ANTICANDIDAL ACTIVITY OF TWO ESSENTIAL OILS FROM MOROCCO: *THYMUS SATUREIOIDES* AND *MENTHA PULEGIUM L.*

EL MOSTAFA ZAHRAOUI[1], SOUAD AMGHAR[2], BOUCHRA ABABOU[1], KHADIJA BOUKACHABINE[1]

Abstract: Due to the side effects of antifungal medications and the resistance that has limited the use of these molecules, several studies on the antifungal activity of essential oils have been conducted to find natural alternatives to conventional antifungal drugs. The anticandidal activity and chemical composition of *Thymus satureioides* and *Mentha pulegium L.* essential oils were evaluated by testing 12 strains of *Candida albicans*, one strain of Candida parapsilosis, and one strain of Candida tropicalis. Two methods were used: the tube dilution method and the poisoned medium method. The use of mathematical modelling allowed for the precise determination of the minimum inhibitory concentration (MIC 80%) of the tested essential oils. Chemical analysis showed that both essential oils have a complex composition, with borneol as the major compound in *Thymus satureioides* essential oil and pulegone in *Mentha pulegium L.* essential oil. Both *Thymus satureioides* and *Mentha pulegium L.* essential oils exhibited anticandidal activity against all 14 tested Candida strains, with MIC80% ranging from 1.28433 mg/mL to 5.18543 mg/mL for *Thymus satureioides* essential oil and from 0.96217 mg/mL to 3.19589 mg/mL for *Mentha pulegium L.* essential oil. The results obtained in this study demonstrate that both essential oils have inhibitory and fungicidal action against all tested strains. These findings suggest that *Thymus satureioides* and *Mentha pulegium L.* essential oils may serve as a natural alternative to antifungal agents in the fight against candidiasis.

Keywords: Anticandidal activity, Essential oils, *Thymus satureioides*, *Mentha pulegium L.*, Candidiasis.

INTRODUCTION

In humans, candidiasis is caused by the growth of yeast of the Candida genus, with *Candida albicans* being the most formidable species. In recent years, there has been significant interest in the study of candidiasis, both at the biological and therapeutic levels. This increased attention to candidiasis is also a result of the

[1] Laboratory of Environmental Sciences and Development, University Hassan 1st, Faculty of Sciences and Technology, Settat, Morocco.

[2] Laboratory of Improved Soil Productivity and Environment. Mohammed V University, Higher Normal School, Rabat, Morocco.

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emergence of resistance in Candida strains to commonly used antifungal agents and their associated side effects (Senhaji *et al*., 2005).

The use of extracts from medicinal and aromatic plants for the treatment of diseases has a long history. In recent decades, there has been a renewed interest among the public in the use of phytotherapy.

However, evaluating the antimicrobial and antioxidant phytotherapeutic properties remains a very interesting and useful task. These plants represent a new source of active compounds as substitutes for currently marketed chemical compounds that have notable side effects (Labib, 2005).

This study falls within the same research framework on the antifungal action of essential oils from aromatic and medicinal plants. The aim is to study the chemical composition as well as the anticandidal activity of two Moroccan essential oils: *Thymus satureioides* and *Mentha pulegium L.*

MATERIALS AND METHODS

ESSENTIAL OILS

Two essential oils were used in this study, those of *Thymus satureioides* and *Mentha pulegium L.* These essential oils, extracted by hydrodistillation, were provided by the company SANTIS-SARL.

STRAINS STUDIED

Fourteen strains of pathogenic yeast from the Candida genus were tested, namely 12 strains of *Candida albicans* (CA), one strain of Candida tropicalis (CT), and one strain of Candida parapsilosis (CP). These various strains, originating from the Children's Hospital of Rabat, were isolated from patients suffering from cutaneous candidiasis, oral candidiasis, vaginal candidiasis, and nail candidiasis (Table 1).

Table 1

Origin of tested *Candida* Isolates

Code for Isolates	Patient's Age	Clinical Presentation
CA1	31 days	Cutaneous lesions
CA2	26 years	Onychomycosis (hands)
CA3	39 years	Onychomycosis (hands)
CA4	2 years	Onychomycosis (hands)
CA5	Premature	Urine
CA6	22 years	Onychomycosis (feet)
CA7	26 years	Vulvitis

Table 1 (continued)

Code for Isolates	Patient's Age	Clinical Presentation
CA8	Premature	Cutaneous
CA9	36 years	Paronychia (hands)
CA10	6 years	Throat
CA11	6 years	Tongue
CA12	4.5 years	Mouth
CP	55 years	Onychomycosis (feet)
CT	53 years	Onychomycosis (hands)

CULTURE MEDIUM

The medium used is Sabouraud (both agar and liquid) supplemented with chloramphenicol (Duarte *et al*., 2005; Giordani et Kaloustian, 2006; Yesil Celiktas *et al*., 2007).

DETERMINATION OF THE CHEMICAL COMPOSITION OF ESSENTIAL OILS

The analysis of the chemical composition of essential oils (EO) was performed using gas chromatography-mass spectrometry (GC–MS) at the National Center for Scientific and Technical Research (CNRST) in Rabat, Morocco.

This analysis was conducted using a gas chromatograph (Trace GC ULTRA) coupled with a mass spectrometer (Polaris Q MS with ion trap). The GC was equipped with a VB-5 column (5% phenyl methylpolysiloxane) measuring (30 m * 0.25 mm * 0.25 µm). For detection, an electron ionization system with 70 eV was used, and helium was used as the carrier gas at a flow rate of 1.4 mL/min.

One microliter (1µL) of EO was injected at a temperature of 200°C. Initially, the column was maintained at 40°C for 2 minutes, then gradually increased to 180°C at a rate of 4°C/min and finally raised to 300°C at a rate of 20°C/min, and held at this temperature for 2 minutes.

The results of these analysis allowed the determination of the chemical composition of the tested EO by comparing the relative retention times of each compound and their mass spectra with those of standards.

PREPARATION OF YEAST SUSPENSION

For each yeast strain, a 24-hour-old colony was aseptically picked and homogenized in liquid Sabouraud-chloramphenicol medium. Yeast counting was carried out using a Thomas cell. The yeast suspension was adjusted to 10^5 yeast cells/mL (Cruz *et al*., 2007; Bouamama *et al*., 2006).

STUDY OF ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS

Two methods were used to study and evaluate the activity of the two essential oils: the tube dilution method described by Giordani and Kaloustian (2006) and the poisoned medium technique (Mishra and Dubey, 1994; Belghazi *et al*., 2002; El Ajjouri *et al*., 2008). All assays were repeated three times, with negative controls containing no essential oil.

The tube method involves preparing dilutions of the essential oil in tubes containing liquid Sabouraud medium inoculated with the strain to be tested. Another series of essential oil dilutions is prepared without inoculation. The initial solution of essential oil was prepared at 2.5%, which corresponds to a concentration of 22.7 mg/mL. From this solution, a series of half-dilutions ranging from 11.35 mg/mL to 0.177 mg/mL was prepared. Incubation was carried out at 37°C for 24 hours (Kobak *et al*., 2004).

The poisoned Medium Technique: This is a direct contact method where essential oil is diluted directly into Sabouraud-chloramphenicol agar in a liquid state. Inoculation is done from a 24-hour culture suspension. Cultures are incubated at 37°C for 24 hours. The MIC on solid medium is the lowest concentration of essential oil that does not allow growth (El Ajjouri *et al*., 2008).

MINIMUM INHIBITORY CONCENTRATION (MIC) 80%

MIC 80% is determined using mathematical modeling of absorbance values, based on nonlinear regression using the Newton-Gauss algorithm, allowing adjustment of the graphical representation (Giordani *et al*., 2002). The trend curve of absorbance at 560nm after 24 hours as a function of essential oil concentrations is an exponential curve of the general formula $y=ae^{-(bx)}$, where a corresponds to the maximum OD value, and if y corresponds to 20% of the maximum OD value, the corresponding x value will be equal to MIC 80%.

MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC is evaluated by reading the absorbance values at 560 nm of the dilution series. It is determined as the lowest concentration of essential oil that inhibits growth.

MINIMUM FUNGICIDAL CONCENTRATION (MFC)

After 24 hours of incubation at 37°C, each dilution is inoculated onto Sabouraud-chloramphenicol agar and incubated for an additional 24 hours at 37°C (Bouhdid *et al*., 2008). MFC is the lowest dilution that did not allow growth.

STATISTICAL ANALYSIS

The MIC 80% results were subjected to the Fisher's LSD (Least Significant Difference) statistical test ($p<0.05$) to compare the means of the Minimum Inhibitory Concentration 80% (MIC 80%) for the different strains studied, allowing for the classification of strains in terms of their sensitivity to the studied essential oils.

RESULTS

CHEMICAL COMPOSITION OF ESSENTIAL OILS

Chemical analysis by GC-MS of *Thymus satureioides* and *Mentha pulegium L.* essential oils revealed the presence of several chemical compounds.

Exploiting the data from the chromatographic profile and mass spectrum allowed for the identification of different chemical compounds in *Thymus satureioides* and *Mentha pulegium L.* essential oil (table 2 and table 3). Borneol is the major compound in *Thymus satureioides* essential oil, with a peak area of 26.45%, followed by Phenol, 5-methyl-2-(1-methylethyl) at 11.24%, and α-Terpinenyl acetate at 10.99%. This suggests that it is the Moroccan species *Thymus satureioides* (Ismaili *et al*., 2004, Ouraïni *et al*., 2007; Ouraïni *et al*., 2005).

Table 2

Chemical Composition of *Thymus satureioides* Essential Oils

RT	Aire du pic (%)	SI	RSI	Nom	Prob (%)
15.97	26.45	833	834	Borneol	22.15
		827	827	Borneol L	17.41
20.75	11.24	861	896	Phenol, 5-methyl-2-(1-methylethyl)	19.20
16.86	10.99	855	866	à-Terpinenyl acetate	18.23
24.39	8.27	844	847	Bicyclo[5.2.0]nonan, 2-methylen-4,8,8-trimethyl-4-vinyl- (caryophyllen "v1")	8.10
27.57	1.44	822	831	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)	28.66
		820	829	ë-Cadinene	28.66

The presence in *Mentha pulegium L.* essential oil of Pulegone with a peak area of 78.07% and Isopulegone (1.45%), Caryophyllene, Humulene, and Aromadendrene represent peak areas of 1.19% confirms that it is the Moroccan species *Mentha pulegium L.* (Bouchra *et al*., 2003; Aghel *et al*., 2004; Ouraïni *et al*., 2007; Ouraïni *et al*., 2005).

Table 3

Chemical Composition of *Mentha pulegium L.* Essential Oils

RT	Aire du pic (%)	SI	RSI	Nom	Prob (%)
18.56	78.07	805	817	Cyclohexanone, 5-methyl-2-(1-methylethylidene)	34.88
		804	818	Cyclohexanone, 5-methyl-2-(1-methylethylidene)-	34.88
		797	808	Pulegone	26.02
16.28	1.45	787	809	Cyclohexanone, 5-methyl-2-(1-methylethenyl)-, trans-	40.76
		781	802	Isopulegone	40.76
		757	808	CIS-Isopulegone	11.33
		770	813	à-Caryophyllene	5.67
25.44	1.19	770	805	à-Humulene	5.67
		770	804	Aromadendrene	5.67

DETERMINATION OF INHIBITION PARAMETERS

Spectrophotometric analysis at 560 nm determined the MIC80% for each strain. Table 4, Figure 1, and 2 show the variations in optical density (OD560nm) for all Candida strains as a function of *Thymus satureioides* and *Mentha pulegium L.*, oil concentration.

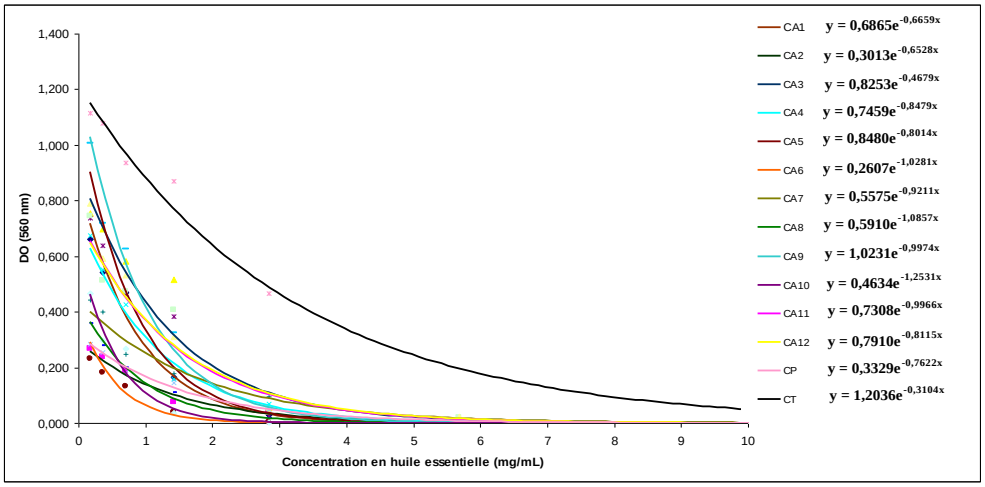

Figure 1. The variation in optical density (at 560nm) as a function of the concentration of *Thymus satureioides* essential oil.

The analysis of this comparison shows that *Mentha pulegium L.* essential oil has more inhibitory action on strains CA1, CA2, CA3, CA5, CA7, CA8, CA10, and CT compared to *Thymus satureioides* essential oil. However, strains CA4, CA6, CA9, CA11, CA12, and CP proved to be less sensitive to *Mentha pulegium L.* essential oil compared to *Thymus satureioides* essential oil.

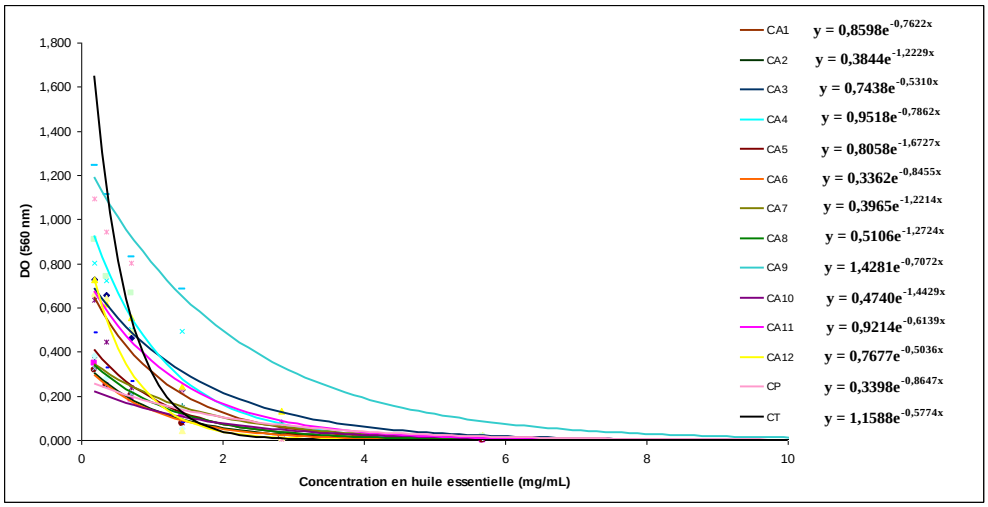

Figure 2. The variation in optical density (at 560nm) as a function of the concentration of *Mentha pulegium L.* essential oil.

Based on the enumeration results, the MFCs were determined (Table 4). The analysis of these results shows that *Thymus satureioides* and *Mentha pulegium L.* essential oil has a fungicidal action on all studied Candida strains, consistent with the MIC80% results.

The fungicidal action of the two essential oils is equivalent for strains CA2, CA3, CA6, CA10, and CP. *Thymus satureioides* essential oil has a lower fungicidal activity than *Mentha pulegium L.* essential oil against strains CA1, CA4, CA9, CA11, and CA12. In contrast, *Mentha pulegium L.* essential oil has a more significant fungicidal action against strains CA5, CA7, CA8, and CT.

Analysis of the MIC of *Thymus satureioides* essential oil shows that most strains have the same MIC (2.838 mg/mL), except CA10, which has the lowest MIC of 1.419 mg/mL, while CA3 and CT have the highest MIC at 5.675 mg/mL (Table 4). The MIC of *Mentha pulegium L.* essential oil shows that most strains have the same MIC (2.838 mg/mL), except for CA3 and CA12, which have the highest MIC at 5.675 mg/mL, while CA2, CA5, CA7, CA8, and CA10 have a MIC of 1.419 mg/mL (Table 4).

Table 4

Inhibition parameters

	Thymus satureioides			***Mentha pulegium L.***		
Strains	**MIC 80% (mg/mL)**	**MFC (mg/mL)**	**MIC (mg/mL)**	**MIC 80% (mg/mL)**	**MFC (mg/mL)**	**MIC (mg/mL)**
CA1	2,41704^{b}	2,838^{c}	2,838^{b}	2,11158^{b}	5,675^{a}	2,838^{b}
CA2	2,4655^{b}	2,838^{c}	2,838^{b}	1,31604^{c}	2,838^{b}	1,419^{c}
CA3	3,43965^{a}	5,675^{b}	5,675a	3,03122^{a}	5,675^{a}	5,675^{a}
CA4	1,89813^{c}	2,838^{c}	2,838^{b}	2,04699^{b}	5,675^{a}	2,838^{b}
CA5	2,00834^{c}	5,675^{b}	2,838^{b}	0,96217^{c}	2,838^{b}	1,419^{c}
CA6	1,56541^{d}	2,838^{c}	2,838^{b}	1,90356^{b}	2,838^{b}	2,838^{b}
CA7	1,74721^{d}	5,675^{b}	2,838^{b}	1,31766^{c}	2,838^{b}	1,419^{c}
CA8	1,48233^{d}	5,675^{b}	2,838^{b}	1,26487^{c}	1,419^{c}	1,419^{c}
CA9	1,61356^{d}	2,838^{c}	2,838^{b}	2,27575^{b}	5,675^{a}	2,838^{b}
CA10	1,28433^{d}	2,838^{c}	1,419^{c}	1,11544^{c}	2,838^{b}	1,419^{c}
CA11	1,61486^{d}	2,838^{c}	2,838^{b}	2,62173^{a}	5,675^{a}	2,838^{b}
CA12	1,98336^{c}	2,838^{c}	2,838^{b}	3,19589^{a}	5,675^{a}	5,675^{a}
CP	2,11146^{c}	5,675^{b}	2,838^{b}	1,86135^{b}	5,675^{a}	2,838^{b}
CT	5,18543^{a}	11,35^{a}	5,675^{a}	2,78715^{a}	5,675^{a}	2,838^{b}

NB. The values in the same column followed by the same letter are not significantly different according to Duncan test at 5% probability.

Both essential oils have similar inhibitory activities against strains CA1, CA3, CA4, CA6, CA9, CA11, CA12, and CP. *Thymus satureioides* essential oil has a lower inhibitory activity than *Mentha pulegium L.* essential oil against strains CA2, CA5, CA7, CA8, CA10, and CT.

The statistical analysis allowed the classification of the 14 Candida strains studied based on their sensitivity to *Thymus satureioides* essential oil into three classes (Figure 3). The first class comprises six *Candida albicans* strains, CA10, CA8, CA6, CA9, CA11, and CA7 (causing vaginal candidiasis, throat infections,

oral thrush, skin infections, toenail fungus, and fingernail infections), which are the most sensitive to the action of *Thymus satureioides* essential oil, with CMI80% ranging from 1.28433 mg/mL to 1.74721 mg/mL.

The second class includes the strains CA4, CA12, CA5, CP, CA1, and CA2 (causing skin lesions, urinary tract infections, and oral cavity infections) with CMI80% values ranging from 1.89813 mg/mL to 2.4655 mg/mL, indicating that they are moderately sensitive to the action of *Thymus satureioides*.

The two strains, CT and CA3 (causing fingernail infections), exhibit the highest resistance to the action of this essential oil, with CMI80% values of 5.18543 mg/mL and 3.43965 mg/mL, respectively.

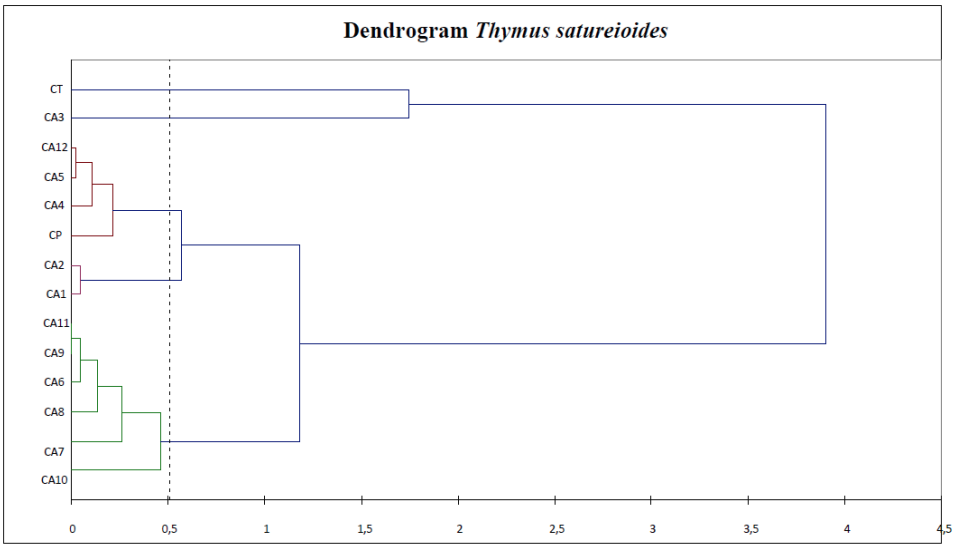

Figure 3. The Euclidean distances of MIC80% of the Candida strains in the presence of *Thymus satureioides* essential oil.

The classification of the 14 Candida strains studied based on their sensitivity to *Mentha pulegium L.* essential oil into three classes (Figure 4). The first class, comprising CA2, CA5, CA7, CA8, and CA10 (causing skin candidiasis, vaginal candidiasis, urinary tract infections, and throat infections), consists of strains most sensitive to the action of *Mentha pulegium L.* essential oil, with CMI80% values ranging from 0.96217 mg/mL to 1.31766 mg/mL.

Strains CA1, CA4, CA6, CA9, and CP (causing toenail fungus, skin lesions, and fingernail infections) are classified as moderately sensitive to the antifungal action of *Mentha pulegium L.* essential oil, with CMI80% values ranging from 1.86135 mg/mL to 2.27575 mg/mL.

The third class includes strains CA3, CA11, CA12, and CT (2.62173 mg/mL, 2.78715 mg/mL, 3.03122 mg/mL, and 3.19589 mg/mL, respectively) (causing

fingernail infections and oral candidiasis) and is composed of strains that exhibit the highest resistance to the action of this essential oil.

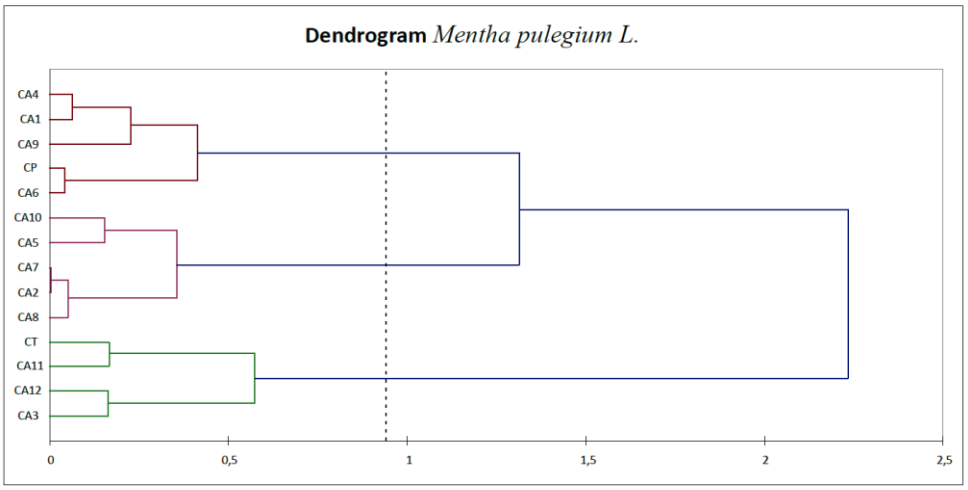

Figure 4. The Euclidean distances of MIC80% of the Candida strains in the presence of Thymus. *Mentha pulegium L.* essential oil.

DISCUSSION

The chemical analysis conducted in this study demonstrates that Borneol is the major compound in *Thymus satureioides* essential oil, constituting 26.45% of its composition. This finding aligns with the results of Ouraïni *et al.* (2005), who reported that Borneol makes up 29.4% of the chemical composition of *Thymus satureioides* essential oil. Additionally, the work of Ismaili *et al*. (2004) supports that Borneol is the predominant compound in *Thymus satureioides* essential oil.

The results obtained show that Pulegone is the major compound, representing 78.07% of the chemical composition of *Mentha pulegium L.* essential oil. These findings are consistent with the research of Ouraïni *et al.* (2005), who reported that Pulegone is the major compound with a content of 88%, and 85.4% according to the study conducted by Bouchra *et al*. (2003).

Mahboubi and Haghi (2008) demonstrated that *Mentha pulegium L.* essential oil from Iran is rich in piperitone and piperitenone compared to Moroccan *Mentha pulegium L.* essential oil, which is rich in Pulegone. This suggests that the plant's origin affects the chemical composition of the extracted essential oil.

The antimicrobial activity of Borneol has been reported by Kelen and Tepe (2008), suggesting that the anticandidal activity of *Thymus satureioides* essential oil is attributed to the presence of Borneol. Studies conducted by Shunying *et al*.

(2005) and Unlu *et al.* (2002) have shown that Borneol inhibits the growth of *Candida albicans*.

According to the study by Tepe *et al*. (2005), the compound Pulegone inhibits the growth of *Candida albicans*. Duru *et al*. (2004) demonstrated that Pulegone has highly significant antimicrobial activity. The antifungal activity is also attributed to the contribution of minor compounds in essential oils alongside major compounds (Randrianarivelo *et al*., 2009).

The results obtained through the tube dilution method indicate that both essential oils, *Thymus satureioides* and *Mentha pulegium L.*, exhibit inhibitory and fungicidal anticandidal activity against various tested Candida strains. Lisin *et al.* (1999) reported that Thymus vulgaris essential oil (borneol type) inhibits the growth of *Candida albicans*, which is consistent with our study's results.

Similarly, the study conducted by Omran and Esmailzadeh (2009) showed that *Mentha pulegium L.* essential oil has inhibitory activity against strains of *Candida albicans*, Candida glabrata, and Candida krusei.

The MIC80% results for *Thymus satureioides* essential oil range from 1.28433 mg/mL for strain CA10, which is the most sensitive, to 5.18543 mg/mL for the most resistant strain, CT. *Mentha pulegium L.* essential oil also exhibits anticandidal activity, with MIC80% values ranging from 0.96217 mg/mL (strain CA5) to 3.19589 mg/mL (strain CA12).

These findings align with the work of Giordani and Kaloustian (2006), where they demonstrated that Thymus vulgaris essential oil of the Borneol chemotype has moderate anticandidal activity compared to other essential oils studied.

Statistical analysis of the MIC80% results using the LSD test for the two essential oils allowed for the classification of different Candida strains based on their sensitivity to the action of *Thymus satureioides* and *Mentha pulegium L.* essential oils.

The most sensitive strains to *Thymus satureioides* essential oil are CA6, CA7, CA8, CA9, CA10, and CA11, with MIC80% values ranging from 1.28433 mg/mL (CA10) to 1.74721 mg/mL (CA7). On the other hand, CA2, CA5, CA7, CA8, and CA10 are the most sensitive to *Mentha pulegium L.* essential oil, with MIC80% values ranging from 0.96217 mg/mL (CA5) to 1.31766 mg/mL (CA7).

Regarding the most resistant strains, CT and CA3 exhibit MIC80% values of 5.18543 mg/mL and 3.43965 mg/mL, respectively, against *Thymus satureioides* essential oil. CA3, CA11, CA12, and CT are the strains that are more resistant to the activity of *Mentha pulegium L.* essential oil, with MIC80% values ranging from 2.62173 mg/mL (CA11) to 3.19589 mg/mL (CA12).

A study by Duarte *et al.* in 2005 showed that *Mentha pulegium L.* essential oil has activity against *Candida albicans* CBMAI 0475 (ATCC 10231) with an average MIC of 0.74 mg/mL, which is consistent with our study's results.

Ouraïni *et al*. in 2007 reported that *Thymus satureioides* essential oil and *Mentha pulegium L.* essential oil has antifungal activity against strains of *Candida albicans*, which is in line with our results.

The comparison of the MIC80% results of the two studied essential oils shows that *Mentha pulegium L.* essential oil is more active against the tested Candida strains compared to *Thymus satureioides* essential oil. This difference observed in the antifungal activities of the two essential oils can be attributed to differences in their active fractions in terms of chemical composition.

The MIC values obtained using the poisoned medium technique are the same (1.419 mg/mL) for both essential oils and all strains, suggesting that this technique may not be reliable for studying the anticandidal activity of essential oils.

CONCLUSION

The results of the chemical analysis by gas chromatography coupled with mass spectrometry of the studied essential oils have shown the abundance of borneol in *Thymus satureioides* essential oil and pulegone in *Mentha pulegium L.* essential oil.

The in vitro study of the anti-candidal activity of both *Thymus satureioides* and *Mentha pulegium L.* essential oils demonstrated that both essential oils possess inhibitory and fungicidal properties against various tested Candida strains.

The MIC (Minimum Inhibitory Concentration) values of 80% obtained for *Thymus satureioides* essential oil range between 1.28433 mg/mL and 5.18543 mg/mL, while for *Mentha pulegium L.*, the MIC80% values range from 0.96217 mg/mL to 3.19589 mg/mL.

The tube dilution method yielded significantly better results compared to the poisoned medium technique.

These findings suggest that *Thymus satureioides* essential oil and *Mentha pulegium L.* essential oil could serve as a natural alternative solution for replacing synthetic antifungal agents that are commercially available and have known health side effects.

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