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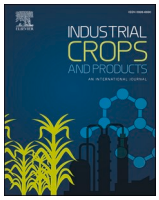

Comparing the effect of *Thymus* spp. essential oils on *Candida auris*

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ABSTRACT

Candida auris is an emergent yeast pathogen responsible for many invasive fungal infections due to its multidrug-resistant character and its huge transmission ability. Essential oils (EOs) obtained from several aromatic plants have been regarded as an alternative treatment upon to fungal infections. For example, *Thymus* spp. are known by their antifungal effect due to the presence of some volatile compounds in their EOs, such as carvacrol, thymol, linalool and γ-terpinene. So, the main goal of this work was to compare the effect of several EOs from *Thymus* spp. on *C. auris* biofilm.

The antifungal activity of *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina* against planktonic cells of *C. auris* NCPF 8971 was assessed by agar disk diffusion method. The effect of these EOs with direct or vapour phases on preformed biofilms was evaluated by colony-forming units' enumeration. Importantly it was noticed a completely different range of action between the EOs from the same genus. While *T. vulgaris* showed the biggest antifungal effect with a halo of 59.75 ± 15.75 mm, *T. mastichina* for the other side, presented a halo of 13.13 ± 1.36 mm, showing a very low activity. In addition, the direct application of *T. vulgaris* and *T. zygis* EOs demonstrated higher antifungal activity against *C. auris* biofilms than vapour phase application. However, *T. vulgaris* also showed significant action in the vapour phase mode. The chemical composition of *Thymus* EOs was analysed by GC and GC-MS, and was demonstrated that they have different chemical profiles, namely in the amount of phenolic compounds, which justify the different antifungal actions.

In conclusion, *T. vulgaris* and *T. zygis* oils, can be pointed out as a great contribution to the treatment of *C. auris* infections, being promising alternatives to conventional therapy. However, the efficiency of the EOs differs substantially between the four species of *Thymus*. Therefore, the selection of natural therapies should always have in account EOs composition.

1. Introduction

In recent years, the incidence and prevalence of fungal infections became a worrying problem in hospital environments. This invasive fungal infections is reported to be one of the most common diseases in humans, affecting nearly 25% of the population (Karpiński, 2020). In the last decade, *Candida auris* has emerged as fungal human pathogen that causes hospital outbreaks, especially in intensive care units (ICU) (Ruiz-Gaitán et al., 2018; Shastri et al., 2020). Since its discovery in 2009, more than 30 countries have already identified this *Candida* spp. as a frightening pathogen (Wang et al., 2020; Welsh et al., 2018).

The virulence factors of *C. auris* include surface's adhesion, thermo and salt tolerance, and the ability to develop biofilms, which are responsible to the enhancement of antifungal resistance (Bravo Ruiz and Lorenz, 2021; Chow et al., 2020; Oh et al., 2020; Shyni and Lavanya, 2021). The persistence of *C. auris* on several equipment and medical device's surfaces increases the risk of transmission (Horton and Nett, 2020). In hospital environment, the fungal spread may occur between patients or via contact of health care workers with patients (de Cássia Orlandi Sardi et al., 2018; Shastri et al., 2020). The ability of *C. auris* to persist on the patients' skin and its complex identification make the outbreaks more difficult to contain (Chaabane et al., 2019; Horton et al.,

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2020; Lockhart, 2019; Ruiz-Gaitán et al., 2018).

The conventional treatment of fungal infections consists of the administration or application of antifungal drugs. However, these agents have some disadvantages, such as lack of selectivity, toxicity and high antimicrobial resistance, mainly against azoles (Bona et al., 2016; Leal et al., 2017). Essential oils (EOs) are complex mixtures of several volatile compounds produced by aromatic plants that can be used as an alternative therapy due to their multiple helpful properties for human complications, including antiviral, anti-inflammatory or antifungal activity. However, their antifungal properties are difficult to associate to a specific compound. Generally, the antifungal activity could be a result of the principal bioactive compounds of the EOs or due to a synergistic action between the major and the minor compounds (Ballester-Costa et al., 2013). EOs of several *Thymus* species are recognized by their antifungal activity (Ballester-Costa et al., 2013; Salehi et al., 2019). Some of these oils are widely used, such as *Thymus vulgaris* and *Thymus zygis* (Karpiński, 2020). The mechanism of action of EOs can include disruption of the wall or membrane of fungi cells, enhancing the permeability of these structures, and inhibition of the synthesis of DNA, RNA, proteins and polysaccharides (Karpiński, 2020). Although there are several studies on the antifungal activity of *Thymus* spp. oils, there are still no reports of the effect of these oils against *C. auris*. Thus, this study evaluated the antifungal properties of EOs from *T. vulgaris* L., *T. zygis subsp. sylvestris* Loefl. ex L., *Thymus satureioides* Coss. and *Thymus mastichina* L. against *C. auris* biofilms and related the activities with their composition.

2. Materials and methods

2.1. Microorganisms and culture conditions

C. auris NCPF 8971 was stored in broth medium with 20% (v/v) glycerol at −80 ± 2 °C until required. Before testing, cells were activated by thawing at room temperature. Then they were subcultured into Sabouraud Dextrose Agar (SDA; Liofilchem) and incubated at 37 °C for 24 h. The inoculum was prepared on Sabouraud Dextrose Broth (SDB; Liofilchem) and was incubated at 37 °C for 18 h with agitation. After, the inoculum was centrifuged (6000 rpm, 10 min, 4 °C) and washed twice with Phosphate Buffered Saline (PBS 0.1 M; pH 7.5). The concentration was adjusted in PBS to obtain 1×10^8 cells/mL.

2.2. Essential oils

This study evaluated and compared the antifungal properties of EOs from *T. vulgaris* (florame®, France), *T. satureioides* (florame®, France), *T. mastichina* (florame®, France), and *T. zygis subsp. sylvestris* (Parque Natural das Serras de Aire e Candeeiros, Central Portugal), all with 100% purity. All EOs samples were stored in the dark at room temperature.

EO of *T. zygis* subsp. *sylvestris* was isolated by hydrodistillation during 3 h using a Clevenger-type apparatus, from flowering serial parts of the plants collected in Parque Natural das Serras de Aire e Candeeiros (Central Portugal). The rest of EOs in study were isolated by florame® (Saint-Rémy-de-Provence, France), through steam distillation of flower cups from Spain (origin of *T. vulgaris* and *T. mastichina*), and Morocco (origin of *T. satureioides*).

2.3. Chromatographic analysis of essential oils

The compositions of the EOs were determined by combination of gas chromatography with FID detectors (GC-FID) and gas chromatography-mass spectroscopy (GC/MS) analysis. GC-FID analysis was performed in a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) set with a single injector and two flame ionization detectors (FID). A divider (Agilent Technologies, part no. 5021–7148) was used for simultaneous sampling on two fused silica capillary columns: a SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm) and a SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70–220 °C (3 °C/min), 220 °C (15 min); injector temperature: 250 °C; mobile phase: helium, with flow adjusted to maintain a linear velocity of 30 cm/s; split ratio 1:40; detectors temperature: 250 °C.

For GC-MS analysis was used a Hewlett-Packard 6890 gas chromatograph interfaced with a Hewlett-Packard mass selective detector MSD 5973 (Agilent Technologies). An HP1 (Agilent Technologies) fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. GC parameters as described above; interface temperature: 250 °C; MSD parameters: interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans/s: 4.51. The compounds were identified by considering, concurrently: 1) the acquired retention indices on both SPB-1 and SupelcoWax-10 columns determined by linear interpolation relative to the retention times of C_8–C_{23} of *n*-alkanes and compared with reference data from authentic products (available in the laboratory database of the Faculty of Pharmacy, University of Coimbra) and literature data (Wallace, 2021); 2) the acquired mass spectra compared with reference data from the laboratory database, the Wiley / NIST library (Mc Lafferty, 2009) and literature (Adams, 2007). Relative amount of each component was calculated from GC peaks areas.

2.4. Agar disk diffusion assay

The agar disk diffusion assay was performed based on European Committee on Antimicrobial Susceptibility Testing (EUCAST E.DEF 7.3.2)(Arendrup et al., 2020). The *C. auris* suspension was spread onto SDA plates in three different directions. After the inoculum was dry, a sterile filter paper disk (6 mm) was impregnated with 10 μL of each EO from *T. vulgaris*, *T. zygis*, *T. satureioides* and *T. mastichina*. One plate with cell suspension without EO was used as a positive control. The SDA culture plates were incubated at 37 ºC for 24 h. The diameter of the inhibition zone was measured in mm. All experiments were performed in triplicate, in three independent assays.

2.5. Antifungal activity of essential oil from Thymus spp.

The antifungal activity of EOs from four *Thymus* spp. against preformed biofilms (24 h old biofilms) of *C. auris* NCPF 8971 was assessed. *C. auris* biofilms were developed according to the method described by Silva et al., (2009), with some adaptations. The *C. auris* cell concentration, from liquid culture, was adjusted to 1×10^5 cells/mL in SDB. Then, under aseptic conditions, 1 mL of cell suspension was transferred to glass wells inside a glass petri plates. The biofilm culture was incubated aerobically for 24 h at 37 °C, under agitation at 120 rpm. After 24 h of incubation, medium of the biofilm culture was renewed. The effect of the EOs (100% or 50%, diluted in almond oil) was evaluated directly and indirectly. For this, 10 μL of *T. vulgaris*, *T. satureioides*, *T. zygis* and *T. mastichina* EO (100% or 50%) were added to the glass wells in two different ways, direct application by addition in the medium (1% or 0.5%) and indirect application by placement, in paper discs which was placed near the wells (the set was kept inside a glass plate), respectively. Positive (biofilm without treatment) and negative (SBD medium) controls were included, as well as almond oil control. All glass petri plates were incubated for 24 h, at 37 °C and 120 rpm. All experiments were performed in triplicate, in three independent assays.

Cell viability was determined by colony-forming units (CFU). The biofilms were washed with PBS, then scraped from the wells with PBS (1 mL), serially diluted in PBS and plated onto SDA. After incubated for 24 h, at 37 °C, the number of grown colonies was counted. The results were presented as CFU per milliliter (Log10 (CFU/mL)).

2.6. Statistical analysis

Statistical evaluation was realized using GraphPad Software (version 6.01 for Macintosh). One-way ANOVA test was applied, and multiple comparisons of means of each treatment with EOs were done by Tukey test. The measures of central tendency and dispersion used were the mean and standard deviation, respectively. A *p-value* < 0.05 was considered significative.

3. Results and discussion

The qualitative and quantitative composition of the oils are shown in Table 1, where the compounds are listed by order of their elution on a polydimethylsiloxane column.

Briefly, *T. vulgaris and T. zygis* oils are mainly composed by phenolic compounds, being thymol the main compound of *T. vulgaris* (63.1%) and thymol (26.5%) and carvacrol (22.7%) the main compounds of *T. zygis* oil, whereas *T. satureioides* oil contains high amounts of borneol (29.3%) and α-terpineol (15.9%), and *T. mastichina* is characterized by high amounts of linalool (31.9%).

The *in vitro* antifungal activity of EOs from *T. vulgaris*, *T. zygis*, *T. satureioides* and *T. mastichina* on *C. auris* planktonic cells was evaluated by zones of inhibition (D_{halo}) using the agar disk diffusion method. The analysis of the results obtained shows that all oils, regardless of the *Thymus* species, had inhibitory effects (Table 2).

As previously demonstrated, *Thymus* spp. are potential antifungal agents because they have a strong action against fungal pathogenic microorganisms (De Lira Mota et al., 2012; Leal et al., 2017). Although the EOs belong to same plant genus, their effect in *C. auris* is significantly different. *T. vulgaris* had the largest inhibition areas (42.33 ± 3.77 mm), being the tested EO with the highest activity. Some of major components of this oil (Table 1) are thymol (63.1%), 1,8-cineole (10.0%), linalool (7.2%), and carvacrol (5.5%), which could be the reason for its high antifungal activity (Leal et al., 2017). In fact, several researchers evaluated the biological properties of *T. vulgaris* and verified that EO from this plant could inhibit fungal development (Rajkowska et al., 2019; Sharifzadeh et al., 2016). These authors sustained that the remarkably antifungal potential was related to the high percentage of thymol that was the main active component of *T. vulgaris* EO. Thus, thymol can be the principal responsible compound for activity of *T. vulgaris* against *C. auris*. In fact, *T. zygis* was the second most potent oil and also contains a significant percentage of thymol (26.5%), besides carvacrol (22.7%), 1,8-cineole (7.2%) and linalool (6.6%). Previous investigations have claimed that antifungal activity of *T. zygis* could be related to the effect of their main compounds, thymol and p-cymene (De Lira Mota et al., 2012; Gonçalves et al., 2010).

Even though *T. satureioides* and *T. mastichina* EOs have presented inhibition zones of 20.00 ± 0.63 mm and 13.60 ± 1.36 mm, respectively, their action against *C. auris* cells was not as significant as that of the other oils tested. The percentage of thymol of these oils (1.7% and 4.4%, respectively) is significantly lower compared to the *T. vulgaris* and *T. zygis* EOs (63.1% and 26.5%, respectively). The slight antifungal action may also be related to the high amount of the borneol, α-terpineol, and camphene in case of *T. satureioides*, and linalool, α-terpineol, and 1,8-cineole regarding *T. mastichina*. Although the activity of linalool, and other non-phenolic compounds is known (Dias et al., 2018), the interactions between the various constituents of the EOs may increase or decrease the action of these agents, due to synergistic or antagonistic effects (Rodrigues et al., 2020). Faleiro et al. (2003) studied *T. mastichina* antifungal properties and demonstrated that *C. albicans* was not susceptible to 1,8-cineole, but the mixture of this compound plus linalool slightly increased the antifungal activity. Research carried out by Pina-Vaz et al. (2004) validates the results obtained in this work. They investigated the effect of *Thymus* oils (*T. vulgaris*, *T. zygis* and *T. mastichina)* against *Candida* spp. fungal cells (*C. albicans, C. glabrata, C. krusei, C. guillermondii, C. parapsilosis,* and *C. tropicalis*) and found that *T. vulgaris* and *T. zygis* displayed better antifungal activity than *T. mastichina*.

Due to the positive effect found in the halo test, the activity of *T. vulgaris*, *T. zygis T. satureioides* and *T. mastichina* against preformed biofilms of *C. auris* was also assessed (Fig. 1). Each EO was tested in both direct or indirect application and in different concentrations.

Overall, the direct application of *Thymus* EOs, on *C. auris* biofilms, had a higher effect than the indirect application, with statistically significant differences ($p < 0.001$) (except with 50% of concentration of *T. mastichina,* not showing antifungal activity either when applied directly or indirectly). The antifungal effect of EOs at 100% of concentration was equal or superior to action of EOs at 50% of concentration, as expected.

The evaluation of indirect application had as main purpose to study the potential antifungal effect of vapour phase of EOs (VP-EOs) from *Thymus* spp., but it was noticed that *C. auris* biofilms were less susceptible to VP-EOs than when EOs were applied directly. Differently, other researches have demonstrated that VP-EOs possesses greater antifungal activity than their liquid phase (Boukhatem et al., 2020; Tullio et al., 2007). According to Boukhatem et al. (2020) that analysed the activity of EOs in liquid and vapour phase, the vapor phase of *T. vulgaris* (phenotype carvacrol) had greater action against planktonic cells of *Candida* spp., namely *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, than liquid phase. A possible explanation for the divergence between results can be related with testing conditions. In this study, the EOs were tested on biofilms that could be less susceptible to VP-EOs, contrasting with high susceptibility of the planktonic cells. Beside this, the *Candida* strain and the chemotype of *T. vulgaris* tested, in this study, are different from those previously studied by Boukhatem et al. (2020). All these factors can change the effectivity of antifungal activity of the both liquid and VP-EOs (Dias et al., 2018).

Preformed biofilms of *C. auris* demonstrated higher susceptibility to direct application of *T. vulgaris* and *T. zygis* EOs than *T. satureioides* and *T. mastichina* for both concentrations tested (Fig. 1). The constituents of EOs have different levels of lipophilicity according to the amount of polar functional groups in their constitution. EOs, due to their lipophilic character, can interact with lipid structures of the fungal cell membrane. This allows an easy penetration of EOs compound, modifying the cell membrane properties, such as permeability and fluidity. As result, fungal cells suffer structural damage that can result in cell death (Ferreira et al., 2021; Ballester-Costa et al., 2013). When added to a liquid medium, the distribution of the EO across medium depends on its hydrophilicity. Hence, the EOs with compounds with low solubility should have little distribution in aqueous medium and, therefore, should display poor activity on biofilms, which have an aqueous extracellular matrix. Thus, physical and chemical properties of the EOs, such as volatility and solubility, can be an important role on theirs activity (Tullio et al., 2007). Considering these properties, since linalool possesses low water solubility contrasting with thymol and carvacrol that have moderate solubility, the EOs with high amount of these last compounds, namely *T. vulgaris* and *T. zygis* EOs, presented great antifungal activity when applied directly, as observed in outcomes obtained (Boukhatem et al., 2020; Tullio et al., 2007). The intrinsic activity of the constituents of EOs is also a factor to considerate in the evaluation of its efficacy. In this sense, the EOs with high amount of phenol monoterpenes or aldehyde are known to have high inhibitory effect on fungal cells, including in vapor phase, followed by alcohol, ketone and ether (Tullio et al., 2007). In this context, the results obtained in this study (Fig. 1A) are in accordance with the expected, as *Thymus* EOs with major percentage of thymol and carvacrol (*T. vulgaris* and *T. zygis* EOs, respectively) demonstrated greater antifungal activity in both applications.

Among the tested *Thymus* oils, *T. vulgaris* was the one that showed significant action in the vapor phase against *C. auris* biofilms at both concentrations (Fig. 1B). Comparing the percentage of these compounds in each *Thymus*, approximately 70% of *T. vulgaris* EO are phenolic

Table 1
Chromatography analysis of the composition of essential oils from *T. vulgaris*, *T. zygis*, *T. satureioides* and *T. mastichina*, here shown in percentage.

RI[a]	RI[b]	Compound	Percent (%) in oil samples			
			T. satureioides	*T. mastichina*	*T. vulgaris*	*T. zygis*
921	1030	Tricyclene	0.5		t	t
929	1030	α-Pinene	3.0	0.2	0.2	0.2
943	1075	Camphene	5.9	0.3	0.3	0.4
959	1442	Oct-1-en-3-ol	0.2	–	0.3	0.4
962	1255	Octan-3-one	–	–	–	0.2
967	1382	Octan-3-ol	–	–	–	0.1
970	1118	β-Pinene	0.8	0.1	t	0.2
964	1124	Sabinene	0.1	t	t	t
980	1162	Myrcene	0.6	0.3	0.2	0.1
997	1171	α-Phellandrene	0.1	t	t	t
1010	1187	α-Terpinene	0.0	t	t	t
1011	1275	*p*-Cymene	3.8	0.8	2.2	4.3
1020	1215	1,8-cineole	2.7	**8.5**	**10.0**	**7.2**
1020	1204	Limonene	0.8	0.3	t	t
1046	1249	γ-Terpinene	0.8	0.2	0.4	0.9
1050	1459	*E*-Sabinene hydrate	0.1	0.6	0.4	–
1055	1438	Z-Linalool oxide (THF)	0.1	0.4	0.1	–
1070	1466	E-Llinalool oxide (THF)	–	–	0.2	–
1071	1439	Cymenene	0.1	–	–	0.1
1076	1288	Terpinolene	0.2	0.4	t	0.1
1082	1543	Linalool	4.5	**31.9**	**7.2**	6.6
1106	1555	*cis-p*-Menth-2-en-1-ol	–	0.2	–	–
1118	1515	Camphor	2.5	1.6	0.7	1.8
1119	1647	*E*-Pinocarveol	–	0.4	–	0.9
1125	1673	*E*-Verbenol	–	0.2	–	–
1144	1664	Isoborneol	0.1	3.3	–	–
1145	1695	Borneol	**29.3**	5.8	3.4	4.9
1158	1597	Terpinen-4-ol	2.3	2.3	1.7	3.9
1165	1622	Myrtenal	–	0.5	–	–
1169	1692	α-Terpineol	**15.9**	**10.0**	0.5	1.2
1176	1786	Myrtenol	–	0.2	–	–
1175	n.d	*trans*-Dihydrocarvone	–	–	–	0.3
1195	1830	*trans*-Carveol	0.1	0.2	–	–
1206	n.d	Bornyl formiate	0.6	0.3	–	–
1214	1679	Neral	–	0.3	–	–
1223	1601	Carvacrylmethyl oxide	0.9		0.2	0.1
1233	1842	Geraniol	–	0.6	–	–
1240	1555	Linalyl acetate	t	3.8	t	t
1264	1574	Bornyl acetate	1.9	0.8	t	t
1263	2183	Thymol	1.7	4.4	**63.1**	**26.5**
1277	2205	Carvacrol	**7.0**	0.5	5.5	**22.7**
1301	1683	Myrtenyl acetate	–	1.2	–	–
1328	1688	α-Terpenyl acetate	–	4.5	–	0.1
1342	1722	Neryl acetate	–	0.2	–	–
1342	1455	α-Cubebene	t	–	–	–
1359	1755	Geranyl acetate	–	0.5	–	–
1369	1487	α-Copaene	0.2	–	–	–
1376	1517	β-Bourbonene	0.1	0.2	–	–
1397	n.d.	Isocaryophyllene	t	–	–	–
1401	1526	α-Gurjunene	0.1	–	–	0.1
1409	1590	*E*-Caryophyllene	5.3	1.9	1.1	2.3
1427	1600	Aromadendrene	0.1	t	0.1	1.0
1442	1662	α-Humulene	0.2	t	t	t
1447	1636	*allo*-Aromadendrene	0.2	0.4	–	0.3
1466	1699	Germacrene D	0.1	0.2	–	–
1470	1715	β-Selinene	t	–	–	
1479	1726	Bicyclogermacrene	0.1	0.7	–	–
1486	1724	α-Muurolene	0.1	0.1	–	–
1488	1720	*Z*-α − Bisabolene	–	–	–	0.8
1498	1751	γ-Cadinene	0.3	0.3	–	–
1502	1825	*Z*-Calamenene	0.1	0.4	–	0.1
1508	1751	δ-Cadinene	0.3	–	0.1	0.2
1557	1975	Caryophyllene oxide	0.2	1.0	0.3	–
1553	2113	Spathulenol	t	1.5	–	1.0
1562	2063	Globulol	–	–	–	1.7
1607	2153	γ-Eudesmol	–	0.3	–	–
1607	2277	Caryophylla-2(12),6(13)-dien-5-α-ol	0.1	–	–	–
1615	2172	τ-Muurolol	t	–	–	–
1615	2153	τ-Cadinol	–	0.3	–	–
1622	2215	β-Eudesmol		0.8		0.2
		Monoterpene hydrocarbons	16.7	2.6	3.3	6.3
		Oxygen containing hydrocarbons	69.7	83.2	93	76.2
		Sesquiterpene hydrocarbons	7.2	4.2	1.3	4.8

(*continued on next page*)

Table 1 (*continued*)

RI[a]	RI[b]	Compound	Percent (%) in oil samples			
			T. satureioides	*T. mastichina*	*T. vulgaris*	*T. zygis*
		Oxygen containing sesquiterpenes	0.3	3.9	0.3	1.9
		Other compounds	0.2	0	0.3	0.7
		Total identified	**94.1**	**93.9**	**98.2**	**89.9**

Compounds listed in order of their elution on the SPB-1column.
t = traces (≤ 0.05%).
[a] Retention index on the SPB-1 column relative to C8 - C24 n-alkanes.
[b] Retention indices on the SupelcoWax-10 column relative to C8 - C24 n-alkanes.

Table 2
Diameter of the inhibition zone, in mm, of the essential oils from different plants of *Thymus* genus (10 μL) for *C. auris* NCPF 8971.

Inhibition zone diameter, mm			
Thymus vulgaris	*Thymus zygis*	*Thymus satureioides*	*Thymus mastichina*
42.33 ± 3.77	28.25 ± 1.09****	20.00 ± 0.63**,****	13.60 ± 1.36****

**P < 0.01 when compared with *T. mastichina,* and ****P < 0.0001 when compared with *T. vulgaris*.

compounds, namely thymol (63.1%) and carvacrol (5.5%). This rate is relatively superior to percentages verified for other *Thymus* tested, justifying the significant differences obtained between the activity of vapour phase of *T. vulgaris* EO and the other EOs on *C. auris* biofilms. However, also the minor compounds of EOs may change, positive or negatively, the antifungal action of the main compounds due to synergistic and/or antagonistic effect resultant of their interactions (Ballester-Costa et al., 2013).

According to the outcomes obtained, there are significant differences in antifungal activity of *Thymus* EOs on planktonic and biofilm cells of *C. auris* NCPF 8971, ranging considerably between species and according with their chemical profile. In this sense, the antifungal activity of *Thymus* EOs should not be widespread, and it is crucial specify the species as well as the main compounds of their EOs before attribute the antifungal potential.

4. Conclusion

EOs may be of future relevance to the treatment of multi-drug resistant fungi, including *Candida* infections. In this study, the antifungal effect of the EOs from *Thymus* spp. against planktonic and biofilms cells of *C. auris* was confirmed. However, the antifungal activity differs between the plant species, according to the chemical profile of their EOs. Thus, the properties of each species should not be expended to all the *Thymus* spp. and the specification of species and chemotype is crucial. Among the *Thymus* spp. tested, *T. vulgaris* and *T. mastichina* had the highest and the slightest antifungal activity, respectively. In the future, studies to analyse the mechanism of action of EOs in liquid and vapour phase on *C. auris* cells will be important.

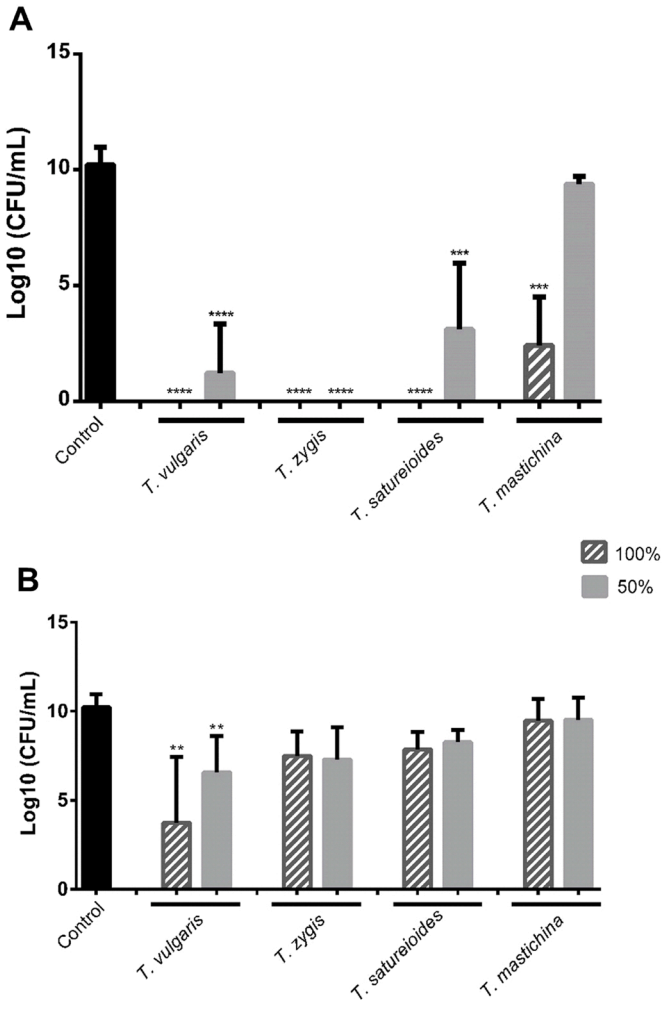

Fig. 1. Assessment of the susceptibility of *C. auris* NCPF 8971 24 h old biofilms to different essential oils from *Thymus* genus by quantification of CFUs per mL. Each essential oil was tested at 100% and at 50% with both direct (A) and indirect (B) application. Error bars indicate the respective standard derivation (SD). Differences in mean Log10 CFU/mL of the different treatments were compared to the control using one-way ANOVA with a post hoc Tukey test (significance at P < 0.05), with ** P < 0.01, *** P < 0.001 and **** P < 0.0001.

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CRediT authorship contribution statement

Rita Ribeiro: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Liliana Fernandes:** Conceptualization, Formal analysis, Writing – original draft. **Raquel Costa:** Resources. **Carlos Cavaleiro:** Methodology, Writing – review & editing. **Lígia Salgueiro:** Methodology, Resources, Writing – review & editing. **Mariana Henriques:** Conceptualization, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Maria Elisa Rodrigues:** Conceptualization, Validation,

Formal analysis, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest in this manuscript.

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