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ORIGINAL ARTICLE

Antifungal Activity of Essential Oils Against *Candida* Species Isolated from Clinical Samples

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Abstract We evaluated the in vitro antifungal activity of essential oils obtained from the aromatic plants *Laurus nobilis*, *Thymus vulgaris*, *Mentha piperita*, *Cymbopogon citratus* and *Lippia junelliana* against the following *Candida* species isolated from clinical samples: *C. krusei* ($n = 10$); *C. albicans* ($n = 50$); *C. glabrata* ($n = 70$) and *C. parapsilosis* ($n = 80$). The minimal inhibitory concentration (MIC) was determined according to EDef 7.3.1 document from EUCAST. Amphotericin B and fluconazole were the antifungal drugs used as inhibition control. The concentration ranges evaluated were 0.4–800 and 0.03–128 mg l^{-1} for essential oils and antifungal drugs, respectively. MIC_{50} and MIC_{90}, mode and ranges were calculated. All the *Candida* spp. evaluated were susceptible to amphotericin B (MIC $\leq$ 1 mg l^{-1}), while fluconazole was inactive for *C. krusei* (MIC $\geq$ 32 mg l^{-1}) and intermediate for *C. glabrata* (MIC$\leq$ 32 mg l^{-1}). The essential oils showed antifungal activity on *Candida* spp. tested with MIC_{90} values ranging from 0.8 to 800 mg l^{-1}. In general, the most active essential oils were *L. nobilis* and *T. vulgaris* (MIC_{90} 0.8–0.16 mg l^{-1}), and the least active was *C. officinalis* (MIC_{90} 400–800 mg l^{-1}). *C. krusei* was inhibited by 5/6 of the essential oils evaluated, and *C. glabrata* was the least susceptible one. This in vitro study confirms the antifungal activity of these six essential oils assayed which could be a potential source of new molecules useful to control fungal infections caused by some *Candida* species, including those resistant to antifungal drugs.

Keywords Essential oils · *Candida* spp. · *Laurus nobilis* · *Thymus vulgaris* · *Mentha piperita* · *Cymbopogon citratus* · *Lippia junelliana*

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Introduction

Candidemia, vulvovaginitis and oral candidiasis are infections caused by *Candida* spp. Candidemia has a significant impact on the outcome of patients; thus, the associated mortality has been estimated to be 15–54% for adults and 10–13% for neonates and children

[1–4]. Species distributions of *Candida* species causing candidemia vary according to geographical origin, although *Candida albicans* remains the most frequent species followed by non-*C. albicans* species such as *C. parapsilosis*, *C. tropicalis, C. glabrata* and *C. krusei,* among others [5, 6]. The antifungal drug chosen for treatment varies according to the yeast species causing infection; thus, echinocandins, amphotericin B and azoles are the antifungal drugs recommended for the treating patients with candidemia, while azole drugs are commonly used for treating vulvovaginitis and oral candidiasis [7]. These antifungal drugs are potentially toxic to humans and are not always efficient enough to control these fungal infections [8]. Currently, the emergence of antifungal resistance is reported worldwide and is still an unresolved problem. [9, 10].

Hence, it is necessary to study other molecules with possible antifungal activity, focusing on the election of natural compounds not based on existing synthetic drugs, in order to avoid the emergence of resistance.

For centuries, several aromatic plants have been used in folk medicine to treat common diseases caused by bacteria, parasites, viruses and fungi [11, 12]. More than 80% of the world population uses different natural compounds obtained from plants for treating human diseases [13]. In addition, the aromatic plants are used as flavoring agents in pharmaceutical and cosmetic industries, and as food preservatives [14].

Among aromatic plants, the essential oils of *Thymus vulgaris*, *Mentha piperita*, *Calamintha officinalis, Lippia junelliana, Laurus nobilis* and *Cymbopogon citratus* have been widely used as antimicrobial agents with promissory results [14, 15]. These six aromatic herbs are present in Argentina's soil and are commonly used as folk medicine by several communities all around the country [16, 17]. In general, these aromatic herbs have been used for treatment of bronchitis, tonsillitis, sore throat, influenza, rheumatism, dermatitis, as antispasmodics, as antioxidants, to prevent cataracts development and as antimicrobials against bacteria, fungi, viruses, and parasites [18–25]. The effectiveness of essential oils as antimicrobial agents has been attributed to the presence and activity of some constituents, such as carvacrol, thymol, eucalyptol, linalool, these constituents can act alone or together, being frequently synergistic among them [14, 26, 27].

Although essential oils show antimicrobial activity against a broad spectrum of microorganisms, in Argentina there are only few studies evaluating the efficacy in fungi isolated from clinical samples.

Aim

The aim of this study was to evaluate the in vitro antifungal activity of essential oils extracted from six aromatic plants on *Candida* species isolated from human clinical samples.

Materials and Methods

Two hundred and ten isolates of *Candida* spp. were studied. The yeasts strains were isolated from different clinical sources of patients living in Argentina (blood, biopsies, oral swab and vaginal fluids). The identification of the species was performed in previous studies [5, 28]. The strains were deposited at the Culture Collection of the Departamento de Micologia (DMic), Instituto Nacional de Enfermedades Infecciosas Agudas, "Dr. Carlos G. Malbrán," Buenos Aires, Argentina and were conserved at − 20 °C in 20% glycerol. For determining assays, an aliquot of each original strain was conserved at 4 °C until use.

Species studied were *C. krusei* $n = 10$; *C. albicans* $n = 50$; *C. glabrata* $n = 70$ and *C. parapsilosis* $n = 80$.

Susceptibility Tests

The minimal inhibitory concentration (MIC) was determined following the reference method E.Def 7.3.1 from European Committee on Antimicrobial Susceptibility Testing [29] using Roswell Park Memorial Institute medium (RPMI) and 3-(*N*-morpholino) propane sulfonic acid buffer (MOPS) plus 2% glucose; these reagents were purchased from Merck, Co., Buenos Aires, Argentina.

Antifungal Drugs

Amphotericin B (Merck, Co.) and fluconazole (Pfizer, Buenos Aires, Argentina) were obtained as powders of known potency from manufacturers and were used as inhibition controls. The final range concentration evaluated was 16–0.03 mg l^{-1} for amphotericin B

and 128–0.25 mg l^{-1} for fluconazole. Drugs were stored at − 20 °C until used.

Essential Oils

The essential oils of *L. nobilis* L. (Lauraceae), *T. vulgaris* L. (Lamiaceae), *M. piperita* L. (Lamiaceae), *C. citratus* (D.C.) Stapf. (Poaceae), *L. junelliana* (Mold.) Tronc. (Verbenaceae) and *C. officinalis* Moench. (Lamiaceae) were extracted by hydro-distillation according to Clevenger (IRAM 18729) (humidity 10–15%) of flowers, leaves or stems. These plants are present in Argentina's soil.

The essential oil of *L. junelliana* was provided for the Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina as stock solution, and the other essential oils were provided by the Curso Bioquímica y Fitoquímica, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina.

The principal constituents of the essential oils tested were determined as follows: Volatile organic compounds (VOC) of the essential oil were sampled by headspace solid-phase microextraction (SPME). A polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65-µm fiber (Supelco, Bellefonte, PA, USA) was conditioned and exposed for 10 min to 1 µl of extract placed in a 4-ml vial sealed with a septum, at two different temperatures (25 and 50 °C to quantify and identify, respectively). Quantitative analysis was performed using a Hewlett Packard 6850 gas chromatograph (GC) employing a nonpolar HP-5MS capillary column (30 m, 0.25 mm I.D., 0.25 µm film thickness) (J&W, Folsom, CA, USA) coupled to flame ionization detector set at 280 °C. The injector was operated in splitless mode set at 250 °C, and the oven was programmed from 40 °C for 2 min, 10 °C/min to 200 °C, 15 °C/min to 250 °C with a holding time of 5 min at the final temperature. VOC identification was performed in similar gas chromatography (GC) conditions with a GC HP 6890 coupled to a mass selective detector Agilent 5975C VL operated at 70 eV. Their mass fragmentation patterns were compared with commercial mass database [30, 31] and Kovats retention index (KI) [32, 33].

Twofold dilutions of essential oils from each aromatic plant were prepared from stock solutions. The final range concentration evaluated was 0.4–800 mg l^{-1}. Then, 100 µl of each dilution was placed on 96-well cell culture plates. Essential oils were stored in a brown glass bottle at 4 °C until used.

The principal constituents of the essential oils tested are summarized in Table 1.

Inoculum

Yeasts were cultured on YM agar (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1%, agar 2%) during 24 h, at 30 °C; then, a suspension 0.5 McFarland (1–5 × 10^6 CFU/ml) was prepared in 0.15 M sterile sodium chloride solution (saline solution 0.85%). The final inoculum was 1–5 × 10^5 CFU/ml^{-1}. Microplates were incubated on static condition at 35 ± 2 °C, 24 ± 2 h. The reading was performed to 405 nm using a spectrophotometer (Labsystems Multiskan Multisoft, Basingstoke, UK).

Quality Control Strains

Candida parapsilosis ATCC 22019 and *Candida krusei* ATCC 6258 were included in all the susceptibility tests performed.

Endpoints

For amphotericin B, the MIC endpoint was defined as the lowest concentration of drug that caused a prominent reduction (MIC ≥ 90%) of growth compared with that of a drug-free growth control well. For fluconazole, the MIC_{50} was considered, that is, the lowest concentration of drug that caused a reduction of ≥ 50% of growth compared to the control well. For these antifungal drugs, the categorical interpretation proposed by EUCAST was used [36].

For essential oils, no break points have been defined; thus, the MIC_{50}, MIC_{90}, range and mode were calculated.

Results

Candida species tested exhibited different susceptibility profiles such as susceptible, intermediate and resistant against the antifungal drugs assayed.

All strains were susceptible to amphotericin B (MIC values ≤ 1 mg l^{-1}).

Table 1 Percentage of the main constituents of essential oils assayed

Main constituents	*Laurus nobilis*[1]	*Lippia junelliana*[2]	*Thymus vulgaris*[3]	*Mentha piperita*[3]	*Cymbopogon citratus*[2]	*Calamintha officinalis*[2]
Limonene	1.3	1.0–2.5				1.9
Linalool	31.3		2.4			
Carvacrol			2.4			
Myrcene	1.1	9.1–10.5			38.3	
Carvone		1.6–1.7				38.7
Sabineno	4.2					1.5
Neral (β-citral)					17.5	
Geranial (α-citral)					18.6	
Cis-davanone		17.7–20.1				
Γ-terpinene			13.1			
p-cymene			18.1			
1.8, cineole	29.8					6.4
Myrcenone		11.6–14.8				
Z-ocimenone		7.0–8.1				
E-ocimenone		7.7–9.3				
Terpinen-4-ol	1.5					
β-caryophyllene	1.0	4.4–6.0	1.5			
Trans-β-ocimene					2.3	
Thymol			39.9			
Germacrene-β	2.8					
Bicyclogermacrene		1.6–2.4				
β-pinene	2.1		2			0.9
Cis-β-ocimene					3.6	
α-pinene	2.1					
Camphor		2.8–5.1				
Espatulenol		2.3–3.7				
Caryophyllene oxide		2.0–3.7				
Camphene		1.7–2.5				
Geraniol					2.3	
α-terpinene			1.8			
Trans-nerolidol		1.8–1.9				
α –humulene		1.0–1.3				
α –terpineol	3	1.1–1.4				
Δ terpineol	1.5					
Cis-oxide linalool	8					
Menthol				44.6		
Menthofuran				10		
Mentone				20		
Methyl acetate				7.4		
Neo-dihydrocarveol						9.9
Dihydrocarveol						6.9
Acetate dihydrocarveol						7.6
Cis-carvyl acetate						6.2

Table 1 continued

Main constituents	*Laurus nobilis*[1]	*Lippia junelliana*[2]	*Thymus vulgaris*[3]	*Mentha piperita*[3]	*Cymbopogon citratus*[2]	*Calamintha officinalis*[2]
Pulegone						4.1

[1]*L. nobilis:* chemical analysis of essential oils was performed by Larran et al. [33]

[2]*L. junelliana, C. citratus* and *C. officinalis*: chemical analysis of essential oils was performed by INIBIOLP [34]

[3]*T. vulgaris* and *M. piperita*: chemical analysis of essential oils was performed by Allippi et al. [35]

For fluconazole, *C. albicans* and *C. parapsilosis* were susceptible; on the contrary, *C. krusei* is intrinsically resistant to this azole and showed MIC value 32 mg l^{-1}, while *C. glabrata* was categorized as intermediate (MIC value 0.125–32 mg l^{-1}).

The essential oils showed different degrees of antifungal activity on *Candida* spp. tested with MIC_{90} values ranging from 0.8 to 800 mg l^{-1}.

In general, the most active essential oils were those obtained from *L. nobilis* and *T. vulgaris* (MIC_{90} 0.8–0.16 mg l^{-1}) and the least active was from *C. officinalis* (MIC_{90} 400–800 mg l^{-1}).

Among the species studied, *C. glabrata* was the least susceptible against all the essentials oils tested (MIC_{90} 200–800 mg l^{-1}) and *C. krusei* was inhibited at MIC_{90} values range of 0.8–3.12 mg l^{-1} by 5/6 of the essential oils evaluated.

For the quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, the MIC value obtained was similar to those of *Candida* spp. evaluated.

The MIC values of essential oils and antifungal drugs evaluated are summarized in Table 2.

Discussion

Despite the biodiversity of several aromatic herbs present in Argentina's soil and the use of them as folk medicine all around the country, no previous reports have evaluated and compared the in vitro antifungal activity of essential oils extracted from *L. nobilis*, *T. vulgaris*, *M. piperita*, *C. citratus*, *L. junelliana* and *C. officinalis* against *Candida* species isolated from patients living in Argentina.

We used the EDEF 7.3.1 EUCAST reference microdilution method to determine the MIC value of antifungal drugs and essential oils in order to obtain reproducible, comparable and trustworthy results.

In the present study, we included four of the most frequent *Candida* species recognized as causal agents of fungal infections: *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei.* These *Candida* species exhibited different susceptibility profiles such as susceptible, intermediate and resistant against the antifungal drugs assayed as controls. This wide profile of in vitro susceptibility to antifungal drugs allowed us to compare the effectiveness of essential oils to inhibit the *Candida* spp. that were categorized as not susceptible to antifungal agents.

Among the essential oils tested, *L. nobilis* and *T. vulgaris* oils were the most potent inhibitors of *Candida* spp. growth.

L. nobilis essential oils had linalool (alcohol monoterpene) and 1.8 cineole (sesquiterpene) as principal compounds. Both compounds showed to alter the formation of biofilm, which would favor the access of antifungal agents to the fungal cell [27, 37].

Our *Candida* spp., where in vitro inhibited at 0.8 mg l^{-1}, this concentration is slightly lower than the one reported by other authors who observed not only high inhibitory activity of *L. nobilis* essential oils at 1 mg l^{-1} against *Candida* spp., but also a significant inhibition of adhesion property and formation of immature biofilm [37].

In this study, *T. vulgaris* oils inhibited the growth of *C. albicans, C. krusei, C. parapsilosis* and *C. tropicalis* at low MIC value of 0.8–1.6 mg l^{-1}. Similar results were communicated by Sokovic et al., [14] who observed a strong antifungal activity of *T. vulgaris* (MIC value 0.25 µl/ml^{-1}) against different species of micelial fungi such as *Alternaria alternata, Fusarium tricinctum, Aspergillus* species and dermatophytes. To remark, the principal compounds of essential oils of *T. vulgaris* used by Sokovic were thymol (48.9%) and

Table 2 MIC values of essential oils and antifungal drugs against *Candida* species

	Essential oils mg l^{-1}						Antifungal drugs mg l^{-1}	
Candida species n = 210	*Laurus nobilis*	*Thymus vulgaris*	*Mentha piperita*	*Cymbopogon citratus*	*Lippia junelliana*	*Calamintha officinalis*	Amphotericin B	Fluconazole
C. krusei n = 10								
MIC_{50}	0.8	0.16	1.25	0.16	3.12	400	0.5	32
MIC_{90}	0.8	0.16	0.16	25.83	3.12	800	0.6	76.8
Mode	0.8	0.16	1.6	1.6	3.12	200	0.5	32
Range	0.8	0.8–1.6	0.4–1.6	0.8–50	1.6–3.12	200–800	0.125–1	32–128
C. albicans n = 50								
MIC_{50}	0.8	0.16	6.12	25	6.12	800	0.25	0.13
MIC_{90}	0.8	0.16	800	25	400	800	0.5	1
Mode	0.8	0.16	800	25	400	800	0.5	0.125
Range	0.8	0.16	0.4–800	1.6–25	3.12–400	12.5–800	0.032–1	0.016–2
C. glabrata n = 70								
MIC_{50}	25	200	400	100	800	800	0.5	2
MIC_{90}	800	200	800	200	800	800	1	16
Mode	12.5	200	400	100	800	800	0.5	1
Range	0.8–800	50–400	25–800	12.5–800	400–800	100–800	0.032–1	0.125–32
C. parapsilosis n = 80								
MIC_{50}	0.8	0.8	1.6	0.8	1.6	400	0.5	1
MIC_{90}	0.8	1.6	110	3.94	3.12	400	1	2
Mode	0.8	0.8	0.8	0.8	1.6	400	0.5	0.5
Range	0.8–800	0.8–1.6	0.8–800	0.8–100	1.6–400	100–400	0.032–1	0.016–2
Quality control strains								
C. krusei ATCC 6258	0.8	1.6	1.6	1.6	3.12	800	0.5	32
C. parapsilosis ATCC 22019	0.8	0.8	0.8	50	1.6	200	0.25	1

MIC: minimal inhibitory concentration; MIC_{90}: MIC at which 90% of the isolates tested are inhibited; MIC_{50}: MIC at which 50% of the isolates tested are inhibited

p-cymene (19.0%), that composition was higher than the one found in our essential oil, 39.9% and 18.1%, respectively. Thymol is an oxygenated phenolic monoterpene and may cause dysfunction of enzymes present in cell membrane and cell wall; therefore, permeability of cell membrane is altered leading to wall disruption and cell death [38]. Some features of thymol are important to highlight, the first one is that the activity of other *T. vulgaris* chemotypes, such us linalool chemotype, geraniol chemotype and sabinene hydrate chemotype varies according to the species tested, in fact, these chemotypes did not inhibit the growth of *Aspergillus niger*, while for *C. albicans* and *Cryptococcus neoformans* they showed low antifungal activity [39]. The second one is the synergistic antifungal effect of thymol in combination with the antifungal drug fluconazole since it can inhibit the over-expression of efflux pump genes CDR1 and MDR1 in *C. albicans*. [27].

For *M. piperita* essential oil, the main inhibitory activity was observed against *C. krusei,* while for *C. albicans* and *C. parapsilosis* the range of MIC values was 0.4–800 mg l^{-1}, indicating that the susceptibility is variable and species dependant. Perhaps, this potent in vitro inhibitory activity against *C. krusei* can be taken into account for the design of new molecules with antifungal properties, since this species is intrinsically resistant to fluconazole and can develop cross-

resistance to other azole antifungal drugs. Menthol is a monoterpene alcohol; it is the main constituent of *M. piperita* essential oil and causes disruption of the cell membrane, increase in membrane permeability and subsequent exit of the cytoplasmic content. This mechanism of action could explain the high antifungal activity of *M. piperita* against some yeasts and micelial fungi [14, 24].

C. citratus oils showed major activity against *Candida* spp. forming hyphae and pseudohyphae (*C. albicans*, *C. krusei* and *C. parapsilosis*). Similar results were observed by other researchers who demonstrated that monoterpene aldehydes such as geranial and neral (∞ and β citral, respectively) have not only anti-inflammatory properties but also inhibit the transition from budding to hyphal form of the yeasts [40]. Such morphological alterations of *Candida* spp. are critical and reduce their possibility to adhere to the host cell; thus, a diminution of virulence can occur. The antifungal activity of these monoterpenes has been related to their highly lipophilic nature and low molecular weight; thus, they cause disruption of the cell membrane, leading to cell death. To remark, this inhibitory activity is lower when the monoterpene are used as single compounds. Therefore, *C. citratus* essential oils could be potentially useful for local treatment of cutaneous infections by *Candida* spp. [18].

For *L. junelliana,* we observed moderate inhibition on *Candida* spp. growth (MIC values of 3.12–400 mg l^{-1}). Similar results were observed by Zigadlo et al. [41] who observed inhibition of micelial elongation of *Alternaria solani* at 100 mg l^{-1}. The low antifungal activity of *L. junelliana* oils can be due to the presence of monoterpene ketones in its composition.

To consider, the available information about the antifungal activity of *L. junelliana* essential oils is scarce; therefore, this was a limitation because we could not compare our results with others.

Regarding to *C. officinalis* essential oil, MIC values were higher (400–800 mg l^{-1}) than the ones obtained with the other five essential oils tested. In the present study, the principal constituent of *C. officinalis* essential oil tested was the ketone carvone (38.7%). However, this composition was not enough to inhibit *Candida* spp. growth as expected. The lack of efficacy of *C. officinalis* to inhibit the fungal growth was also observed by Nostro et al. [42] who reported the inefficacy as preserving cosmetics cream when *Candida* sp. and *Aspergillus* sp. caused contamination. On the contrary, Monforte et al., [26] evaluated the in vitro activity of *C. officinalis* essential oils whose principal compound was carvone (38.7%) and observed inhibitory activity against *Candida* sp. and *Aspergillus* sp. Although the percentage of carvone was the same in our work and in that of Monforte, differences between results could be due to the possible interaction among compounds that are present in smaller quantities [27].

We recognize our limitations since we did not evaluate the in vitro susceptibility of each essential oil compounds separately, although we know the majority of compounds do not exert the antimicrobial action by themselves, on the contrary, the minority of compounds would act in interaction improving the antimicrobial activity [43]. Despite these limitations, data obtained here could represent the susceptibility profiles of at least the four most common *Candida* spp. circulating in Argentina.

In summary, we observed an acceptable antifungal activity of *L. nobilis*, *T. vulgaris*, *M. piperita*, *C. citratus* and *L. junelliana* essential oils against *C. albicans*, *C. parapsilosis* and *C. krusei,* while for *C. officinalis* the activity was low for all *Candida* spp. tested.

Regarding *C. glabrata,* all the essential oils tested failed to inhibit its growth.

Finally, our in vitro results seem to be promissory and could be considered as potential utility for choosing an alternative antifungal treatment.

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Author Contributions SC designed the study and prepared the manuscript; WV and WS performed the in vitro test; GNA analyzed the data.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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