2. Microorganisms and media

The test organisms used in this study were as follows: *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231), *Aspergillus brasiliensis (niger*) (ATCC 16404) (all purchased from Oxoid-Thermofisher Scientific, Rodano, Italy), and three *Candida* spp. clinical strains (respectively isolated from a vaginal, rectal and throat swab, kindly supplied by Dr. M. Tidore, Laboratory of Clinical and Microbiological Analysis of Policlinic Hospital of Sassari, Italy). Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Liquid Medium (SLM), Sabouraud Dextrose Agar (SDA) and phosphate-buffered saline tablets (PBS, Dulbecco A, pH 7.3) were purchased from Oxoid-Thermofisher Scientific (Rodano, Italy). Culture media, PBS and other solutions were prepared with MilliQ water.

2.4.3. Antibacterial activity of H. Microphyllum subsp. Tyrrhenicum essential oil sample 1

Antimicrobial activity of *H. microphyllum* subsp. *tyrrhenicum* essential oil sample 1 was determined as Minimum Inhibitory Concentration (M.I.C.) by using a broth microdilution test performed in 96-well microplates (Thrupp, 1986). Twofold dilutions of the stock solution of the oil, ranging from 2 mg ml^{-1} to 0.063 mg ml^{-1}, were prepared in MHB; control wells contained only liquid medium and liquid medium with the highest concentration of PEG-200 used. As positive control we used chlorhexidine diacetate (Sigma, St. Louis, MO, USA; mother solution 10 mg/ml in distilled water), a broad-spectrum synthetic antimicrobial agent used primarily as a topical antiseptic and disinfectant. All assays were performed in triplicate. Microplates were inoculated with about 1×10^4 bacteria/well and aerobically incubated at 35 °C for 24 h. After incubation, plates were visually checked for bacterial growth and the MIC of the oil was defined as the lowest concentration at which no growth was observed. PEG-200 had no inhibitory effect on the growth of microorganisms at all concentrations tested. To determine the M.B.C. (Minimum Bactericidal Concentration), aliquots of 10 µl of medium from each well with no visible growth were subcultured onto MHA plates, which were then incubated at 35 °C for 24 h; MBC was defined as the lowest concentration at which no growth was detectable. Results are reported in Table 2.

2.4.4. Antifungal activity of H. microphyllum subsp. tyrrhenicum essential oils

Antifungal activity of *H. microphyllum* subsp. *tyrrhenicum* essential oils was assessed on *Candida albicans* and *Aspergillus brasiliensis* standard strains and on *Candida* spp. clinical isolates. *Candida* strains and *Aspergillus brasiliensis* were grown on SDA plates at 35 °C and 25 °C respectively, and stock cultures were maintained at 4 °C during all the experiments.

Table 2
Minimum Inhibitory Concentration (M.I.C.) and Minimum Bactericidal Concentration (in brackets; M.B.C.) of essential oil 1 and chlorhexidine diacetate against a panel of standard bacteria.

	Essential oil Sample 1	Chlorhexidine diacetate
Staphylococcus aureus ATCC 6538	2 (4) mg ml^{-1}	0.90 (3.90) µg ml^{-1}
Escherichia coli ATCC 8739	>4 mg ml^{-1}	1.96 (15.6) µg ml^{-1}
Pseudomonas aeruginosa ATCC 9027	>4 mg ml^{-1}	21.9 (125) µg ml^{-1}

Table 3
Minimum Inhibitory Concentration (M.I.C.) and Minimum Candidacidal Concentration (in brackets; M.C.C.) of samples of *H. microphyllum* subsp. *tyrrhenicum* essential oils and chlorhexidine diacetate.

	1	2	3	4	Chlorhexidine diacetate
Candida albicans ATCC 10,231	0.75 (1.75) mg ml^{-1}	1 (2) mg ml^{-1}	2 (2) mg ml^{-1}	2 (4) mg ml^{-1}	7.8 (7.8) μg ml^{-1}
Candida spp. clinical isolate 1 (rectal swab)	0.5 (2) mg ml^{-1}	0.5 (2) mg ml^{-1}	4 (4) mg ml^{-1}	2 (4) mg ml^{-1}	7.8 (7.8) μg ml^{-1}
Candida spp. clinical isolate 2 (throat swab)	1 (2) mg ml^{-1}	1 (2) mg ml^{-1}	4 (>4) mg ml^{-1}	2 (>4) mg ml^{-1}	7.8 (7.8) μg ml^{-1}
Candida spp. clinical isolate 3 (vaginal swab)	1 (2) mg ml^{-1}	1 (2) m ml^{-1}	4 (4) mg ml^{-1}	4 (4) mg ml^{-1}	7.8 (7.8) μg ml^{-1}

The M.I.C. of *H. microphyllum* subsp. *tyrrhenicum* essential oils for *Candida* strains was determined by using the same broth microdilution assay described in the previous paragraph; twofold dilutions of the stock solution of the oil were prepared in SLM. All assays were performed in triplicate. Microplates were inoculated with about 1x10^4 yeasts/well and aerobically incubated at 35 °C for 24 h. After incubation, plates were visually checked for fungal growth, and the MIC of the oil was defined as the lowest concentration at which no growth was observed. PEG-200 had no inhibitory effect on the growth of microorganisms at all the concentrations tested. To determine the M.C.C. (Minimum Candidacidal Concentration), aliquots of 10 μL of medium from each well with no visible growth were subcultured onto SDA plates, which were then incubated at 35 °C for 24 h; MCC was defined as the lowest concentration at which no growth was detectable. Results are reported in Table 3.

The MIC of *H. microphyllum* subsp. *tyrrhenicum* essential oil sample 1 against *Aspergillus brasiliensis* was determined using an agar macrodilution method (McGinnis and Rinaldi 1986). Twofold serial dilutions of essential oil in SDA were made in 5 mm-Petri dished (total volume 10 mL) in order to obtain a final concentration of 2 mg ml^{-1}, 1 mg ml^{-1}, 0.5 mg ml^{-1} and 0.25 mg ml^{-1}. The experiments were all performed in triplicate. Control plates, containing only SDA and SDA + PEG-200, were run simultaneously. The agar surface of the plates was then inoculated into the center with 1–3 μL of a conidial suspension prepared in sterile distilled water +0.05% Tween-80, containing 10^3-10^4 conidia. Plates, wrapped with Parafilm® to maintain the correct water activity in the medium, were inverted and incubated at room temperature (about 25 °C).

5 days later, mycelial diameter was then measured in plates where a growth was observable; results are reported in Table 4.

Table 4
Effect of *H. microphyllum* subsp. *tyrrhenicum* 1 essential oil on mycelial growth of *Aspergillus brasiliensis* ATCC 16,404 (after a week of incubation).

Control	100%
Control PEG 200	100%
E.O. 0.25 mg/ml	76%
E.O. 0.50 mg/ml	70%
E.O. 1 mg/ml	51%
E.O. 2 mg/ml	0%

2.4.5. Killing curve determination

The anticandidal activity of essential oil of *H. microphyllum* subsp. *tyrrhenicum* sample 1 (chosen because of its more marked inhibitory effect) was also characterized by a "Time-kill" assay performed on *C. albicans* ATCC 10,231 strain; this test evaluates the reduction of viable yeast count when a standardized inoculum is incubated with different oil concentrations in a liquid medium not supporting cell growth. The assay was performed in agreement to Juliano et al. (2000).

Yeasts in the logarithmic phase of growth were centrifuged at 1500 *rpm* for 10 min, washed in PBS and then resuspended at a density of 5x10^5-1x10^6 colony-forming units (cfu) ml^{-1} in appropriate volumes of PBS containing a concentration of essential oil, ranging from 0.25 mg ml^{-1} to 2 mg ml^{-1}. Control tubes (microorganisms suspended in PBS + PEG at highest concentration used) were included in each assay. Tween 80 (Sigma Aldrich) at 0.02% vol/vol was added to each test tube (controls included). Test tubes were incubated at 35 °C; at time zero and at predetermined intervals; 0.5 mL of the suspensions were removed and subjected to serial 10-fold dilution in PBS; aliquots of 0.5 mL of the appropriate dilutions were thoroughly mixed in Petri plates (50 mm diameter) with molten SDA (45 °C). Plates were then incubated for 24 h at 35 °C; after this time, the number of viable yeasts at each time was evaluated by counting plates with 30–300 colonies. The results of the test are summarized as shown in Fig. 1.

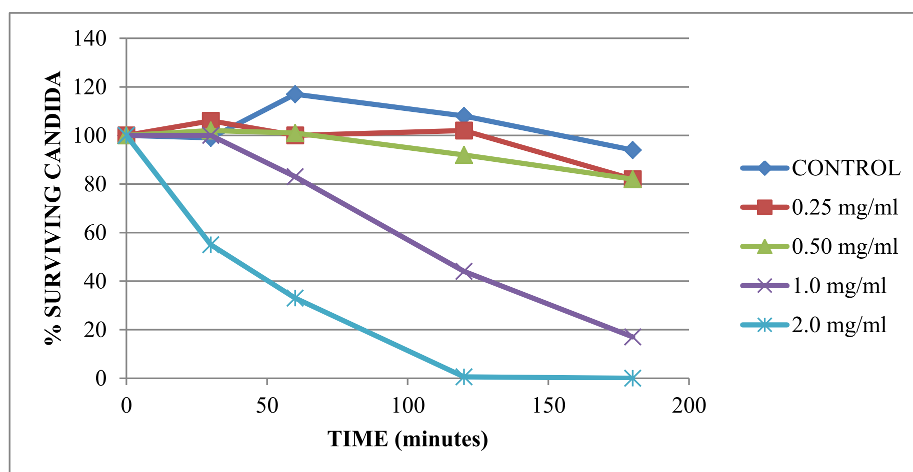


Fig. 1. Time-kill curves of *C. albicans* ATCC 10,231 in PBS in the presence of different *H. microphyllum* subsp. *tyrrhenicum* 1 essential oils concentrations (mean of 3 experiments).

Table 5
Minimum Inhibitory Concentration (M.IC.) and Minimum Candidacidal Concentration (M.C.C.) of chitosan, *H. microphyllum* subsp. *tyrrhenicum* essential oil 1, and *H. microphyllum* subsp. *tyrrhenicum* essential oil 1 in the presence of sub-inhibitory chitosan concentrations.

	M.I.C.	M.C.C.
E.O. *H. microphyllum* subsp. *tyrrhenicum* essential oil 1	0.75 mg ml^{-1}	1.75 mg ml^{-1}
CHITOSAN	1 mg ml^{-1}	1 mg ml^{-1}
E.O. *H. microphyllum* subsp. *tyrrhenicum* essential oil 1 + CHITOSAN 0.25 mg/ml	0.094 mg ml^{-1}	0.125 mg ml^{-1}
E.O. *H. microphyllum* subsp. *tyrrhenicum* essential oil 1 + CHITOSAN 0.125 mg/ml	0.250 mg ml^{-1}	0.375 mg ml^{-1}

2.4.6. Antifungal activity of essential oil H. microphyllum subsp. tyrrhenicum sample 1 in the presence of sub-M.I.C. concentrations of chitosan

The inhibitory activity of chitosan solution against *Candida* strains was previously evaluated as M.I.C. and M.C.C. by using the techniques described in the section 2.4.4. Chitosan solution was tested at twofold serial dilutions ranging from 2 mg ml^{-1} to 0.0039 mg ml^{-1}in SLM (whose pH value did not change after chitosan solution addition; data not shown). The results are reported in Table 5.

Afterwards, M.I.C. and M.C.C. of essential oil 1 were evaluated as previously described in the presence of chitosan concentrations of 0.25 mg ml^{-1} and 0.125 mg ml^{-1}, i.e. respectively one-half and one-quarter of its MIC value. Results of these experiments are reported in Table 5.

3. Results and discussion

3.1. Chemical composition of essential oils

A yellow liquid oil is the result of the hydrodistillation of the aerial parts of *H. microphyllum* subsp. *tyrrhenicum* plants collected in South-West of Sardinia. Overall, numerous compounds were identified for every sample, representing from a minimum amount of 88.97% to a maximum of 99.73% of the total composition. Fifty-three compounds were detected and quantified by GC and GC/MS methods in the oils we studied. All these constituents were identified on the basis of their mass spectra, retention indices and/or by comparison with authentic compounds (National Institute of Standards and Technology, 1999; Adams, 2007). The analytical data reported in Table 1 are the average of 3 injections plus ± SD.

Each one of the four samples shows a very peculiar chromatographic profile. The sample 4, coming from the Sinis Peninsula (Oristano), has a very typical composition referred to some previous analyses carried out on Sardinian *H. microphyllum* (Usai et al., 1999; Chessa et al., 2000; Usai et al., 2010). In fact, it is possible to note a very high concentration in neryl acetate (33.6%) followed by γ-curcumene (11.53%), farnesene (7.38%) and 5-eudesmen-11-ol (4.34%).

The oils coming from the plants collected in the area named Sulcis-Iglesiente (sample 1, 2 and 3) show very different profiles, when compared to sample 4. Samples 1 and 2 are almost similar. It is possible to note that in this case the most represented compounds are: γ-curcumene (about 28%), linalool (between 14 and 11%), 5-eudesmen-11-ol (between 9,8 and 7%). Another evident characteristic is the total absence of neryl acetate that is unusual for *H. microphyllum* subsp. *tyrrhenicum.*

The last sample (3) comes from plants growing on the west coast, in front of Carloforte Island. Again, we didn't found here any trace of neryl acetate and the major constituents were: γ-curcumene (12%), linalool (11%), 5-eudesmen-11-ol (10%), limonene and δ-cadinene (both 4%). Another think that is to underlined is the low content of diones, in fact we found only 4,6-dimethyloctan-3,5-dione (0.9–1.6%) is a mixture of diastereoisomers as reported in Bianchini et al., 2003.

We carried out an analysis of principal components (PCA), using the analytical data derived from essential oil. The score plot (a) and the loading plot (b) are reported in Fig. 2. The score plot shows that the samples 1 and 2, both collected in Iglesias area and characterized from highest percentage of γ-curcumene, are practically comparable; sample 4, deriving from Oristano area, is quite different from the other samples because it is the only one that contains neryl acetate.

3.2. Antifungal activity of H. microphyllum subsp. tyrrhenicum essential oils

The data of the anticandidal activity of *H. microphyllum* subsp. *tyrrhenicum* oils examined are summarized in Table 3.

The four oils showed different levels of inhibition against *Candida* strains; oils obtained from plants collected in stations 1 and 2 were able to inhibit *Candida* ATCC and *Candida* spp. 1 at concentrations of 0.5–1 mg ml^{-1}, while essential oils obtained from *H. microphyllum* subsp. *tyrrhenicum* collected in stations 4 and 3 presented a weaker anticandidal effect.

Essential oil coming from station 1 was also tested against the filamentous fungus *Aspergillus brasiliensis* ATCC and showed an antifungal dose-dependent activity; the mycelial diameter was halved by an essential oil concentration of 1 mg/mL and essential oil completely suppressed fungal growth at 2 mg ml^{-1} (MIC = mg ml^{-1}) (Table 4).

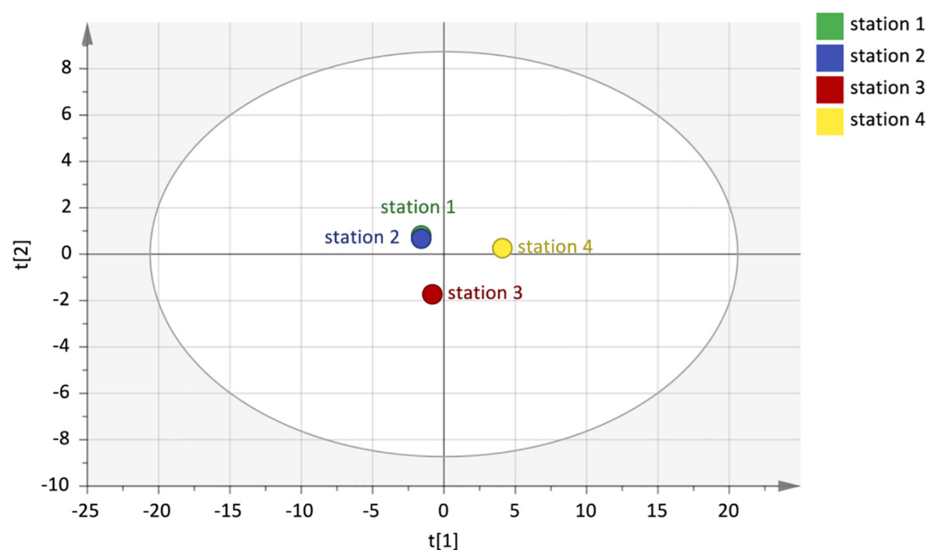
Time-kill assays showed that *H. microphyllum* subsp. *tyrrhenicum* 1 essential oil was able to reduce viability of a *Candida albicans* ATCC 10231 inoculum in a dose-dependent manner, as in Fig. 1. After a 3 h-long contact, concentrations of the oil of 0.25 and 0.50 mg ml^{-1} did not affect significantly yeast inoculum viability in comparison to control, but only 17% of cells survived to the contact with 1 mg ml^{-1}, and no viable cells could be detected after 3 h of contact with 2 mg ml^{-1} (with a viability of only 0.6% after 2 h of contact).

When evaluated in presence of sub-inhibitory concentrations of chitosan, MIC and MBC of *H. microphyllum* subsp. *tyrrhenicum* 1 essential oil were markedly reduced (Table 5), showing that, in our experimental conditions, their combination leads to an improvement of anticandidal oil efficiency.

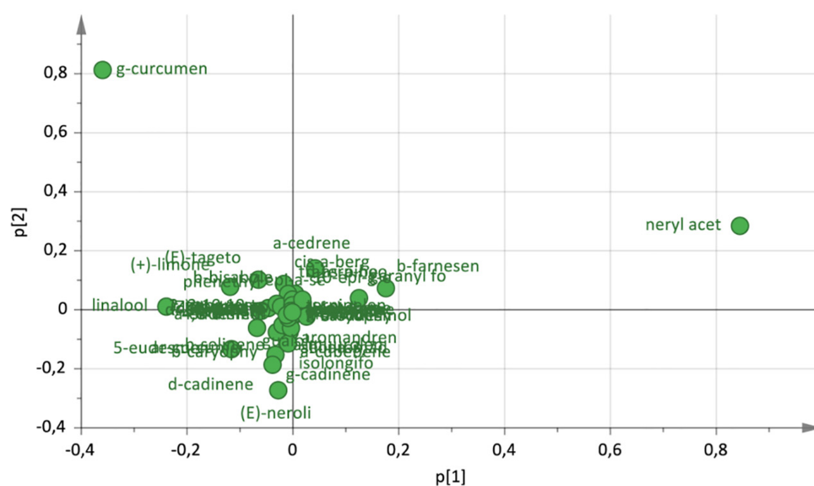
This study allows us to conclude that the essential oils coming from the four stations located in the Sulcis-Iglesiente area show different chemical profiles; in particular, essential oils from station 1 and 2 are very rich in γ-curcumene. This is a very unusual chorotype that is reported for Italy only in *H. italicum* subsp. *italicum* by locality Centopozzi (Foggia) with 23% and locality Torre Gattarella, Vieste (Foggia) with 27% (Morone-Fortunato et al., 2010), but no researcher published data on this chemotype for *H. microphyllum* subsp. *tyrrhenicum.*

Samples coming from station 3 present a good concentration of γ and ar-curcumene and linalool. In these three stations we didn't found neryl acetate that, on the contrary, is present in samples coming from station 4, and this can be considered as the typical composition, referred to *H. microphyllum* subsp. *tyrrhenicum* growing in the north and in the center of Sardinia.

Several *in vitro* studies demonstrated an antimicrobial activity of *H. microphyllum* subsp. *tyrrhenicum* (Angioni et al., 2003; Ornano et al., 2015) and this paper confirms and completes these data; however, it is difficult to compare the results of different studies since different extracts and different methods are used for the evaluation of the antimicrobial activity. Furthermore, the



Score plot of PCA (a)



Loading plot of PCA (b)

Fig. 2. PCA analysis and loading plot of PCA of essential oil of listed *H. microphyllum* subsp. *tyrrhenicum* samples.

subspecies used in such studies are frequently not clearly indicated and the relative comparisons are simply not possible.

Our results point out that the *H. microphyllum* subsp. *tyrrhenicum* oils examined are mostly characterized by an antifungal activity, whereas antibacterial efficacy, albeit assessed only for essential oil 1, proved to be modest and only limited to Gram+ microorganism *Staphylococcus aureus*.

The inhibition of *Candida* is interesting, because the infections caused by this yeast are a highly topical issue. Oral candidiasis is one of the most common opportunistic infections associated with HIV/AIDS (Pakfetrat et al., 2015) and in patients receiving radiotherapy for head and neck malignancies (Deng et al., 2010); the yeasts often become refractory to triazole antifungal drugs, prescribed for treating oral candidiasis in these patients (Fichtenbaum et al., 2000), and extracts and natural products from plants can offer an efficient alternative for the treatment of *Candida* infections.

Our data also suggest that the differences found in the anticandidal activity of these oils can be related to some differences in their chemical composition (Table 3). We could state that the prevalence of terpenes and alcohols such as γ-curcumene and linalool (with discreet presence of 5-eudesmen-11-of, for example) identify the essential oil of *H. microphyllum* examined in the study as a mixed chemotype (triple) essential oil, as an anti-microbial species with an interesting anti-fungal activity, as well as the well-known anti-inflammatory, antioxidant, skin-venous decongestant and cicatrizing activities. The idea of a topical use in an infected dermatitis showing a strong inflammatory component, in acne or rosacea, in different kinds of dermatitis or even (conveyed in a suitable vegetable oil) during a massage session in post-traumatic hematoma, or in so-called chilblains, appears very likely and effective; however, it requires the prudential methodology that belongs to almost all essential oils (Campagna, 2016).

Interestingly, the anticandidal oil efficiency was strengthened by sub-inhibitory concentrations of chitosan; the mechanism of this effect is still not known, but a role could be played by some of the peculiar biological properties of this biopolymer, such as: chelating capacity for various metal ions (Rabea et al., 2003);

electrostatic interaction of protonated amino groups of chitosan with negatively charged surface components of fungi and bacteria, with consequent cell surface alterations, leakage of intracellular substances and loss of microbial viability (Rafaat and Sahl, 2009; Kong et al., 2010); deposition of chitosan onto the surface of microorganisms, with blockage of nutrient flow and suppression of metabolic activity of bacteria (Kumar et al., 2005).

This enhancement is noteworthy because it could lead to the development of innovative formulations of *H. microphyllum* subsp. *tyrrhenicum* essential oils exploiting bioadhesion and water solubility of chitosan; polymer bioadhesive properties could allow more prolonged retention times of the oil on skin and mucosae, and water solubility would avoid the delivery of oils in alcoholic solutions, which can often be pretty irritant or painful for patients with skin or mucosal lesions. On the basis of these considerations, we believe that it might be interesting to investigate a possible applicability of the synergism chitosan-essential oil in clinical situations.

The phytochemical composition of *H. microphyllum* subsp. *tyrrhenicum* essential oil shows once again that an eventual chemotypization of the various species examined could lead the therapist to a targeted clinical use of the specific plant, in a concept of a rational scientific aromatherapy, according to chemical, pharmacological and clinical criteria, which are increasingly present in literature.

Conflict of interest

No conflict of interest declared.

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