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# Chemical polymorphism of populations of *Thymus leptobotrys L.* harvested from the Argan tree regions of Morocco, assessed by analysis of their essential oils, and its impact on their anticandidal and antioxidant activity

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**ABSTRACT :** *The composition of the essential oils isolated from six endemic populations of Thymus leptobotrys L. Collected from six geographical provenances of the Argan tree in south-western of Morocco were studied by GC–MS. All the essential oils analysed were dominated by their monoterpene fraction (80.76-97.38%), where the proportion of the oxygenated monoterpenes (70.07-77.74%) was higher than that of the monoterpene hydrocarbons (10.69-19.64%) of the total essential oils from the populations grown in Souss-Massa region. Cluster analysis of the identified components with a concentration ≥1% grouped the oils that corresponded with their main components in five chemotypes : Carvacrol/β-caryophyllene/p-cymene type (S1 and S5), carvacrol/p-cymene/γ-terpinene type (S2), carvacrol/p-cymene/borneol type (S3),carvacrol/p-cymene type (S4) and carvacrol/β-caryophyllene type (S6). In addition, the antifungal activities of essential oils from five chemotypes of endemic thyme were investigated in this study. Results from the antifungal tests demonstrated that the essential oils belonging to all chemotypes found showed marked antifungal activity against all Candida species studied compared to conventional antifungal.*

**KEYWORDS:** *Thymus leptobotrys L.; Essential oils; Chemotypes; Anticandidal activity; Antioxidant activity*

## I. INTRODUCTION

Anti-Candida activity of essential oils has been studied extensively, and they are being investigated as alternative or complementary therapeutic agents in the treatment of candidiasis [1-3]. Indeed, the scientific interest of these substances has increased today in search of new antifungal compounds from plant sources, due to Emerging fungal infections who are a major challenge for health professionals, which have a poor prognosis [4, 5], and which have a resistance to conventional antifungal used in current therapy [6-8].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 Candida albicans. Therefore, it is necessary to isolate new antifungal agents, mainly from plant extracts. In recent years, some researchers have focused on the use of components made from extracts of plants that exhibit biological activity in vitro and in vivo, thereby justifying based research on traditional medicine for their characterization activity looking for molecules that are both highly effective antifungal, with fewer side effects, very tolerable by the human body and less costly [1, 2]. Indeed, the concomitant use of an essential oil and Amphotericin B has significantly reduced the Minimum Inhibitory Concentration of the latter, leaving hope for a reduction of side effects associated with the use of this molecule drug in a therapeutic protocol [9].Therefore increasing the resistance to conventional antifungal, toxicity and the costs involved justified the search for new therapeutic approaches. Among these new approaches, essential oils are one of the groups of promising natural compounds for use in the prevention and treatment of fungal infections. Thus we are focused on the study of essential oils of *Thymus leptobotrys L.* which is an endemic that characterizes the air of the Argan tree of southwestern of Morocco [10].

Is an evergreen shrub belonging to the family of Lamiaceae [11]. These species are used in folk medicine as a powder, decoction or infusion to relieve some pains and to treat several disturbances such as gastro-intestinal infections, whooping coughs, bronchitis, flue and infections of throat and mouth [12]. Pharmacological studies with the Moroccan species have confirmed the medicinal properties of thyme described in folk medicine. These studies have confirmed the antimicrobial effect of *Thymus broussonetii, Thymus zygis* and *Thymus satureioides* [13, 14].The goal of this study was to study the composition of the essential oils from six populations (Table I) of *Thymus leptobotrys L.* collected from several locations in the area of the Argan tree of southwestern of Morocco, and to evaluate their antifungal activity against Candida species resistant to conventional antifungal agents, taken from patients.

**Table I:** Sites of collection of six populations of *Thymus leptobotrys L.* growing on Argan tree region of Morocco.

| **Scientific name** | | *Thymus leptobotrys L.* | **Local name** | Azoukni |
|---|---|---|---|---|
| **Family** | | *labiatae* | **part used** | flowering tops and leaves |
| **Collection site** | **designation of the area** | **Latitude/longitude** | **Altitude** | **EO Yield** |
| Ikhsayn | S1 | 29°46'38,159" N/9°9'33,455" W | 989m | 2.00±0.01% |
| Isk | S2 | 30°41'52" N/9°27'50,2" W | 1350m | 2.12±0.02% |
| Asgharkiss | S3 | 29°49'22,56" N/9°12'4,7" W | 734m | 1.82±0.01% |
| Ait Lbacha | S4 | 29°35'37,53" N/9°6'59,73" W | 1039m | 2.03±0.021% |
| Fizrane | S5 | 29°53'48,35" N/9°11'6,61"W | 853m | 1.73±0.01% |
| Près de Tamanar | S6 | 30°57’14.07’’ N/9°32’57.04’’ W | 981m | 1.51±0.01% |

## II. MATERIALS AND METHODS

**Plant materials**

Fresh plant samples were collected from six regions of area of Argan tree in Souss-Massa- Draa Valley, Southwestern of Morocco, between March and June 2012. The taxonomic identification of the species of the 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 I.

**Essential oils extraction**

Essential oils were isolated by hydrodistillation for 4 h from air dried material, using a Clevenger-type apparatus, according to the European Pharmacopoeia method [15, 16], obtained oils were weighed and stored at 4 ◦C in a sealed brown vial until use. The Yields of essential oils extracted were noted in Table I.

**Gas Chromatography-Mass Spectrometry (GC-MS)**

GC-MS 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 C8-C32 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 [17].

**Drugs preparation**

The used drugs usually eradicate fungal infections such as those belonging to the family of Imidazole agents like Fluconazole, and those belonging to the family of Polyenes, agents such as Amphotericin B. These antifungal agents are dissolved in 2 ml DMSO 10% to give the following stock solutions: Fluconazole 75 mg/ml and Amphotericin B 33 mg/ml.

**Isolation of the microorganisms**

In this study, thirty clinical isolates of *Candida species*, including *Candida parapsilosis* (n=10), *Candida lusitaniae* (n=4), and *Candida famata* (n=5), *Candida tropicalis* (n=11), were clinically isolated from patients suffering from nosocomial candidiases. These strains were isolated in the laboratory of parasitology-mycology and bacteriology Avicenna military hospital –Marrakech MOROCCO, on Sabouraud chloramphenicol agar plates and identified by the germ tube test, API 20 C AUX (Bio-merieux, marcy-l'etoile, 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 chloramphenicole (Bio-RAD), and then incubated at 30°C for 48hours.

**Antioxidant Activity**

To evaluate the antioxidant activity of the essential oil we used the method of DPPH (1,1-diphenyl-di-picrylhydrazyl) proposed by Chen et al. [18] and Leitao et al. [19], with some modifications. The DPPH solution is obtained by dissolving 4 mg of the powder in 100 ml of ethanol. Samples of essential oils were prepared by dissolving in ethanol as 0.75ml of essential oil in 1.5ml of Ethanol/eau (50%).Those solutions, called stock solutions were then undergone dilutions for having the following concentrations: for *Thymus leptobotrysL.* : 0,0664;0,0332;0,0166;0,0083;0,0041mg/ml. The test is performed by mixing 4 ml of the above solution of DPPH with 1ml of each essential oil for the six origins, each essential oil to be tested at different concentrations. The antioxidant reference or positive control (Quercetin) was also prepared by the same method. Thereby Inhibition of free radical DPPH by Quercetin was also analyzed at the same concentration of each essential oil for comparison. Measuring the variation of absorbance was made after shaking and incubation period of 30 min at room temperature after introduction of the tanks in the spectrophotometer at the 514 nm wavelength. The values obtained are then processed in percent inhibition using the following formula [19, 20]:

$$\mathbf{I\% = 100 \times (A_{control} - A_{test})/A_{control}} \quad \text{(i.e,(1))}$$

With $A_{control}$ is the absorbance of the control (containing all reagents without the test product) and $A_{test}$ is the absorbance of the test.

The graph of the variation in the percentage of inhibition versus the concentration of the essential oil is used to determine the IC50 (otherwise known as EC50, concentration at 50% inhibition, which is the antioxidant activity of the essential oil). This value is compared to that found for the reference compound (Quercetin). All tests were performed in triplicate for each concentration.

**Antifungal testing**

**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 hour at 37°C to ensure that yeast cells were actively dividing, then adjusted between: 2.16 x 105 Cells/ml to 5.22 x 105 Cells/ml for fungal strains with counting with haemocytometer for each repetition.

**Antifungal screening**

Antifungal activity against *Candida* species of essential oils from *Thymus leptobotrys L.* harvested from different region of south west of Morocco was assessed using the agar diffusion method [21]. Thirty 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 hours. 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 is poured into a container for infectious waste. Therefore said natural essential oil of *Thymus leptobotrys* (521 mg/mL), were applied on filter paper (10 µL/disk) disks of 5 mm in diameter separately. 10µL of Fluconazole (75 mg/mL) and 10µL 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 calliper. Studies were performed in triplicate, and mean values were calculated. The methods used to assess the antifungal activity of EO are different and can give different results from one technique to another[22].

**Statistical analyses and cluster analysis**

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. The percentage composition of the essential oil samples was used to determine the relationship between the different populations of *T. leptobotrys* by cluster analysis using the same statistical package software. Euclidean distance was selected as a measure of similarity.

## III. RESULTS AND DISCUSSION

### 3.1. Chemical composition of thyme essential oils

Chemical composition of *Thymus leptobotrys L.* essential oils was determined by the GC-MS analyses. The results are presented in Table II.

**Table II.** The chemical composition of the essential oil of *Thymus leptobotrys L.* endemic of the Argan forest in southwestern Morocco, by region

| Compounds | R.T | KI | S1 | S2 | S3 | S4 | S5 | S6 |
|---|---|---|---|---|---|---|---|---|
| **Monoterpene hydrocarbons** | | | **12.689** | **18.808** | **19.641** | **14.662** | **19.61** | **10.694** |
| Tricyclene | 6.77 | 926 | 0.11 | − | 0.12 | 0.07 | 0.08 | − |
| α-Thujene | 6.97 | 930 | 0.238 | 1.47 | 0.12 | 0.751 | 1.198 | 0.246 |
| **α-Pinene** | 7.35 | 939 | **1.86** | **1.845** | **2.69** | **1.912** | **2.11** | **1.062** |
| Camphene | 7.9 | 954 | 0.191 | 0.307 | 2.7 | 1.304 | 1.204 | 0.226 |
| Thuja 2,4(10)diene | 8.07 | 960 | 0.041 | 0.029 | 0.04 | 0.027 | 0.043 | 0.032 |
| Sabinene | 8.78 | 975 | 0.041 | 0.063 | 0.121 | 0.063 | 0.059 | 0.021 |
| β-Pinene | 8.94 | 978 | 0.199 | 0.417 | 0.27 | 0.325 | 0.411 | 0.113 |
| Myrcene | 9.55 | 990 | 0.964 | 1.892 | 0.99 | 1.216 | 1.47 | 1.42 |
| α-Phellandrene | 10.06 | 1002 | 0.154 | 0.236 | 0.12 | 0.148 | 0.191 | 0.056 |
| δ-3-carene | 10.31 | 1011 | 0.072 | 0.05 | 0.08 | 0.053 | 0.064 | 0.043 |
| α-Terpinene | 10.63 | 1017 | 0.764 | 1.085 | 0.84 | 0.498 | 0.792 | 0.931 |
| **ρ-Cymene** | 11.15 | 1024 | **5.283** | **5.541** | **8.34** | **4.607** | **7.829** | **3.782** |
| Limonene | 11.29 | 1029 | 0.349 | 0.456 | 0.48 | 1.604 | 0.381 | 0.245 |
| Cis-Ocimene | 11.64 | 1037 | 0.016 | 0.01 | 0.02 | 0.021 | 0.014 | 0.012 |
| **□-Terpinene** | 12.65 | 1059 | **2.277** | **5.322** | **2.52** | **1.973** | **3.663** | **2.437** |
| Terpinolene | 13.92 | 1088 | 0.13 | 0.085 | 0.19 | 0.09 | 0.101 | 0.068 |
| **Oxygenated monoterpenes** | | | **76.917** | **72.382** | **75.14** | **77.74** | **70.068** | **76.822** |
| 1,8-Cineole | 11.35 | 1031 | 0.228 | 0.275 | 0.04 | 0.228 | 0.267 | 0.076 |
| Cis-Linalool oxide | 13.19 | 1072 | 0.056 | 0.04 | 0.06 | 0.061 | 0.056 | 0.025 |
| **Linalool** | 14.72 | 1096 | **0.762** | **1.505** | **3.12** | **2.607** | **1.89** | **0.648** |
| Cis-Thujone | 14.8 | 1102 | 0.038 | 0.021 | 0.04 | 0.031 | 0.027 | 0.019 |
| Trans-Pinocarveol | 16.43 | 1135 | 0.234 | 0.07 | 0.1 | 0.094 | 0.1 | 0.088 |
| Camphor | 16.63 | 1146 | 0.156 | 0.02 | - | 0.043 | 0.023 | 0.038 |
| **Borneol** | 18.52 | 1169 | **3.205** | **0.506** | **5.85** | **1.482** | **2.387** | **0.388** |
| Terpinen-4-ol | 18.64 | 1177 | 0.702 | 0.441 | 0.85 | 0.457 | 0.521 | 0.428 |
| α-Terpineol | 19.7 | 1188 | 0.214 | 0.111 | 0.16 | 0.168 | 0.119 | 0.12 |
| Carvacrol methyl ether | 21.36 | 1244 | 0.432 | 0.963 | 0.85 | 0.328 | 0.216 | 0.423 |
| Thymol | 23.55 | 1290 | 0.28 | 0.211 | 0.61 | 0.305 | 2.234 | 0.269 |
| **Carvacrol** | 24.06 | 1299 | **70.543** | **68.192** | **63.38** | **71.865** | **62.16** | **74.269** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Eugenol | 26.86 | 1359 | 0.067 | 0.027 | 0.08 | 0.071 | 0.068 | 0.031 |
| **Sesquiterpene hydrocarbons** | | | **8.701** | **7.472** | **4.1** | **6.217** | **8.631** | **11.106** |
| α-Copaene | 27.06 | 1376 | 0.01 | – | 0.02 | 0.014 | 0.018 | 0.01 |
| β-Bourbonene | 27.44 | 1388 | – | – | 0.01 | 0.01 | – | – |
| α-Gurjunene | 28.49 | 1409 | – | – | 0.01 | 0.011 | 0.01 | – |
| **β-Caryophyllene** | 29.04 | 1419 | **5.447** | **3.892** | **2.46** | **3.721** | **4.887** | **7.059** |
| β-Copaene | 29.29 | 1432 | – | – | 0.02 | – | 0.015 | 0.01 |
| Aromadendrene | 29.68 | 1441 | 1.57 | 0.576 | 0.75 | 1.214 | 0.577 | 1.994 |
| α-Humulene | 30.29 | 1454 | 0.241 | 0.176 | 0.08 | 0.188 | 0.209 | 0.315 |
| allo-aromadendrene | 30.57 | 1460 | 0.4 | 0.253 | 0.27 | 0.347 | 0.31 | 0.505 |
| □-Muurolene | 31.25 | 1479 | 0.036 | 0.031 | 0.04 | 0.038 | 0.027 | 0.03 |
| Bicyclogermacrene | 32.03 | 1500 | 0.185 | 2.072 | - | 0.199 | 1.665 | 0.371 |
| α-Muurolene | 32.2 | 1500 | 0.032 | 0.029 | 0.03 | 0.024 | 0.015 | 0.027 |
| □-Cadinene | 32.76 | 1513 | 0.153 | 0.077 | 0.06 | 0.115 | 0.102 | 0.193 |
| δ-Cadinene | 33.13 | 1523 | 0.627 | 0.366 | 0.35 | 0.336 | 0.796 | 0.592 |
| **Oxygenated sesquiterpenes** | | | **0.701** | **0.406** | **0.54** | **0.509** | **0.75** | **0.383** |
| Spathulenol | 35.21 | 1578 | 0.3 | 0.303 | 0.04 | 0.357 | 0.402 | 0.222 |
| Caryophyllene oxide | 35.5 | 1583 | 0.401 | 0.103 | 0.5 | 0.152 | 0.348 | 0.161 |
| **Total percentage of identified components** | | | **99.008** | **99.068** | **99.421** | **99.128** | **99.059** | **99.005** |

In this study, the essential oils content in the flowering tops and leaves of *Thymus leptobotrys L.*, were obtained from plants harvested in six different regions of Souss-Massa part of the Argan tree of southwestern Morocco (harvest sites S1-S6), as described in Table I.The essential oils of *Thymus leptobotrys L.* collected in six regions had an average yield from 1.51 ± 0.01% to 2.12 ± 0.02 %. The highest essential oil yield was found in S2 (2.12%). As regards the content of essential oils of *Thymus leptobotrys L.*, we notice the variety of yield based on the area of the harvest.Compositions of the six essential oils are also reported in Table 2. A total of 44 compounds were identified, thus either 99.01% to 99.42% of volatile constituents of essential oils coming from *Thymus leptobotrys L.* of different harvest sites, respectively.Essential oils from all origins indicate a chemical composition marked mainly by oxygenated monoterpenes (70.07 at 77.74%), followed by monoterpenes hydrocarbons (10.69-19.64%), followed by sesquiterpenes hydrocarbons (4.10 to 11.11%) and finally by the sequiterpenes oxygenated (0.38 to 0.75%), as seen in table II and Fig.I.

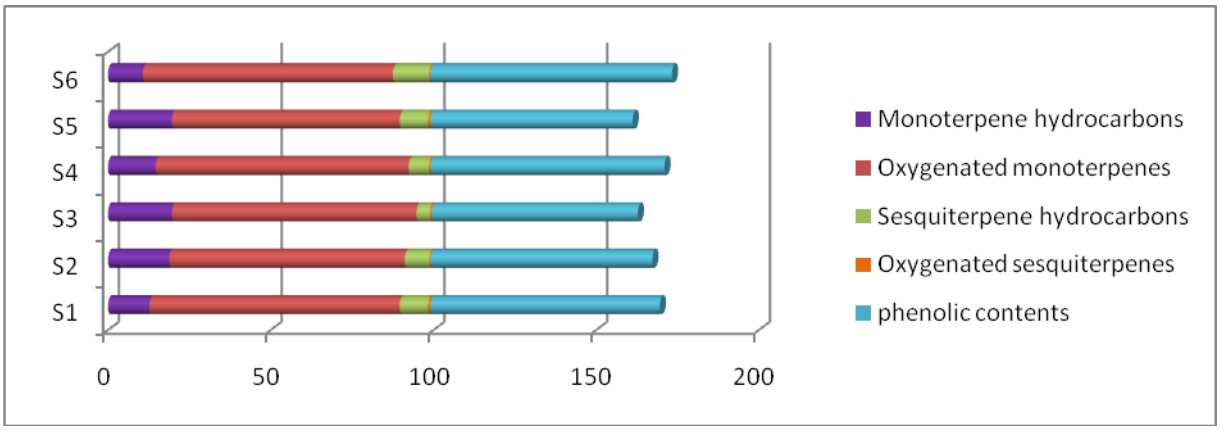


**Figure I.** Distribution of the compound and phenolic contents in T. leptobotrys essential oils of different harvest sites.

The sesquiterpene fraction was rather small (4.48-11.85%), being always dominated by the hydrocarbons compounds (4.10-11.11%). The presence of a low percentage of sesquiterpenes, found in the present study, is,

according Stahl-Biskup [23].In addition, significant differences in the amounts of the major constituents of essential oils and their phenolic content were found within the region of collection samples of *Thymus leptobotrys L.* (Table II and Fig.II).These differences let us suppose the influence of the geographical area, the type of soil, genetic factors in plants and climate on the composition of the essential oil of *Thymus leptobotrys L.*

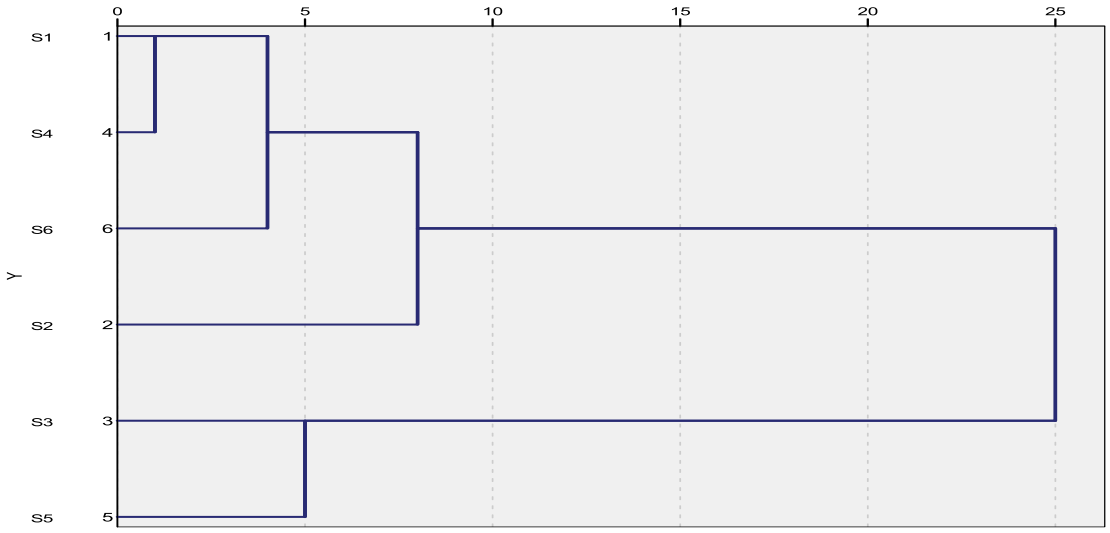


**Figure II.** Dendrogram obtained by cluster analysis of the percentage composition of essential oils from six provenances of T.leptobotrys.

Carvacrol was the major constituents of the essential oil of *T. leptobotrys* in the six regions of the Argan tree studied. Other minor compounds identified were α-Pinene, ρ-Cymene, γ-Terpinene, Linalool, Borneol and β-Caryophyllene (Table II). The present data reveals qualitative and quantitative variation in composition of essential oils of *Thymus leptobotrys L.* However, the GC-MS analyses show the predominance of the monoterpene in all the oils under investigation (Fig.I). The chemical analyses carried out reveal the richness and variability of Moroccan *Thymus* essential oils. Carvacrol was found as a predominant component in *T. leptobotrys.* The high content of carvacrol in this thyme species has also been reported by other authors [24, 25], and carvacrol has been suggested as the main component responsible for the cytotoxic activity of this species [24]. From the aforementioned results it is obvious that this specie under investigation could be regarded as rich natural sources of some important substances such as Carvacrol. It would also be noteworthy to point out that the composition of any plant essential oil studied is influenced by the presence of several factors, such as local climatic seasonal, altitude and experimental conditions [26]; there by altering the biological activities studied [27]. Further, the oils chemical variability attributed mainly to environmental conditions has been previously reported on some other *Thymus* species [28].The cluster analysis was performed with SPSS statistical package software to identify relatively homogeneous groups of six provenances (S1–S6) of *Thymus leptobotrys L.* based on percent composition of their essential oil samples. Euclidean distance was selected as a measure of similarity, was used for cluster definition [29].The differences in the percent composition of the essential oils among six provenances of *T. leptobotrys* were determined by using cluster analysis (Fig. 2). Following the results obtained herein, we have classified the essential oils into five main groups, carvacrol/β-caryophyllene/p-cymene type (S1 and S5), carvacrol/p-cymene/γ-terpinene type (S2), carvacrol/p-cymene/borneol type (S3), carvacrol/p-cymene type (S4) and carvacrol/β-caryophyllene type (S6).The Argan tree populations of *T. leptobotrys* under study show a clear chemical polymorphism. However, regarding their main components, they are more or less homogeneous within Souss-Massa regions.

In essential oil-bearing plants, the yield of oil and its chemical composition vary considerably due to both intrinsic (sexual, seasonal, ontogenetic and genetic variations) and extrinsic (ecological and environmental aspects) factors [23, 30]. Since it was not possible to find any correlation between the chemical composition of the oils and the altitudes of the collection sites, or the longitude and the climate of the Souss-Massa region, the polymorphism recorded in the present study may result either from the genetic variability of the populations or from the influence of edaphic factors.

**Antioxidant Activity**

The antioxidant activity of the *Thymus leptobotrys L.* essential oils was assessed by antioxidant DPPH* assay [31]; i.e. evaluating the H-donating or radical- scavenging ability of the essential oil using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH*) as a reagent. From the absorbance values obtained, we calculated the percentage of DPPH-scavenging using the formula given in material and methods section (antioxidant activity)(i.e,(1)). The results obtained during the test measurement of the percentage of inhibition of DPPH are recorded in Fig.III. It shows that this percentage inhibition increases with increasing concentration for each essential oils or Quercetin (reference antioxidant compound).

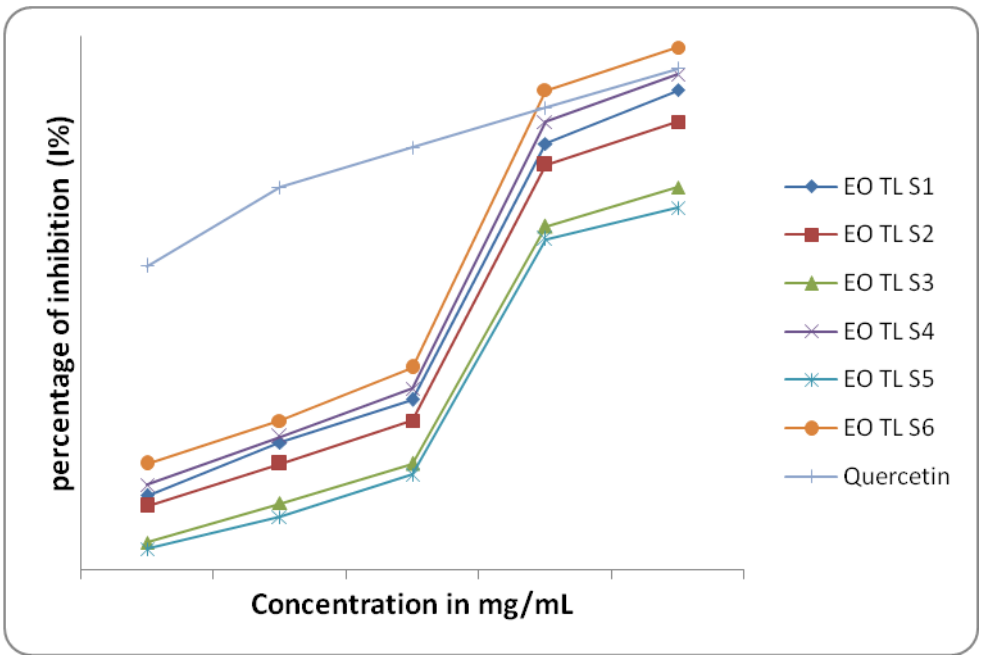


**Figure III.**Inhibition percentage of essential oil of *Thymus leptobotrys L.* collected from several locations and Quercetin.

The percentage inhibition of free radical for essential oils studied is slightly lower than that of Quercetin for all concentrations used, except for the essential oil of *Thymus leptobotrys* collected in the S6 Site (Near Tamanar) for concentrations of 0.0212 and 0.0425 mg /mL for what is higher than that of control. For example, a concentration of 0.0425 mg/mL of *Thymus leptobotrys* essential oil, showed a percentage inhibition of 74% while Quercetin showed a percentage inhibition of 87.04%We determined graphically the concentration corresponding to 50% inhibition of DPPH (IC50), which represents the antioxidant activity of the essential oils studied. The essential oils of *Thymus leptobotrys L.* of each origins gave values of CI50 respectively: S1: 0.015 mg/mL, S2: 0.016mg/mL, S3: 0.017mg/mL, S4: 0.015mg/mL, S5: 0.018mg/mL and S6: 0.014 mg/mL. These values are determined by reference to the value of the reference IC50 (Quercetin), which is 0.0011 mg/mL (Table III). These results show that the essential oil studied arouses the interest for that intéressent antioxidant capacity to use immediately.

**Table III.** DDPH*-Scavenging Activity (IC50DPPH) of the six *Thymus* Essential Oils and the Reference Antioxidants Quercetin:

| **Samples** | *TL EO* $_{S1}$ | *TL EO* $_{S2}$ | *TL EO* $_{S3}$ | *TL EO* $_{S4}$ | *TL EO* $_{S5}$ | *TL EO* $_{S6}$ | Quercetin |
|---|---|---|---|---|---|---|---|
| **$IC_{50DPPH}$** (mg/mL)[a] | **0.015** | **0.016** | **0.017** | **0.015** | **0.018** | **0.014** | **0.0011** |

[a)] Values are means (n=3).

The antioxidant activity detected in *Thymus leptobotrys L.* essential oils of each origins may be due to major compounds, Carvacrol, Borneol, alone or together with other minority compounds that could act synergistically In this assay [32, 33], a very strong positive and linear correlation was observed across the oils between their antioxidant activity and the content of oxygenated monoterpenes [34]. These results are consistent with the close relationship between the carvacrol content and high antioxidant potential reported by many

authors [35, 36]. Furthermore we find that the essential oil carvacrol /β-caryophyllene type (S6) is the one who gave the best antioxidant activity against other essential oils. However, before any hasty conclusion, it is suggested that the antioxidant activity of this essential oil should be also evaluated by other methods and also to evaluate the antioxidant activity of these components separately.It is often very difficult to explain the activity profile of essential oils with a very complexe chemical composition, although the antioxidant activity of some phenolic compounds and other pure compounds are well known [37]. Generally the antioxidant activity of phenols is confirmed [38], especially Thymol and Carvacrol are mainly responsible for the antioxidant potential of essential oils containing them [39], also Terpinolene and γ-Terpinene could also be responsible for antioxidant activity observed, but obviously the Thymol and Carvacrol are the main compounds responsible for this activity, the presence of the methyl group is probably the reason for this behavior. Depending on the chemical composition listed in table2. Indeed, the antioxidant capacity is strongly correlated with the concentration of Carvacrol in essential oils studied which could attribute to this activity.

### 3.3. Anticandidal Activity

#### 3.3.1. Disk diffusion method

The results obtained for the anticandidal disc-diffusion assay are summarized in Table IV. All thirty strains of Candida species tested were inhibited by the *Thymus leptobotrys L.* essential oils collected from several locations in the area of the Argan tree of southwestern of Morocco to a varying degree, with the diameters of the inhibition zone ranging from 24 mm to 80 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.

**Table IV.** Inhibition-Zone Diameters Determined with the Disc-Diffusion Method of the *Thymus leptobotrys L.* Essential Oils from different regions, Fluconazole and Amphotericin B against thirty Candida Species.

| Strains | Inhibition-zone diameter [mm][a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number of strains | TL $EO_{S1}$ 10µL | TL $EO_{S2}$ 10µL | TL $EO_{S3}$ 10µL | TL $EO_{S4}$ 10µL | TL $EO_{S5}$ 10µL | TL $EO_{S6}$ 10µL | AMB 10µL | FLC 10µL |
| *Candida parapsilosis* | *10* | $60^d$ | $62^d$ | $48^c$ | $64^d$ | $50^c$ | $66^b$ | $10^c$ | $21^d$ |
| *Candida tropicalis* | *11* | $80^a$ | $80^a$ | $80^a$ | $80^a$ | $80^a$ | $80^a$ | $8^d$ | $25^c$ |
| *Candida famata* | *5* | $74^b$ | $70^c$ | $24^d$ | $78^b$ | $42^d$ | $80^a$ | $25^a$ | $30^b$ |
| *Candida lusitaniae* | *4* | $70^c$ | $72^b$ | $61^b$ | $75^c$ | $60^b$ | $78^a$ | $12^b$ | $32^a$ |

[a]) Inhibition zone diameter including the disc diameter of 5mm determined by the agar disc-diffusion method at a concentration of 10 µL of essential oils/disc, 10µL of fluconazole/disc and 10µL of Amphotericin B.
Thymus leptobotrys= TL, EO = essential oil.
[a]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 student Newman and Keuls test.

However, the activity against the tested Candida species was highly correlated with its chemical composition. In this study we find that the essential oil carvacrol /β-caryophyllene type (S6) is the one who gave the best anticandidal activity against all Candida species studied compared to essential oils from other regions. It is possible that the wider range in the sensitivity profile shown by the essential oils of T.leptobotrys collected from several locations may be advantageous, because these are widely available and demonstrate a wide action spectrum against pathogenic fungi. Moreover, the human therapeutic response to medicine is not uniform, as suggested by the in vitro assays with Fluconazole and Amphotericin B, which showed poor activity against Candida species studied.The difference in activity between the six essential oils tested can be correlated with the difference in their chemical composition as noted in Table II. [40].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 [41]. The occurrence of these phenols in Thymus essential oils have been of great interest for some time. The essential oils of the T. leptobotrys is rich in oxygenated monoterpenes, especially carvacrol and borneol, it can be used for medicinal purposes and other biological applications. In addition, the *T.leptobotrys* may be a potential carvacrol rich source for commercial cultivation [42] . In fact, a study conducted by Salgueiro et al [43], 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 [43-47]. A study on antimicrobial activity of essential oils of four samples of *Origanum vulgare subsp. virens* recently published [48] 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 [48]. This may explain the strong antifungal activity of the essential oils of *Thymus leptobotrys* which it contained a high content of carvacrol in our study [49]. A study led by Pinto et al [50] 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 [51-53]. 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 [53, 54]. 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 were associated with high carvacrol and thymol content [50].

It has been reported that carvacrol causes perturbations in the bacterial membrane and thus potentially can exert antibacterial activity also at intracellular sites [47, 55]. Furthermore, other Thyme essential oils rich in carvacrol have demonstrated potent antimicrobial activities in vitro [56, 57]. Among the major constituents of the investigated essential oils, γ-terpinene, p-cymene and α-pinene were reported to have weaker antibacterial activities [52, 58]. Synergy effect between carvacrol and its biogenetic precursor p-cymene has been noted [47]. 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, that's probably why all chemotypes studied showed marked activity in vitro on all species of *Candida*.

## IV. CONCLUSION

According to GC–MS and cluster analyses the thyme essential oils of the six provenances and their relative contents could be classified in five chemotypes: Carvacrol/β-caryophyllene/p-cymene type (S1 and S5), carvacrol/p-cymene/γ-terpinene type (S2), carvacrol/p-cymene/borneol type (S3),carvacrol/p-cymene type (S4) and carvacrol/β-caryophyllene type (S6). The polymorphism recorded in the present study may result either from the genetic variability of the populations or from the influence of edaphic factors. In addition, this study demonstrates that all essential oils studied have excellent antifungal and antioxidant activities. Despite the fact that in vitro studies cannot be directly extrapolated to in vivo effects, the results suggests that the use of essential oils such as thyme are renewable natural products and may be further explored as a potential source for the development of new antifungal against *Candida* species could be a viable alternative, alone or combined with antifungal agents, for therapeutic and/or preventive purposes against candidiasis.

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