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Chemical Composition and Anticandidal Effect of Three *Thymus* Species Essential Oils from Southwest of Morocco against the Emerging Nosocomial Fluconazole-Resistant Strains

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Abstract

The purpose of this present work is to study the essential oils composition of three endemic *Thymus* species from southwest of Morocco: *Thymus satureioides* Coss., *Thymus pallidus* Batt. and *Thymus leptobotrys* Murb., as well as their antifungal activity towards nineteen strains of emerging nosocomial Fluconazole-resistant *Candida* species. The chemical composition of the essential oils was determined by capillary gas chromatographic-mass spectrometry analysis. The results reveal qualitative and quantitative variation in composition of *Thymus* species. Seventy-three different compounds, 56 for *T. satureioides* accounting for 99.97% of the total essential oil, 52 for *T. pallidus*, accounting for 98.94% of the total essential oil, and 40 for *T. leptobotrys* accounting for 99.20%, were determined. The results obtained for the anticandidal disc-diffusion assay shows that the 19 strains of *Candida* species tested were inhibited by the Moroccan *Thymus* essential oils to a varying degree, with the diameters of the inhibition zone ranging from 49±1.00 to 85±1.15 mm. There were significant differences ($p \leq 0.05$) in the antifungal activities of the essential oils on all species tested who showed larger inhibition zones than the positive control fluconazole and amphotericine B. *Candida albicans* showed a high sensitivity to essential oils of *Thymus pallidus* and *Thymus leptobotrys* compared with essential oil of *Thymus satureioides* and controls. While non- *albicans Candida* species showed less sensitivity to essential oils of *Thymus pallidus* and *Thymus leptobotrys* and are more sensitive to essential oils of *Thymus satureioides* than *Candida albicans*. Interestingly *C. krusei*, *C. dubliniensis* and *C. glabrata* were found to be resistant to conventional antifungal (fluconazole and amphotericine B), while our essential oils tested were able to inhibit the growth of *Candida* strains resistant to antifungal agents. The value of Minimal inhibitory concentration (MIC) and Minimal Fungicidal Concentration (MFC) of the *Thymus* essential oils studied ranges from 0.33 mg/mL to 0.91 mg/mL. All the essential oils possessed higher antifungal potential than classical fungicide.

Keywords: *Thymus* species, essential oils, anticandidal activity, Nosocomial fluconazole-resistant strains.

1. Introduction

During the past two decades, we attend a considerable increase in candidiasis, both surface and deep, as well as the patient population at risk (organ transplantation, long-term antibiotic therapy with broad-spectrum, immunosuppressive therapy, AIDS) (Robert 1997; Ascioglu *et al.* 2002; Buitrón García-Figueroa *et al.* 2009; Chabasse *et al.* 2009; Garnica and Nucci 2009; Silva *et al.* 2011; Toubas 2013). Systemic candidiasis are fourth among nosocomial infections with a percentage of 10 to 15% (Eggimann & Pittet 2002; Yera *et al.* 2001). *Candida albicans* is the species most frequently incriminated (60%), followed by *Candida glabrata* (20%) whose incidence has increased in recent years, under the pressure of azole antifungals subsequently by *Candida tropicalis* (10%) and *Candida parapsilosis* (5%) (Eggimann & Pittet 2002; Rimek *et al.* 2003; Hanna *et al.* 2003; Anane *et al.* 2007; Vazquez & Sobel 2011; Bouchoux 2012; Toubas 2013). It should also be mentioned that the emergence of *Candida krusei* was attributed to its primary resistance to fluconazole (Develoux & Bretagne 2005; Aoufi & Agoumi 2007; Vazquez & Sobel 2011; Dannaoui 2013; Toubas 2013). A new species of *Candida* with similar phenotypic characteristics to those of *C. albicans* was identified in 1995 and was named *Candida dubliniensis* (Sullivan *et al.* 1995; Perfect & Shell 1996; Vazquez and Sobel 2011). The majority of isolates of *C. dubliniensis* were found in the oral cavity of HIV-infected subjects and at other sites including the lung, vagina, blood ... as well as in subjects infected with HIV than subjects not infected with this virus (Fotedar & Hedaithy 2003). *C. dubliniensis* is less sensitive than *C. albicans* to azole antifungals especially fluconazole.

Moreover, it has been observed over the past fifteen years, a decrease in the prevalence of *C. albicans* for the

benefit of other species, so-called emerging (Garnica & Nucci 2009). The new species differ in their pathogenic profile and antifungal susceptibility profile (Develoux & Bretagne 2005). This change in the distribution of the genus *Candida* is accompanied by an increase in the severity of candidiasis. However, these *Candida* species are opportunistic fungi, which exert their pathogenicity in the presence of predisposing factors. Moreover, some *Candida* species are commensal of the digestive tract and urogenital tract of humans as *C. albicans* and *C. glabrata* and other species are saprophytes of the skin such as *C. parapsilosis* which is involved in septicemia caused by contaminated catheters (Eggimann & Pittet 2002; Yera *et al*. 2001; Sendid *et al*. 2003). Emerging fungal infections are therefore a major challenge for health professionals. These infections can lead to a poor prognosis (Anane & Khalfallah 2007; Pihet & Marot 2013), and resistance to conventional antifungal used in current hospital practice (Develoux & Bretagne 2005; Dannaoui 2013).

Despite therapeutic advances marked by the emergence of new antifungal molecules, together the high cost (Toubas 2013), the mortality rate is still around 50% (Eggimann & Pittet 2002; Granier 2000; Mishra *et al*. 2010; Poulain 2000). Fluconazole and amphotericin B are still the antifungal agents of choice commonly used in infections related to *Candida* species; however they are known to have side effects and high toxicity, in addition to emerging resistance among clinical isolates of *C. albicans*. Therefore, it is necessary to isolate new antifungal agents, mainly from natural vegetal sources. In recent years, some researchers have focused on the use of components obtained from extracts of plants that exhibit antifungal activity *in vitro* and *in vivo*. These plants are selected according to traditional medicine and their compounds have fewer side effects than conventional antifungal drugs and both high tolerance for human body and less cost (Agarwal *et al*. 2010; Kumar *et al*. 2012). The concomitant use of an essential oil and amphotericin B has significantly reduced the Minimum Inhibitory Concentration (MIC) of this latter, leading a reduction of side effects associated with the use of this drug in a therapeutic protocol (Giordani & Kaloustian 2006).

Therefore the increase of resistance to conventional antifungal, toxicity and the costs involved are the principal reasons to search new therapeutic approaches. Among these new approaches, essential oils are promising natural compounds for use in the prevention and treatment of fungal infections. In this study, we attempted to evaluate the antifungal activity of the essential oils extracted from three *Thymus* of southwest of Morocco: *Thymus leptobotrys*, *T. satureioides* and *T. pallidus* against *Candida* species isolated from patients with nosocomial candidiasis.

2. Materials and methods

2.1. Plant materials

Three fresh plant samples were collected from several regions of area of Argan tree in Souss-Massa Draa Valley, Southwest of Morocco, between March and April 2012. The taxonomic identification of the species of all samples were confirmed by Pr. Ahmed Ouhammou, a plant taxonomist in the Laboratory of Ecology and Environment of University Cadi Ayyad of Marrakech (Morocco), and were deposited in the herbarium of the laboratory of Plant Biotechnology, Planta Sud unity, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Plant samples were cleaned air-dried in the shade in the laboratory at room temperature. The botanical name, family, and parts used of plant samples are summarized in Table 1.

Table 1: The botanical name, family, and parts used of plant samples

Scientific name	Family	Collection site	Local name	Latitude/longitude	Altitude (m)	EO Yield (%)	Medicinal use
Thymus leptobotrys	*Labiatae*	Asgharkiss	Azoukni	N29°82'/W09°19'	760	2.00±0.01	Anti-infective, expectorant, coughing
Thymus satureioides	*Labiatae*	Imouzzer Idaoutanane	Tazouknit	N30°67'/W09°50'	1038	2.62±0.02	Anti-infective, expectorant, coughing
Thymus pallidus	*Labiatae*	Imouzzer Idaoutanane	Ajellabi	N30°71'/W09°42'	1618	2.10±0.02	Anti-infective, expectorant, coughing

2.2. Essential oils extraction

Essential oils were obtained by hydrodistillation for 4 h from air dried material, using a Clevenger-type apparatus, according to the European Pharmacopoeia method (Anonymous 1975; Council of Europe 1997). These essential oils were weighed and stored at 4 °C in a sealed brown vial until biological assays. The yields of essential oils extracted are showed in Table 1.

2.2.1. GC-MS analysis

Gas Chromatography-Mass Spectrometry analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60°C during 5 min, with 3°C/min increases to 180°C, then 20°C/min increases to 280°C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30–500 range with an ionizing voltage of 70 eV. Kovat's retention index was calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by MS and their identity was confirmed by comparison of their RIs, relative to C_8-C_{32} *n*-alkanes, and mass spectra with those of authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature (Adams 2007).

2.3. Drugs preparation

The drugs usually employed for fungal infections belong to the family of imidazole agents such as fluconazole and to the polyenes family agents such as amphotericin B. These antifungal agents were dissolved in 2 mL dimethylsulfoxyde (DMSO) 10% to give the following stock solutions: fluconazole 75 mg/mL and amphotericine B 33 mg/mL.

2.4. Isolation of the microorganisms

In this study, nineteen clinical strains isolates of *Candida* species, including *C. albicans* (n = 12), *C. dubliniensis* (n = 1), *C. glabrata* (n = 3), *C. krusei* (n = 3), were isolated from patients suffering from nosocomial candidiasis. The isolation was done in the laboratory of Parasitology-Mycology and Bacteriology Avicenna Military Hospital, Marrakech, Morrocco, on Sabouraud chloramphenicol agar plates and identified by the germ tube test, API 20 C AUX (bioMérieux, Marcy-l'Étoile, France) according to the Manufacturer's recommendations and chromogenic medium CandiSelect 4 (Bio-Rad, Marnes-la-Coquette, France). For this experiment, all isolated strains were tested and yeast cells were cultured on Sabouraud dextrose agar (SDA), supplemented with chloramphenicol (Bio-Rad), and then incubated at 30°C for 48 h.

2.5. Antifungal testing

2.5.1. Fungal suspension

A fresh overnight culture, in log phase, of the tested yeasts was used to prepare the cell suspension by inoculating 5 mL of serum glucose 5% broth with an appropriate yeast strain and incubating for 24 h at 37°C to ensure that yeast cells were actively dividing (Manohar *et al*. 2001), then were adjusted between: 2.16 x 10^5 cells/mL to 5.22 x 10^5 cells/mL for fungal strains counting with haemocytometer for each repetition.

2.5.2. Antifungal screening

2.5.2.1. Disk diffusion method

Natural essential oils of *T. leptobotrys*, *T. satureioides* and *T. pallidus* were tested for their anticandidal activity using the disk diffusion method (Jirovetz *et al*. 2012). Nineteen strains of *Candida* species were used as described in detail above. The cultures of *Candida* spp. were cultivated on Sabouraud Dextrose Agar (SDA), supplemented with chloramphenicol at 37ºC±1°C for 48 h. Seeded agar plates were prepared by pouring 20 mL of SDA into each sterile plate. After solidification of medium, each plate was overlaid with 5 mL of the suspensions of yeasts. The excess of the suspension was poured into a container for infectious waste. After that, the natural essential oils of *T. leptobotrys* (521 mg/mL), *T. satureioides* (725 mg/mL) and *T. pallidus* (627 mg/mL) were applied on filter paper (10 µL/disk) disks of 5 mm in diameter separately. Ten µL of fluconazole (75 mg/mL) and 10 µL of amphotericin B (33 mg/mL) were used as positive control. These disks were placed on the surface of seeded agar. All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 60 min at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 48 h. After the incubation period, the zone of inhibition was measured in millimeters with a caliper. Studies were performed in triplicate, and mean values were calculated.

The methods used to assess the antifungal activity of essential oils are different and can give different results from one technique to another (Chami 2005). For each essential oil their minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) were determined using the broth macrodilution method.

2.5.2.2. The broth macrodilution method

The broth macrodilution method was used to determine the Minimum Inhibitory Concentration (MIC) according to Clinical and Laboratory Standards Institute-CLSI reference document (formerly NCCLS-M27-A3 guidelines) (NCCLS 2002) with modifications. The fungal suspension was then used to inoculate the tubes in the test group. The test groups were prepared with 1 mL of medium containing 10 µL of DMSO 10% to enhance essential oil solubility where the final concentration never exceeded 2%, and 200 µL of each strain of yeast suspension previously adjusted. Ten µL of each essential oil obtained respectively from *T. leptobotrys*, *T. satureioides* and *T. pallidus*, 10 µL of fluconazole (75 mg/mL) and 10 µL of amphotericin B (33 mg/mL) were respectively added to tubes containing culture medium in the test groups. Then, a sufficient amount of glucose 5% was added to a final volume of 1 mL and serial dilutions and concentration gradient were established as follows: essential oil of

T. leptobotrys from 5.21 mg/mL to 0.0814 mg/mL, essential oil of *T. satureioides* from 7.25 mg/mL to 0.1133 mg/mL, *T. pallidus* from 6.27 mg/mL to 0.0100 mg/mL, fluconazole 0.75 mg/mL to 0.01172 mg/mL and amphotericin B from 0.33 mg/mL to 0.0052 mg/mL. The test tubes were incubated at 37 °C in an orbital shaking incubator (100 rpm) for 48 h, and the MICs were determined. The MIC was defined as the lowest concentration of the essential oil at which the microorganism did not demonstrate visible growth.

To determine the Minimum Fungicidal Concentration (MFC), aliquots (20 µL) of broth were taken from each tube without fungal growth after reading MIC tube, cultured in the Sabouraud agar plates (SDA) and incubated at 37 °C for 48 h. The MFC was defined as the lowest concentration of the essential oil at which the incubated microorganism was completely killed. Each test was performed in triplicate. Fluconazole and amphotericin B were used as positive antifungal controls, respectively.

For each strain tested, the growth conditions and the sterility of the medium were checked in two control tubes. The safety of DMSO was also checked at the highest tested concentration. All experiments were performed in triplicate.

2.6. Statistical analyses

Statistical analyses were performed using a statistical package; SPSS windows version 19, by applying mean values using one-way analysis of variance (ANOVA) followed by post Student-Newman-Keuls (S-N-K) method. A *P* value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Chemical composition of Thymus essential oils

Hydrodistillation yielded 2.62±0.02%, 2.10±0.02% and 2.00 ± 0.01% of essential oil (on a dry mass basis) for *T. satureioides*, *T. pallidus* and *T. leptobotrys*, respectively. Seventy-three different compounds from three endemic Moroccan *Thymus* were identified by capillary GC-MS analysis. In *T. satureioides* essential oil 56 compounds accounting for 99.97% of the total oil, 52 for *T. pallidus* essential oil (98.94%) and 40 for *T. leptobotrys* essential oil (99.20%), were determined. Components are listed (Table 2) as homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, oxygenated diterpenes, aromatics (C_6-C_1 and C_6-C_3 series) and others.

Table 2. Chemical composition of three endemic *Thymus* essential oil from Morocco: *T. satureiodes*, *T. pallidus* and *T. leptobotrys.*

Compounds	**RT**	**KI**	**% of total**		
			T. satureioides	***T. pallidus***	***T. leptobotrys***
Monoterpene hydrocarbons			**33.94**	**2.03**	**19.44**
Tricyclene	6.77	926	0.54	-	0.12
α-Thujene	6.97	930	0.34	0.01	0.12
α-Pinene	7.35	939	7.85	0.08	2.69
Camphene	7.90	954	15.05	0.03	2.70
Thuja 2,4(10) diene	8.07	960	0.02	0.01	0.04
Sabinene	8.78	975	0.02	-	-
β-Pinene	8.94	978	1.10	0.02	0.27
Myrcene	9.55	990	0.47	0.04	0.92
α-Phellandrene	10.06	1002	0.03	0.01	0.11
δ-3-Carene	10.31	1011	0.01	-	0.08
α-Terpinene	10.63	1017	0.30	0.06	0.84
p-Cymene	11.15	1024	4.90	1.18	8.34
Limonene	11.29	1029	1.34	0.16	0.48
cis-Ocimene	11.64	1037	0.01	-	0.02
γ-Terpinene	12.65	1059	1.74	0.39	2.52
Terpinolene	13.92	1088	0.22	0.04	0.19
Oxygenated monoterpenes			**61.18**	**91.47**	**74.30**
1,8-Cineole	11.35	1031	0.30	0.92	0.04
cis-Sabinene hydrate	12.96	1070	-	0.01	0.02
cis-Linalool oxide	13.19	1072	0.03	0.03	0.05
Camphenilone	13.65	1082	0.01	-	-
Linalool	14.72	1096	2.25	1.08	3.12
cis-Thujone	14.80	1102	-	0.08	-
cis-*p*-Menth-2-en-1-ol	15.55	1121	0.03	-	-
α-Campholenal	15.70	1126	0.02	-	-
trans-Pinocarveol	16.43	1139	0.09	0.05	-
Camphor	16.63	1146	1.42	0.11	-
Camphene hydrate	16.74	1149	0.09	-	-

Borneol	18.52	1169	32.13	0.42	5.85
Terpinen-4-ol	18.64	1177	1.18	0.66	0.85
α-Terpineol	19.70	1188	19.87	0.10	-
cis-dihydro-Carvone	20.52	1192	-	0.12	-
Isobornyl formate	20.69	1239	0.46	-	-
Carvacrol methyl ether	21.36	1244	0.10	0.23	0.28
Carvenone	21.95	1258	0.05	-	-
Bornyl acetate	23.33	1288	1.89	-	0.10
Thymol	23.55	1290	0.06	2.92	0.61
Carvacrol	24.06	1299	1.20	84.74	63.38
Sesquiterpene hydrocarbons			**3.56**	**3.89**	**4.72**
α-Ylangene	27.00	1375	-	0.02	-
Isoledene	27.04	1376	-	-	0.04
α-Copaene	27.06	1376	0.07	-	-
β-Bourbonene	27.44	1388	0.06	0.03	-
α-Gurjunene	28.49	1409	0.04	-	0.07
β-Caryophyllene	29.04	1419	2.59	1.11	2.46
β-Copaene	29.29	1432	0.03	-	-
α-*trans*-Bergamotene	29.43	1434	-	0.02	-
Aromadendrene	29.68	1441	0.04	0.31	0.73
α-Humulene	30.29	1454	0.11	0.05	0.08
trans-β-Farnesene	30.34	1456	-	0.16	-
allo-Aromadendrene	30.57	1460	0.07	0.07	0.27
γ-Muurolene	31.25	1479	0.06	0.08	0.04
α-Curcumene	31.52	1480	0.04	-	-
Viridiflorene	31.94	1496	-	0.23	0.46
Bicyclogermacrene	32.03	1500	0.02	-	-
α-Muurolene	32.20	1500	0.04	0.03	0.07
β-Bisabolene	32.70	1505	-	1.46	-
γ-Cadinene	32.76	1513	0.19	0.06	0.15
δ-Cadinene	33.13	1523	0.20	0.20	0.35
trans-Cadina-1,4-diene	33.31	1534	-	0.01	-
α-Cadinene	33.51	1538	-	0.02	-
Calacorene	33.71	1545	-	0.03	-
Oxygenated sesquiterpenes			**1.25**	**0.70**	**0.54**
Spathulenol	35.21	1578	0.05	0.40	0.04
Caryophyllene oxide	35.50	1583	0.75	0.30	0.50
β-Oplopenone	36.40	1607	0.07	-	-
1,10-*diepi*-Cubenol	36.62	1619	0.04	-	-
tau-Cadinol	37.62	1640	0.30	-	-
α-Cadinol	38.10	1654	0.04	-	-
Oxygenated diterpenes			-	**0.30**	-
Manool oxide	47.50	1987	-	0.20	-
13-*epi*-Manool oxide	47.73	2010	-	0.10	-
Aromatics			**0.02**	**0.07**	**0.08**
Benzaldehyde	8.10	960	-	0.01	-
Eugenol	26.86	1359	0.02	0.06	0.08
Others			**0.02**	**0.48**	**0.12**
1-Octen-3-ol	9.02	979	-	0.22	-
3-Octanone	9.32	983	-	0.10	0.12
3-Octanol	9.72	991	0.02	0.16	-
Total identified			**99.97**	**98.94**	**99.20**

In *T. satureioides* essential oil the monoterpenic fraction reached the highest quantities (95.12%). Both hydrocarbons (33.94%) and oxygenated monoterpenes (61.18%) with 16 and 18 identified compounds respectively, are also qualitatively the principal phytochemical group represented in their essential oil. Borneol (32.13%), α-terpineol (19.87%), camphene (15.05%), α-pinene (7.85%) and *p*-cymene (4.90%) were the main compounds. Sesquiterpenic fraction (both hydrocarbons and oxygenated) were found at lower quantities (4.81%) (Table 2). In this fraction, only large amount of β-caryophyllene (2.59%) was found (Table 2).

In recent paper (El Bouzidi *et al*. 2013) carvacrol (26.5%), followed of borneol (20.1%) was reported as the most abundant compounds in *T. satureioides*. However, in our study, borneol was present at higher percentage of

32.13%, whereas carvacrol only reached 1.20 of the total essential oil.

The oxygenated monoterpenes (91.47%) is quantitatively the main fraction of *T. pallidus* essential oil, following by the sesquiterpene hydrocarbons fraction (3.89%) with 17 identified compounds (Table 2). Phenolic compounds, thymol (2.92%) and mainly carvacrol (84.74%) were the most abundant among the oxygenated monoterpenes. However in previous work (Jamali *et al*. 2012), the authors reported thymol (26.8%) and the biogenetic precursors γ-terpinene (29.6%), and p-cymene (18.9%), as the main compounds in the essential oil of *T. pallidus*, to be very low carvacrol content (1.4%). It is interesting to note the presence of oxygenated diterpenes (manool oxide and 13-*epi*-manool oxide) in *T. pallidus* essential oil here analysed.

Carvacrol (63.38%) also was the main compound in the essential oil of *T. leptobotrys*, followed of *p*-cymene (8.34%), borneol (5.85%), linalool (3.12%), camphene (2.70%), α-pinene (2.69%), γ-terpinene (2.52%) and the sesquiterpene hydrocarbon β-caryophyllene (2.46%). This essential oil also was dominated by the monoterpene fraction that amounted 93.74% (Table 2).

The present data reveals qualitative and quantitative differences in the essential oil composition of Moroccan *Thymus* species. GC-MS analyses showed the predominance of the monoterpenic fraction in all the essential oils under investigation (Table 2).

Carvacrol was found as a predominant component in *T. pallidus* and *T. leptobotrys* while, *T satureioides* was rich in borneol and α-terpineol. The high content of carvacrol in these two *Thymus* species has also been reported by other authors (Jaafari *et al*. 2007; Saad *et al.* 2010), suggesting that this phenolic compound is responsible of the cytotoxic activity (Jaafari *et al*. 2007).

From these results it can be concluded that besides the anatomical characters, the chemical composition of the essential oils of different *Thymus* species (De Lisi *et al*. 2011; Jirovetz *et al*. 2012) could be contributed to the chemotaxonomical identification of this genera. In addition, these species could be considered as rich natural sources of some bioactive substances such as carvacrol, borneol, α-terpineol and camphene. Both essential oils rich in carvacrol and few sesquiterpenoids content can be considered according previous biological studies as important antimicrobial agents.

3.2. Anticandidal Activity

3.2.1. Disk diffusion method

The results obtained for the anticandidal disc-diffusion assay are summarized in Table 3**.** All 19 strains of *Candida* species tested were inhibited by the Moroccan *Thymus* essential oils to a varying degree, with the diameters of the inhibition zone ranging from 49±1.00 to 85±1.15 mm. There were significant differences ($p \leq 0.05$) in the antifungal activities of the essential oils on all species tested which showed larger inhibition zones than the positive control fluconazole and amphotericin B. *Candida albicans* showed a high sensitivity to essential oils of *Thymus pallidus* and *T. leptobotrys* compared with essential oil of *Thymus satureioides* and compared to controls. Each non-albicans *Candida* showed a high sensitivity to essential oils of the three *Thymus* species compared to controls (Table 3).

While non-albicans Candida species showed less sensitivity to essential oils of *Thymus pallidus* and *T. leptobotrys* and are more sensitive to essential oils of *T. satureioides* that *Candida albicans* (Figure 2). Interestingly *C. krusei*, *C. dubliniensis* and *C. glabrata* were found to be resistant to conventional antifungals (fluconazole and amphotericin B), while our essential oils tested were able to inhibit the growth of *Candida* strains resistant to antifungal agents in Figure 2.

Table 3. Inhibition-zone diameters determined with the disc-diffusion method of the three *Thymus* essential oils, fluconazole and amphotericin B against eight *Candida* species and other yeast.

Strains	**Number of strains**	**TL EO 10µl**	**TP EO 10µl**	**TS EO 10µl**	**AMB 10µl**	**FLC 10µl**
Candida albicans	12	85^{a}	85^{a}	53^{b}	13^{d}	22^{c}
Candida dubliniensis	1	85^{a}	85^{a}	85^{a}	6^{c}	9^{b}
Candida glabrata	3	56^{b}	65^{a}	49^{c}	5^{d}	5^{d}
Candida krusei	3	53^{c}	71^{a}	67^{b}	11^{d}	4^{e}

*) Inhibition zone diameter including the disk diameter of 5mm determined by the agar disk-diffusion method at a concentration of 10 µl of essential oils/disk, 10µl of fluconazole/disk and 10µl of amphotericin B/disk.

TL = *Thymus leptobotrys*, TS = *Thymus satureioides*, TP = *Thymus pallidus*, EO = *essential oil. Each value represents the mean of three replicates.*

Means in each line having the same superscript letters are not significantly different at $P \leq 0.05$ according to student Newman and Keuls test.

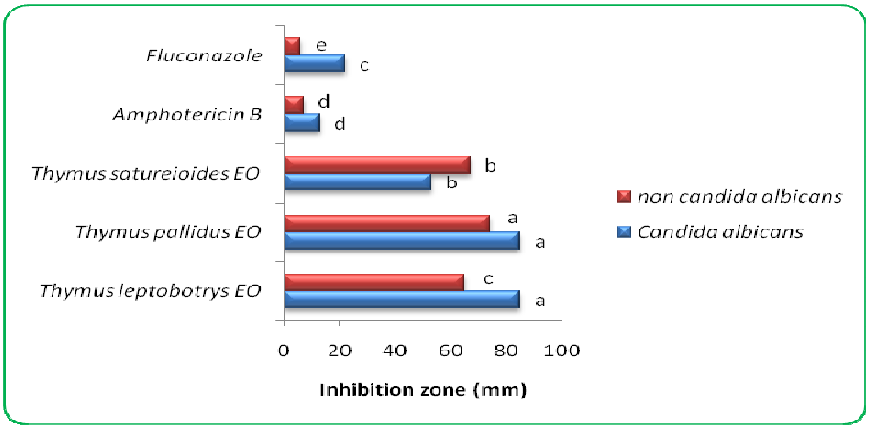

Figure 2. Means of inhibition zone of essential oils against clinical isolates of *Candida albicans* and non-*albicans Candida*.

Each value represents the mean of three replicates. Means followed by different letters in each color are significantly different at $P \leq 0.05$ according to Newman and Keuls test, EO: Essential Oil.

3.2.2. Broth macrodilution method

The results of susceptibility assays with essential oils of three species of Thymus were expressed as MIC and MFC. All essential oils showed an inhibitory activity against 19 strains of *Candida* species tested. The results are presented in Table 4.

Table 4. Antifungal susceptibilities of 19 *Candida* strains isolates from Avicenna Military Hospital determined by the CLSI macrodilution method after 48 h of incubation.

MIC and MFC (mg/mL) at 48 h[L]				
Species (no. of isolates)	**Essential oil and Antifungal agent**	**Range**	**MIC**	**MFC**
***Candida albicans* (n=12)**	EO TL	5.21-0.0814	0.33[a]	0.33[a]
	EO TS	7.25-0.1133	0.9062[a]	0.9062[a]
	EO TP	6.27-0.099	0.7837[a]	0.7837[a]
	FLC	0.75-0.012	0.0234[b]	0.0469[a]
	AMB	0.33-0.0052	0.165[a]	0.165[a]
***Candida dubliniensis* (n=1)**	EO TL	5.21-0.0814	0.3300[a]	0.3300[a]
	EO TS	7.25-0.1133	0.9062[a]	0.9062[a]
	EO TP	6.27-0.099	0.7837[b]	0.7837[a]
	FLC	0.75-0.012	>0.75[c]	>0.75[c]
	AMB	0.33-0.0052	0.0103[a]	0.0103[a]
***Candida glabrata* (n=3)**	EO TL	5.21-0.0814	0.33[b]	0.65[a]
	EO TS	7.25-0.1133	0.9062[a]	0.9062[a]
	EO TP	6.27-0.099	0.7837[a]	0.7837[a]
	FLC	0.75-0.012	>0.7500[d]	>0.7500[d]
	AMB	0.33-0.0052	0.3300[a]	0.3300[a]
***Candida krusei* (n=3)**	EO TL	5.21-0.0814	0.3300[a]	0.3300[a]
	EO TS	7.25-0.1133	0.9062[b]	0.9062[b]
	EO TP	6.27-0.099	0.7837[a]	0.7837[a]
	FLC	0.75-0.012	>0.7500[f]	>0.7500[f]
	AMB	0.33-0.0052	0.3300[a]	0.3300[a]

EO: Essential oil; TL: *Thymus leptobotrys*; TS: *Thymus satureioides*; TP: *Thymus pallidus*; FLC: Fluconazole; AMB: Amphotericin B; n: Number of strains.[L] Each value represents the mean of three replicates. Means followed by different letters in each column are significantly different at $P<0.05$ according to Newman and Keuls test.

The values of Minimal Inhibitory Concentrations (MIC) and Minimal Fungicidal Concentrations (MFC) of the Thyme oils studied range from 0.3300 mg/mL to 0.9062 mg/mL (Table 4). Against some strains the essential oils possessed higher antifungal potential than classical fungicide, MICs values of fluconazole range from 0.0234 mg/mL to more than 0.7500 mg/mL and its MFCs values ranges from 0.0469 mg/mL to more than 0.7500 mg/mL and MICs and MFCs values of amphotericin B ranges from 0.0103 mg/mL to 0.3300 mg/mL.

On the other hand, all strains studied show the same sensitivity towards essential oils of thyme studied with the same MICs and MFCs values (0.3300 mg/mL for *T. leptobotrys*, 0.7837 mg/mL for *T. pallidus*, 0.9062 mg/mL to *T. satureioides*), except for *C. glabrata* which shows a value of 0.65mg/mL for essential oil of *T. leptobotrys*.

As to the standard antifungal drugs used in the test, the MIC values for fluconazole and amphotericin B were 0.0234 and 0.165 mg/mL for *C. albicans*, respectively, >0.75 and 0.0103mg/mL for *C. dubliniensis*, respectively, and >0.75 and 0.33 mg/mL for *C. glabrata* and *C. krusei*, respectively. Essential oils studied showed the highest MIC values, ranging from 7.25-0.1133mg/mL for essential oil of *T. satureioides*, followed by the MIC values, ranging from 6.27-0.099 mg/mL for essential oil from *T. pallidus* and then followed by those ranging from 5.21-0.0814 mg/mL for essential oil of *T. leptobotrys* with MIC values lower indicating a stronger activity than the other essential oils studied.

On the other hand, total inhibition was observed when the MIC of essential oils of *T. pallidus* reaches a value ≥ 0.7837 mg/mL, almost twice as much as the essential oils of *T. leptobotrys*, since no significant difference was recorded; *C. albicans* has the same sensitivity to the three essential oils studied at $P < 0.05$.

The MIC of essential oils of *T. leptobotrys* and those of *T. satureioides*, showed no significant difference with respect to *C. dubliniensis*, on the other hand, this difference is significant compared to the MIC of essential oils of *T. pallidus*.

For *C. glabrata*, complete inhibition was achieved by CMI presenting no significant difference respectively that of essential oils of *T. pallidus* and *T. satureioides*, which are significantly different from the MIC of essential oils of *T. leptobotrys*.

As to, *C. krusei*, the MIC causing complete inhibition without significant (<0.05), are those belonging to essential oils of *T. leptobotrys* and those of *T. pallidus*. We deduce that *C. krusei*, is more sensitive to essential oils of *T. leptobotrys* and *T. pallidus* than to essential oil of *T. satureioides.*

These results agree with previous study reports (Duarte *et al*. 2005; Jamali *et al*. 2012). The difference in activity between the three essential oils tested with different concentrations can be correlated with the difference in their chemical composition as noted in Table 2 (Chebli *et al*. 2003).

Although essential oil values were high when compared with those of the antifungal drugs, the results are of interest because are natural compounds and some authors considered even higher values than for antifungal drugs (Aligiannis *et al*. 2001). The antifungal activity of the essential oils is related to the respective composition of the plant essential oils, the structural configuration of the constituent components and their functional groups and possible synergistic interactions between components (Naeini *et al*. 2009).

It is difficult to attribute the activity of a complex mixture to particular constituents. Nevertheless, it is reasonable to speculate that the activity of these oils can be related to the presence of borneol, α-terpineol, camphene, carvacrol and thymol or their biogenetic precursors like γ-terpinene and *p*-cymene. In general, the cytotoxic activity of essential oils is mostly due to the presence of phenols such as thymol and carvacrol. Several studies showed that thyme essential oils, particularly those of the phenol type, as *Thymus vulgaris* and *T. zygis*, possess the most antimicrobial activity (Bakkali *et al*. 2008). The occurrence of these phenols in *Thymus* essential oils have been of great interest for some time. The essential oils of the *T. pallidus*, *T. leptobotrys* and *T. satureioides* are rich in oxygenated monoterpenes, especially borneol, α-terpineol and carvacrol, and due to the high phenol content in *T. pallidus* and *T. leptobotrys*, they can be used for medicinal purposes and other biological applications. In addition, the Morocan *Thymus pallidus* may be a potential carvacrol rich source for commercial cultivation (Naeini *et al*. 2009). In fact, a study conducted by Salgueiro *et al*. (2003), showed that carvacrol has the highest antifungal activity among all products tested and these results are in agreement with another study of its anti-*Candida* activity (Periago & Moezelaar 2001; Roller & Seedhar 2002; Ultee *et al*. 2002; Salgueiro *et al*. 2003; Kisko & Roller 2005). A study on antimicrobial activity of essential oils of four samples of *Origanum vulgare* subsp. *virens* recently published (Castilho *et al*. 2012) showed a high antifungal activity. The activity in essential oils with low levels of carvacrol seems to be due to a high content of thymol (Castilho *et al*. 2012). This may explain the strong antifungal activity of the essential oil of *Thymus pallidus* which it contained proportionally more carvacrol and thymol than other essential oils in our study (Vale-Silva *et al*. 2012). A study led by Pinto *et al*. (2006) showed that carvacrol and thymol have the lowest MIC values. The importance of the phenolic hydroxyl groups for the antimicrobial activity of the monoterpenoids has previously been reported (Adam *et al*. 1998; Dorman and Deans, 2000; Aligiannis *et al*. 2001). Other species of the genus *Thymus*, such as *T. zygis* and *T. vulgaris*, with high amounts of phenols, showed a broad spectrum of activity against a variety of pathogenic yeasts and filamentous fungi, including fungi with decreased susceptibility to fluconazole (Dorman & Deans 2000; Pina-Vaz *et al*. 2004). Carvacrol proved to be active against dermatophyte strains, in a similar manner to the essential oil. MIC and MFC values were very similar and the fungistatic and fungicidal properties of the essential oil was associated with high carvacrol and thymol content (Pinto *et al*. 2006).

It has been reported that carvacrol causes perturbations in the bacterial membrane and thus potentially can exert antibacterial activity also at intracellular sites (Cristani 2007; Ultee *et al*. 2002).

Furthermore, other *Thyme* essential oils rich in carvacrol have demonstrated potent antimicrobial activities *in vitro* (Jamali *et al*. 2012; El Bouzidi *et al*. 2013). Among the major constituents of the investigated essential oils, γ-terpinene, *p*-cymene and α-pinene were reported to have weaker antibacterial activities (Consentino *et al*.

1999; Aligiannis *et al*. 2001). Synergy effect between carvacrol and its biogenetic precursor *p*-cymene has been noted (Ultee *et al*. 2002). It appears that *p*-cymene swells bacterial cell membranes to a greater extent than carvacrol does and probably by this mechanism enables carvacrol to be more easily transported into the cell, so that a synergistic effect is achieved when the two are used together.

4. Conclusion

This study is part of an overall approach that aims to highlight the antifungal activity of natural floral resources from Morocco Southwest (region of area of the Argan tree), against invasive candidiases due to emerging fluconazole-resistant strains. Thus, three essential oils obtained from *Thymus leptobotrys*, *T. pallidus* and *T. satureioides* have proven their antifungal potential against *Candida albicans* and *non-albicans Candida in vitro*. The interesting antifungal activity demonstrated *in vitro* by these essential oils should encouraged to research the chemical isolation of the active components in order to study possible synergies between these components or between these components and conventional antifungal used, as well as to conduct clinical trials *in vivo*.

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