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Abstract

With the goal of unravelling antimicrobial agents and mixtures inspired by plant defences, we investigated the antibacterial and antifungal efficacy of *Hirtellina lobelii* DC. essential oil (EO), both alone and in combination with antimicrobial drugs.

Hirtellina lobelii DC. EO was analysed by GC, GC-MS and partial fractionation/NMR. It was essentially composed of oxygenated sesquiterpenes (75.2%), with α-bisabolol (34.5%), fokienol (12.0%) and T-muurolol (6.8%) serving as the main components. Microbial susceptibility was determined by the broth microdilution method and was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC). This EO was found to possess remarkable bactericidal (MBC/MIC = 2) and fungicidal (MFC/MIC = 1 - 4) potential, particularly against the Gram (+) bacteria *Staphylococcus aureus*, including its methicillin-resistant forms, the yeast *Cryptococcus neoformans* and dermatophytes from the genus *Trichophyton* (MICs 8 - 128 µg/ml). The examination of the combined effects of the EO with antimicrobial drugs revealed synergisms of the EO with vancomycin against *S. aureus* and of the EO with fluconazole and griseofulvin against dermatophytic fungi (FICI 0.2 - 0.5). The effect of *H. lobelii* EO on the morphologies of fungal hyphae and bacteria, as determined by scanning electronic microscopy (SEM), showed fungal hyphae swelling and bulging.

These results suggest that *H. lobelii* EO and its major constituent, α-bisabolol, have remarkable antimicrobial potential. Combination therapies of this EO with antifungal drugs could offer a promising alternative for treatment of human mycoses caused by filamentous dermatophytic fungi.

Keywords: essential oil; antimicrobial activity; synergy; scanning electronic microscopy; *Hirtellina lobelii*; α-bisabolol

1. Introduction

Antimicrobial resistance has become a major therapeutic challenge, as a variety of multiresistant pathogenic microbes have emerged that defy commonly available treatments. The decline in effectiveness of existing drugs is partly due to natural selection and partly to their intensive use and misuse. As a matter of concern, the number of immunocompromised patients, who frequently develop opportunistic systemic infections, has dramatically increased since the 1990s [1]. Thus, infectious diseases are considered a real global threat, representing 26% of overall mortality in 2001 [2] and almost 30% in 2011 [3].

The development of microbial resistance to conventional treatments along with drug-related toxicities and costs have generated a clear need for new therapeutic strategies, new antimicrobial compounds and, particularly, new combination treatments and active mixtures. Combinatorial therapies can be less vulnerable to the development of drug resistance and can increase therapeutic efficacy [4,5]. Their potential synergistic effects provide broader pharmacological windows and lower toxicities [6]. Thus, efforts toward discovering multi-agent therapies that can overcome the limitations of monotherapies are highly encouraged [5–8].

In the quest for new antimicrobial drugs and bio-inspired mixtures, medicinal plants must not be overlooked. The antimicrobial potential of aromatic plants has been recognized since antiquity and is mainly attributed to their volatile oils, which contain wide chemical diversity [9,10]. Essential oils (EOs) are known for their antiseptic, i.e., bactericidal, fungicidal and

59 virucidal, properties, and some of them have been claimed to cure microbial infections and
60 have been proposed for use in complementary medicine [11]. Thus, EOs could transition from
61 being used solely in traditional medicine to also being used in modern medicine.

62 Although many studies have focused on showing the antimicrobial activity of EOs, few have
63 investigated the origin of their bioactivities and their exact mechanisms of action by examining
64 their effects on the morphologies and ultrastructures of pathogenic strains.

65 *Hirtellina lobelii* DC., formerly known as *Staehelina lobelii* DC., is an herbaceous plant belonging
66 to the Asteraceae (or Compositae) family. The genus *Staehelina* (tribe Cardueae) is an
67 extremely small genus that consists of only a few species worldwide and has rarely been
68 documented. The ascription of this genus is highly problematic, and the subtribal placement of
69 *Staehelina* remains unresolved. Based on Dittrich [12], the two species of *Staehelina* with
70 hirsute pericarps (*S. fruticosa* L. and *S. lobelii* DC.) should be classified in a distinct genus,
71 *Hirtellina*. We followed the classification proposed by Dittrich, which is also accepted by most
72 databases such as Euro+Med PlantBase (http://ww2.bgbm.org/EuroPlusMed) and the plant list
73 (http://www.theplantlist.org).

74 *Hirtellina lobelii* grows on rock crevices in large clumps and is a locally important element of
75 Mediterranean chasmophytic vegetation. Its distribution is restricted to Lebanon, Cyprus, Syria
76 and Asiatic Turkey [13, Euro+Med PlantBase (http://ww2.bgbm.org/EuroPlusMed)].

77 We evaluated the chemical composition of the EO of *H. lobelii*, its antimicrobial potential alone
78 and in combination with antibiotics and antifungal drugs as well as its mechanism of action by
79 observing the morphological alterations to pathogen structures that it caused by scanning

electron microscopy (SEM). To the best of our knowledge, this is the first description of the chemical composition and the antimicrobial potential of *Hirtellina lobelii* DC. EO. In addition, no volatile organic compound has ever been described from species of the *Hirtellina* or *Staehelina* genera.

2. Experimental part

2.1. Plant material and essential oil extraction

The aerial part of the plant (fresh leaves and stems) was collected in June 2012 from Qartaba, Mount Lebanon (34°05'58.70" N 35°48'46.39" E) at an altitude of 1250 m. A voucher specimen was deposited at the Herbarium of the Department of Botany and Medicinal plants, Holy Spirit University, Faculty of Agricultural and Food Sciences (USEK-Lebanon) under the registry number MNV446a. Hydrodistillation of the plant was performed for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia, 1997. The EO was obtained with a yield of 0.1%.

2.2. Essential oils analyses

2.2.1. GC analyses

Analytical gas chromatography was performed using a Thermo Electron Corporation gas chromatograph fitted with a DB-5 MS capillary column (30 m × 0.25 mm, 0.1 µm film thickness) or a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm, 0.20 µm film thickness). Helium was the carrier gas (0.7 ml/min). The column temperature was initially set to

35 °C and was gradually increased to 85 °C at 5 °C/min. It was held at 85 °C for 20 min and then raised to 300 °C at 10 °C/min. Finally, it was held at 300 °C for 5 min. Diluted 1 µl samples (1/100, vol/vol) were manually injected at 250 °C in the splitless mode. Flame ionisation detection (FID) was performed at 310 °C.

2.2.2. GC/MS analyses

The GC/MS analyses were performed using an Agilent gas chromatograph 6890 coupled with a Mass Detector 5975. The 7683 B autosampler injected 1 µL of each oil sample. A fused silica capillary column DB-5 MS (30 m × 0.25 mm internal diameter, 0.1 µm film thickness) or a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm, 0.20 µm film thickness) was used. Helium was the carrier gas (0.7 ml/min). The oven temperature program was identical to that described in 2.2.1. The mass spectra were recorded at 70 eV with an ion source temperature of 310 °C and a transfer line heated to 320 °C. The acquisition was recorded in full scan mode (50 – 400 amu).

2.2.3. Identifications and quantifications

Most constituents were identified by GC and GC/MS by comparing their retention indices (RI) with those from the literature [14,15] or those of authentic compounds obtained from Sigma-Aldrich (Lebanon). The retention indices were determined relative to a homologous series of *n*-alkanes (C8 to C24) that had been analysed under the same operating conditions. Their mass spectra using both columns were compared with those provided in the NIST and Wiley 275 libraries, our home-made library constructed with pure compounds and EOs of known compositions or mass spectra from the literature [14,16]. The relative concentrations of the

components were calculated based on the GC peak areas without correction and are reported in Table 1.

2.2.4. Essential oil fractionation by HPLC

Analytical and preparative HPLCs were conducted using a Gilson system equipped with a 322 pumping device, GX-271 fraction collector, 171 diode array detector, and ELSII preparative electrospray nebulizer detector. Phenomenex Luna C18 columns of two sizes were used for these experiments: a 4.6 × 250 mm column with 5 µm film thickness for analytical HPLC and a 21.2 × 250 mm column with 5 µm film thickness for preparative HPLC. The flow rates were set to 1 and 21 ml/min for analytical and preparative HPLC, respectively, using a linear gradient of water mixed with an increasing proportion of acetonitrile (30/70 to 0/100 over 35 min and then 100% CH_3CN for 24 min). The EO was diluted in acetonitrile at 10 mg/ml for analytical HPLC. The EO was also diluted in acetonitrile for prep-HPLC (60 mg EO in 300 µL CH_3CN), and 250 µL of the diluted solution was injected.

Forty-nine 25 ml fractions were collected between 2 and 59 min and were combined into 22 fractions according to their HPLC profiles. These 22 fractions were analysed by GC/MS. Fraction 23/24 contained 4 compounds, among which the unidentified component was the major one and accounted for 47% of the mixture according to GC/MS integration. The volatile organic compounds were collected from the fraction as follows: the fraction was separated between ether (250 ml) and water (250 ml). The organic layer was washed with water (3 × 50 ml), dried with Na_2SO_4, and evaporated without heating.

2.2.5. NMR spectroscopy

142 The nuclear magnetic resonance (NMR) spectra (^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC and
143 HMBC) of fraction 23/24 allowed us to confirm the identification of one of the main EO
144 constituents (fokienol, **2**, Fig. 1) by comparison with NMR data reported in the literature
145 [17,18]. The NMR spectra were recorded using a Bruker 500 MHz spectrometer equipped with
146 a 5 mm inverse detection probe. Chemical shifts (δ) are reported as ppm based on the TMS
147 signal, with s, d, t, and br standing for singlet, doublet, triplet and broad, respectively. The NMR
148 signals of fokienol as extracted from the NMR analysis of fraction 23/24 are as follows:
149 Fokienol (**2**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29 (3H, s, H-15), 1.61 (3H, s, H-14), 1.56-1.62 (2H, m,
150 H-4), 1.84 (3H, s, H-12), 2.02-2.08 (2H, m, H-5), 2.76 (2H, br d, *J* = 7.2 Hz, H-8), 4.89 (2H, br s, H-
151 13), 5.19 (1H, m, H-6), 5.07 (1H, dd, *J* = 11.0; 1.2 Hz, H-1a), 5.19 (1H, m, H-6), 5.23 (1H, dd, *J* =
152 17.1; 1.2 Hz, H-1b), 5.62 (1H, dt, *J* = 15.9; 7.2 Hz, H-9), 5.93 (1H, dd, *J* = 17.1; 11.0 Hz, H-2), 6.14
153 (1H, br d, *J* = 15.9 Hz, H-10).^{13}C-NMR (125 MHz, $CDCl_3$) δ 16.0 (C-14), 18.5 (C-12), 22.6 (C-5),
154 27.7 (C-15), 41.8 (C-4), 42.8 (C-8), 73.2 (C-3), 111.6 (C-1), 114.4 (C-13), 125.0 (C-6), 128.5 (C-9),
155 133.9 (C-10), 134.1(C-7), 141.9 (C-11), 145.0 (C-2).

156 **2.3. Antimicrobial activity**

157 *2.3.1. Microorganisms*

158 The antimicrobial activity of the EOs against the following microbial strains was investigated:
159 Gram (–) bacterial strain, *Escherichia coli* ATCC 25922; Gram (+) bacterial strains,
160 *Staphylococcus aureus* ATCC 29213 and methicillin-resistant *Staphylococcus aureus* (also called
161 oxacillin-resistant *Staphylococcus aureus*) ATCC 33591; yeasts, *Candida albicans* ATCC 10231,
162 *Candida parapsilosis* ATCC 22019 and *Cryptococcus neoformans* SNB-CN1; and filamentous
163 fungi, *Trichophyton rubrum* SNB-TR1, *Trichophyton violaceum* SNB-TV1, *Trichophyton*

soudanense SNB-TS1, *Trichophyton tonsurans* SNB-TT1, *Trichophyton mentagrophytes* SNB-TM1 and *Aspergillus fumigatus* SNB-AF1. The ATCC strains were purchased, while the other strains were clinical isolates kindly provided by Prof. Philippe Loiseau, Université Paris Sud. These strains were identified by Prof. Philippe Loiseau and Christian Bories; the molecular analyses were conducted by BACTUP. The ITS sequences were deposited in the NCBI GenBank database under the following registry numbers: KF360235 (*C. neoformans* SNB-CN1), KC692746 (*T. rubrum* SNB-TR1), KF360236 (*T. violaceum* SNB-TV1), KF360237 (*T. soudanense* SNB-TS1), KF360238 (*T. tonsurans* SNB-TT1), KF360239 (*T. mentagrophytes* SNB-TM1) and KC692747 (*A. fumigatus* SNB-AF1).

2.3.2. Microdilution method

The broth microdilution method was used to determine the minimal inhibitory concentration (MIC) of the EOs according to the Clinical and Laboratory Standards Institute guidelines [19–22]. The essential oil and its major components diluted in DMSO were tested at concentrations ranging from 512 to 1 µg/ml. Oxacillin, vancomycin and gentamicin (16 - 0.03 µg/ml) were used as reference antibiotics, while itraconazole (16 - 0.03 µg/ml) and fluconazole (64 - 0.125 µg/ml) were used as positive controls for antifungal activity. These antimicrobial standard drugs were purchased from Molekula, and pure terpene was purchased from Sigma-Aldrich. The microplates were incubated at 37 °C for 24 h for bacteria, 48 h for yeasts and *A. fumigatus*, and five days for the other filamentous fungi. The MIC values corresponding to the lowest concentration that prevented visible microbial growth are reported in Table 2.

2.3.3. Minimum bactericidal and fungicidal concentrations (MBCs and MFCs)

After MIC determination, the bactericidal and fungicidal activities of the EO were determined as previously described [23–25]. From each well with no detected microbial growth, 20 µl of the culture medium of bacterial and fungal cultures were subcultured on Mueller-Hinton and Sabouraud dextrose agar plates, respectively; the contents of the wells were not agitated prior to removal of the specified volume. The growth control wells of the microdilution plate were used as the growth control, and oxacillin, itraconazole and fluconazole were used as positive controls. The plates were incubated at 35 °C until growth was observed in the growth control subculture (24 h for bacteria, 48 h for yeasts and 5 days for dermatophytic fungi). The MBC and MFC was defined as the lowest concentration that resulted in no visible bacterial or fungal growth on agar plates, respectively, and those values are reported in Table 2.

2.3.4. Synergy test by Microdilution Checkerboard assay

This test is based on the microdilution method, and the protocol was described by Shin and Lim [26] and Houël et al. [27]. EO activity was tested in combination with the following drugs against the following microorganisms: vancomycin, *S. aureus*; fluconazole, *C. neoformans*; and fluconazole and griseofulvin, dermatophytic fungi *Trichophyton* sp. The drug solutions were diluted along the horizontal orientation so that the plates contained final concentrations of vancomycin and griseofulvin ranging from 16 to 0.03 µg/ml and final concentrations of fluconazole ranging from 64 to 0.125 µg/ml. The EO solution was diluted along the vertical orientation so that the final concentrations ranged from 64 to 1 µg/ml for bacteria and dermatophytes and from 256 to 4 µg/ml for yeast.

Fractional inhibitory concentrations (FIC), which represent the gain in activity of individual components of the mixture, were determined for each point by dividing the MIC of the

207 combinations of the two products by the MIC of the essential oil or the drug alone. The FIC
208 index (FICI), which is a measure of synergy, was obtained by adding both FICs. The FICI was
209 interpreted as follows: values ≤ 0.5 indicated a synergistic effect, values > 0.5 and ≤ 2.0
210 indicated an indifferent effect, and values > 2.0 indicated an antagonistic effect [26,28].
211 The results were also analysed by the isobologram method (see supplementary material, S1).

212 **2.4. Cytotoxicity Assays**

213 Cytotoxicity assays were conducted with MRC5 (human foetal lung fibroblast) and MDA435
214 (melanoma) cell lines according to the procedure described by Rochais et al. [29].

215 **2.5. Scanning electron microscopy observations**

216 To examine the effect of *H. lobelii* EO on the surface topography of *S. aureus*, *T. rubrum* and *T.*
217 *soudanense*, samples for SEM observation were prepared as follows. For bacteria, a
218 conventional broth microdilution assay was performed in a 96-well microtitre plate; then, 40
219 µL of the suspension was sampled and deposited on sterilized glass squares distributed in a 24-
220 well polystyrene plate. Sedimentation lasted three hours. Fungi were directly grown on the
221 same system.
222 Samples treated with a sub-inhibitory concentration were fixed with 2.5% glutaraldehyde in 0.1
223 M sodium cacodylate buffer at pH 7.4 for 1 hour at RT. The untreated control microorganisms
224 were processed in parallel for the SEM analyses. After three washes of 10 min with the same
225 buffer, the samples were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate
226 buffer at pH 7.4 for 1 hour at RT and then washed in sterile water three times for 10 min. The
227 cells were dehydrated with increasing concentrations of ethanol (50%, 70%, 90%, 3x100%) at

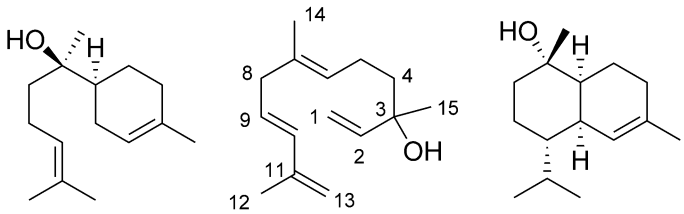
RT for 10 min for each bath. The samples were critical-point dried at 75 bar and 37 °C with liquid CO_2 as the transition fluid and were then depressurized slowly (400 cm^3/min) in a Quorum Technologies K850 device (Elexience, France). Then, the samples were sputter-coated in Argon plasma with Platinum (thickness ≈ 30 nm) in a Polaron SC7640 device (Elexience, France) at 10 mA and 0.8 kV for 200 s. Observations were performed using a FE-SEM Hitachi S4500 (Hitachi, Japan) in a high vacuum with a sample holder tilted at 45° and a low SE detector at 2 kV and 21 mm WD. The experiments were performed using the MIMA2 microscopy platform (http://www6.jouy.inra.fr/mima2).

3. Results and Discussion

3.1. Essential oils analyses

The essential oil of the fresh aerial parts of *Hirtellina lobelii* was obtained by hydrodistillation with a yield of 0.1% (vol/wt, relative to dry weight material). The chemical composition of the EO and the relative proportions of the components are reported in Table 1.

GC, GC-MS, and fractionation/NMR led to the identification of 25 components representing 86.9% of the EO. The EO was essentially composed of oxygenated sesquiterpenes (75.2%), with α-bisabolol (**1**, fig. 1; 34.5%), fokienol (**2**, fig. 1; 12.0%) and T-muurolol (**3**, fig. 1; 6.8%) as the main components. We used NMR to identify the second major component, fokienol, which could not be identified by GC/MS, or by comparison with Kovats Index (KI) and MS databases.



α-Bisabolol (**1**) Fokienol (**2**) T-Muurolol (**3**)

Fig. 1. Major compounds identified in the *H. lobelii* EO

Table 1. Composition of the essential oil of *Hirtellina lobeli*

R_i [a]	R_i [b]	Identification[c]	Compound ID	Leaves EO
1013	1188	R_i, MS, CoGC	α-Phellandrene	t
1030	1203	R_i, MS, CoGC	Limonene	t
1098	1553	R_i, MS, CoGC	Linalool	0.1
1115	1584	R_i, MS	β-Fenchyl alcohol	0.1
1352	1466	R_i, MS	α-Cubebene	0.1
1377	1497	R_i, MS	α-Copaene	0.4
1382	1838	R_i, MS	(E)-β-Damascenone	0.2
1415	1612	R_i, MS, CoGC	β-Caryophyllene	0.7
1455	1689	R_i, MS	α-Humulene	0.3
1483	1784	R_i, MS	α-Curcumene	1.7
1500	1740	R_i, MS	α-Muurolene	1.3
1515	1716	R_i, MS, CoGC	γ -Cadinene	3.0
1526	1773	R_i, MS	δ -Cadinene	3.2
1566	2050	R_i, MS	Nerolidol	4.0
1577	2008	R_i, MS, CoGC	Caryophyllene oxide	2.6
1585	2182	NMR	Fokienol	12.0
1625	2088	R_i, MS	Epi-cubenol	2.4
1640	2188	R_i, MS	T-Cadinol	3.3
1642	2209	R_i, MS	T-Muurolol	6.8
1649	2256	R_i, MS	α-Cadinol	4.3
1669	2229	R_i, MS, CoGC	α-Bisabolol	34.5
1702	2323	R_i, MS	8-Cedren-13-ol	1.2
1758	2355	R_i, MS	Nuciferol	4.1
1750	2655	R_i, MS	Benzyl benzoate	0.3
2118	2603	R_i, MS	Phytol	0.2
			Monoterpene hydrocarbons	0.1
			Oxygenated monoterpenes	0.2
			Sesquiterpenes hydrocarbons	10.9
			Oxygenated sesquiterpenes	75.2
			Other	0.5
			Total identified	86.9

Notes: t = trace, less than 0.05%. [a] Retention index on a HP-5MS column; [b] Retention index on an Innovax column; [c] R_i Retention index identical to bibliography.

MS: identification based on comparison of mass spectra. Co-GC: retention time identical to authentic compounds; NMR: comparison of NMR spectra with those reported in the literature.

3.2. Antimicrobial activity

The minimum inhibitory concentrations (MICs) of *H. lobelii* EO and its main compound, α-bisabolol, as well as the minimum bactericidal and fungicidal concentrations (MBCs and MFCs) of the oil are reported in Table 2. The oil was considered active if the minimal inhibitory concentration was 128 µg/ml or below [30]. Most of the tested pathogens were sensitive to *H. lobelii* EO. The only resistant strains were the bacterium *E. coli*, the *Candida* yeasts, and the clinical isolate *A. fumigatus*. The EO was very active against the five dermatophytic *Trichophyton* species, *T. rubrum*, *T. mentagrophyte, T. violaceum, T. soudanense* and *T. tonsurans* (MIC values ranging from 8 to 64 µg/ml) and against the Gram (+) bacterium *S. aureus* with a MIC value of 32 µg/ml. It was also moderately active against the encapsulated yeast *C. neoformans* with a MIC value of 128 µg/ml. Interestingly, the growth of methicillin-resistant *S. aureus* (MRSA) was also inhibited by the EO, with a MIC value of 128 µg/ml. Although this value is higher than that for the non-resistant *S. aureus*, this result supports the use of combination therapies for MRSA infections that include *H. lobelii* EO.

Table 2. Antimicrobial activity (MIC, MBC or MFC in µg/ml) of *Hirtellina lobelii* essential oil and its major compound.

	Compounds

			H. lobelii EO	α- bisabolol	Oxacillin	Vancomycin	Gentamicin	Itraconazole	Fluconazole
Bacteria	*S. aureus* ATCC 29213	MIC	32	32	0.5	1	-	-	-
		MBC	64	-	0.5	-	-	-	-
		MBC/MIC	2	-	1	-	-	-	-
	MRSA ATCC 33591	MIC	128	-	>16	-	-	-	-
	E. coli ATCC 25922	MIC	> 512	-	-	-	8	-	-
Yeasts	*C. albicans* ATCC 10231	MIC	512	-	-	-	-	4	16
	C. parapsilosis ATCC 22019	MIC	512	-	-	-	-	0.5	2
	C. neoformans SNB-CN1	MIC	128	64	-	-	-	1	8
Filamentous fungi	*A. fumigatus* SNB-AF1	MIC	>512	-	-	-	-	0.5	>512
	T. rubrum SNB-TR1	MIC	64	32	-	-	-	<0.03	2
		MFC	64	-	-	-	-	4	>64
		MFC/MIC	1	-	-	-	-	>128	>32
	T. mentagrophytes SNB-TM1	MIC	32	32	-	-	-	0.125	64
		MFC	32	-	-	-	-	16	>64
		MFC/MIC	1	-	-	-		128	>1
	T. violaceum SNB-TV1	MIC	16	32	-	-		<0.03	4
		MFC	16	-	-	-		0.5	64
		MFC/MIC	1	-	-	-		>16	16
	T. soudanense SNB-TS1	MIC	16	16	-	-		<0.03	4
		MFC	32	-	-	-		4	64
		MFC/MIC	2	-	-	-		>128	16
	T. tonsurans SNB-TT1	MIC	8	8	-	-		0.25	16
		MFC	32	-	-	-		1	64
		MFC/MIC	4	-	-	-		4	4

Next, we investigated the source of the antimicrobial potential of this EO. The major constituent of *H. lobelii* oil, α-bisabolol, represented 34.5% of the total oil, and thus, it was tested on the microorganisms that showed the greatest susceptibility to the EO. The activity of α-bisabolol was roughly equivalent to that of the crude oil, with MIC values ranging from 8 to 64 µg/ml (Table 2). This result suggests a crucial role of α-bisabolol in the observed antimicrobial activity of this EO.

281 In the context of this study, it was interesting to discern whether the EO possesses bactericidal
282 and fungicidal properties capable of destroying bacterial and fungal cells or simple growth
283 inhibition effects (bacteriostatic and fungistatic activities). The bactericidal and fungicidal
284 activities of the oil were evaluated on the most sensitive pathogens (MIC < 128 µg/ml) and
285 were compared to those of the positive control drugs, oxacillin, intraconazole and fluconazole
286 (Table 2). The MBC/MIC and MFC/MIC ratios were calculated for each microorganism;
287 compounds are considered bactericidal or fungicidal when the MBC/MIC or MFC/MIC ratio is ≤
288 4 [31]. *H. lobelii* EO was found to be bactericidal against *S. aureus* (MBC/MIC = 2) and fungicidal
289 against all tested *Trichophyton* spp. (MFC/MIC = 1 to 4). Additionally, the EO was more effective
290 than the positive control antifungal drugs (MFC/MIC = 4 to > 128). Even itraconazole, which
291 inhibited the growth of the dermatophytic fungi at much lower concentrations (MIC 0.25 to <
292 0.03 µg/ml), was essentially fungistatic. The development of fungicidal therapies is crucial
293 because the prophylactic use of fungistatic drugs has been shown to be associated with an
294 increased frequency of acquired drug resistance in clinical isolates [32]; thus, these results add
295 more value to the *H. lobelii* EO.

296 **3.3. Combined effects of *H. lobelii* oil and antimicrobial drugs**

297 Based on the above results, it was relevant to evaluate the combined antimicrobial effects of *H.*
298 *lobelii* EO with various antimicrobial drugs. Synergies between EOs and antimicrobial drugs can
299 shorten the duration of therapies, decelerate the emergence of drug resistance and reduce the
300 possible side effects of current therapies by decreasing the necessary doses of the current
301 drugs and EO. The results of the checkerboard assay (FIC and FICI) are reported in Table 3, and

the isobolograms are presented in Fig. S1 (Supplementary Material). Most of the tested combinations of the oil and the drugs showed synergistic activity.

Table 3. Combined effects of *Hirtellina lobelii* essential oil and antimicrobial drugs

		EO			Drug					MRC5		MDA435	
Path	**Combination**	MIC_a	MIC_c	FIC	MIC_a	MIC_c	FIC	FICI	MIC_M	IC_{50}	SI	IC_{50}	SI
S. a[a]	EO:Vancomycin (1:0.5)	32	1	0.03	1	0.5	0.5	0.5	1.5	8	5.3	9	6
C. n[b]	EO:Fluconazole (1:0.125)	128	64	0.5	8	8	1	1.5					
	EO:Itraconazole(1:0.016)	128	64	0.5	1	1	1	1.5					
T. r[c]	EO:Fluconazole (1:0.5)	64	1	0.01	2	0.5	0.25	0.3					
	EO:Griseofulvin* (1:0.25)	64	1	0.01	1	0.25	0.25	0.3	1.25	7	5.6	8.5	6.8
T. m[c]	EO:Fluconazole (1:8)	32	2	0.06	64	16	0.25	0.3					
	EO:Griseofulvin* (1:0.25)	32	1	0.03	0.5	0.25	0.5	0.5	1.25	7	5.6	8.5	6.8
T. v[c]	EO:Fluconazole (1:0.5)	16	1	0.06	4	0.5	0.13	0.2					
	EO:Griseofulvin* (1:0.03)	16	4	0.25	0.5	0.13	0.25	0.5	4.125	7.5	1.8	8.5	2.1
T. s[c]	EO:Fluconazole (1:0.5)	16	2	0.125	4	1	0.25	0.4					
	EO:Griseofulvin*(1:0.125)	16	1	0.06	0.5	0.13	0.25	0.3	1.125	7.2	6.4	8.5	7.5
T. t[c]	EO:Fluconazole (1:8)	8	1	0.125	16	8	0.5	0.6					
	EO:Griseofulvin*(1:0.125)	8	2	0.25	1	0.25	0.25	0.5	2.25	7.2	3.2	8.5	3.8

[a]Bacteria: *S.a.* = *Staphylococcus aureus* (ATCC 29213), [b]Yeast: *C.n.* = *Cryptococcus neoformans* (SNB-CN1), [c]Filamentous fungi: *T.r.* = *Trichophyton rubrum* (SNB-TR1), *T.m.* = *Trichophyton mentagrophytes* (SNB-TM1), *T.v.* = *Trichophyton violaceum* (SNB-TV1), *T.s.* = *Trichophyton soudanense* (SNB-TS1), *T.t.* = *Trichophyton tonsurans* (SNB-TT1).

MIC_a: MIC of the product alone (in µg/ml); MIC_c: MIC of the drug or the essential oil representing the highest synergy or antagonism (in µg/ml); MIC_M: MIC of the mixture (MICc EO + MICc drug); FIC: fractional inhibitory concentration; FICI: FIC index; SI: selectivity index (SI = IC_{50}/MIC).

**H. lobelii* EO: itraconazole combinations could not be tested on dermatophytic fungi because itraconazole has very low MIC values. Instead, *H. lobelii* EO: griseofulvin combinations were tested, given that griseofulvin is frequently prescribed for dermatophytosis.

317 We observed a synergistic interaction between vancomycin and *H. lobelii* EO against *S. aureus*
318 (FICI = 0.5). For the yeast *C. neoformans*, an additive effect was only detected for the
319 combination of EO and azole drugs (FICI = 1.5). However, the most interesting results of the
320 combination treatments were observed for the dermatophytic fungi. *H. lobelii* EO was
321 synergistic with fluconazole and griseofulvin against all tested *Trichophyton* spp. (FICI values
322 from 0.2 to 0.5), with one exception of an indifferent interaction between the EO and
323 fluconazole against *T. tonsurans* (FICI = 0.6). The greatest synergism was observed for the
324 combination treatment of EO with fluconazole against *T. violaceum*, with a FICI value of 0.2.
325 Overall, the combinations tested in this study would allow for a notable decrease in the
326 necessary concentrations of commercial drugs due to a 2- to 8-fold decrease in MIC values.
327 Likewise, the oil concentration required to inhibit the growth of the pathogens was reduced to
328 very small amounts (MIC_C 1 – 2 µg/ml), thus, limiting the potential cytotoxic effect of the EO
329 (Table 3, see also Fig. S1). Indeed, the selectivity indexes (SI) of the synergetic mixtures at the
330 FICI values showed that the antimicrobial activity did not exceed the cytotoxicity (with the
331 exception of *T. violaceum*). For *S. aureus*, *T. rubrum*, *T. mentagrophytes* and *T. soudanense*, the
332 selectivity indexes of the combination treatments ranged from 5.3 to 7.5, indicating relatively
333 low cytotoxicities. The selectivity indexes were calculated based on the cytotoxicity
334 measurements of the drugs in the skin cancer cell line MDA435, which is a relevant cell line in
335 the context of the topical application of antimicrobial agents.

336 The mechanism by which some EOs have synergistic interactions with antimicrobial drugs is not
337 yet clear. It has been postulated that some terpenes may act as solvents for the antimicrobial
338 drug, facilitating its passage across cell membranes [33]. However, *H. lobelii* EO is strongly

antimicrobial on its own. This may indicate a specific mode of action and could explain why combination treatments with this EO are so effective.

3.4. Scanning electron microscopy analysis of in vitro effects of *H. lobelii* EO

Changes in morphology of bacteria and filamentous fungi caused by antimicrobial compounds have often provided insight into their mechanism of action [34]. To investigate the effect of *H. lobelii* EO on the morphological characteristics of *S. aureus*, *T. rubrum* and *T. soudanenese*, bacterial and fungal samples treated with sub-inhibitory concentrations (sub-MICs) of the oil were observed by SEM. At sub-MICs, microbial growth is not severely affected while morphological alterations can sometimes be detected [35,36].

The morphological characteristics of *S. aureus* bacteria were not affected by EO treatment. No morphological changes were detectable by SEM (data not shown).

Untreated *T. rubrum* and *T. soudanenese* showed typical structures of healthy hyphal elements, including rod-shaped filaments of uniform width with lines of separation (septa) and a smooth surface. Hyphae showed regular branching (fig. 2a, e). In contrast, an unusual pattern of hyphal growth, including alterations in cell shape and size were evident in both *T. rubrum* and *T. soudanenese* hyphae in response to the EO (fig. 2b, c, d, f). The treated hyphae showed aberrant morphologies, such as a loss of linearity with bulging, swollen cells and anomalous branching. A portion of the *T. rubrum* mycelia seemed to be particularly inflated with a rough surface, probably because they were covered by extruded cell material (fig. 2c). Interestingly, fig. 2d shows a flattened and shrivelled hyphal element with partial distortion and a few small vesicles on the surface. At this time point, hyphal or cytoplasmic debris were dispersed on the

surface of the hyphae. These morphological alterations have been previously observed in dermatophyte hyphae treated with terbinafine [37]. These different morphological characteristics could give us a glance on the sequence of events during the exposure of fungi to the EO, starting with cell swelling and cellular leakage, leading to the breakage and collapse of hyphal cells.

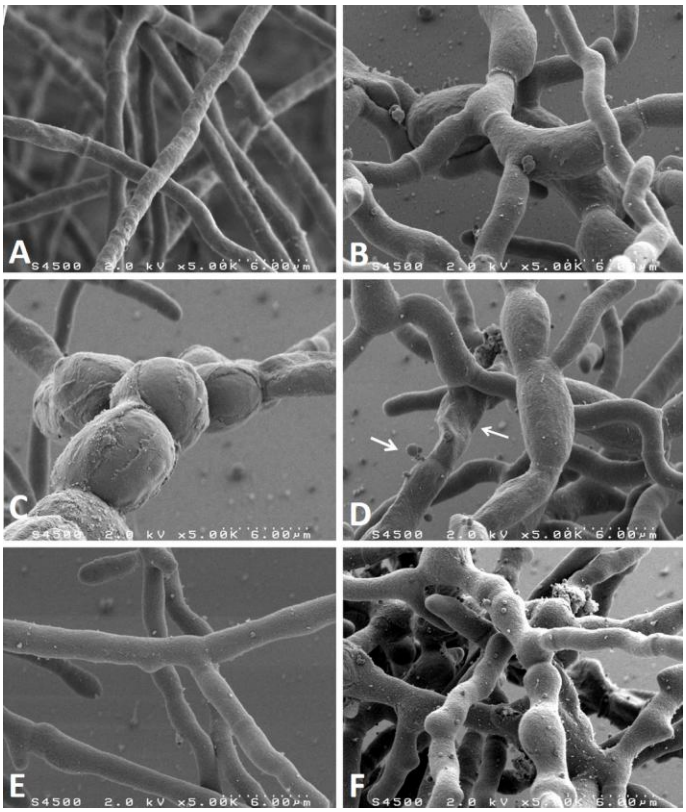


Fig. 2. Scanning electron micrographs of dermatophytic hyphae (5000x magnification)
(A) Untreated control *T. rubrum* hyphae. (B), (C) and (D) *T. rubrum* hyphae treated with 32 µg/ml *H. lobelii* EO; (B) showing swollen cells and anomalous branching, (C) showing highly inflated cells with a rough surface, (D) arrow indicates flattened and shriveled hyphae with vesicles on the surface. (E) Untreated control of *T. soudanense* hyphae. (F) *T. soudanense* hyphae treated with 8 µg/ml *H. lobelii* EO, showing swollen cells and anomalous branching.

In previous studies, similar cell damaging effects were also observed in hyphae exposed to terpenes or plant extracts [38,39]. It has been postulated that terpenoids interfere with the phospholipid bilayers of membranes and increase their permeability [40]. Our SEM observations validate the disruption of the fungal membrane in response to *H. lobelii* EO. Interestingly, our results with *H. lobelii* EO do not necessarily correlates with those obtained with closely related antifungal sesquiterpenes. For example, nerolidol did not induced membrane swelling in *Trichophyton mentagrophytes* [41]. At high concentration (0.11 mg/mL and above), internal vesicles appeared in the cells, ultimately leading to membrane breakdown and abnormal mitochondria structure.

Our results are in agreement with the observed synergetic combinations because alteration in membrane permeability by the EO would facilitate the infiltration of the cell cytoplasm by more hydrophilic molecules, including a wide range of antibiotics and antifungal drugs. However, in this case, *H. lobelii* EO is active at low concentration, lower than that of many terpenes described in the literature. This might indicate an additional specific interaction of EO constituents against cell membrane components, most probably α-bisabolol which was active on its own.

4. Conclusions

Our data demonstrated that *Hirtellina lobelii* EO and its major component, α-bisabolol, have potent antimicrobial activities, as manifested by drastic morphological changes, particularly in dermatophytic fungal cells. Although the mechanism of action of this EO and α-bisabolol

against *S. aureus* remains unresolved, SEM observation on EO-treated fungi revealed alteration of cell membrane permeability, presumably associated with a specific antifungal potential linked to the presence of α-bisabolol. Altogether, these results demonstrate that both *H. lobelii* EO and α-bisabolol could be used in antifungal mixtures of either the EO or the terpene in combination with antifungal drugs, such as fluconazole and griseofulvin. These combinations should improve the efficiency of the drugs in local applications, in particular, when the drugs alone are essentially fungistatic. Combination treatments will also reduce the amount of drug used and restrict the use of newly developed drugs to serious clinical cases, therefore, slowing the development of chemoresistant strains. Additional combinations of this EO with other antifungal drugs are worth testing.

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