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Chemical composition and fungicidal effects of four chemotypes of *Thymus satureioides* Cosson essential oils originated from South-west of Morocco

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Abstract

Results of chemical composition analysis by CG-MS of essential oils of *Thymus satureioides* Cosson extracted by hydrodistillation from leaves samples collected in four locations of Taroudante region (Southwest Morocco), identified fifty components representing over than 98%. The major constituent was borneol with proportions ranging between 33.03 and 41.80%. Essential oils yields varied from 1.35 to 2.32% and were positively correlated with an altitude gradient. Four chemotypes were then distinguished according to the collection site. The antifungal activity of essential oil of this thyme against four wood-decaying fungi, assessed by direct contact on agar medium, showed that 1/500 concentration (v/v) of all EOs was sufficient to inhibit the growth of all tested fungal strains. These results suggest that this oil can be used as wood preservative against wood-destroying fungi.

1. Introduction

The genus *Thymus* belongs to the Lamiaceae family. In Morocco, endemism of this genus reach 46 % and it is represented by seven species [1]. Morocco exports over than 700 tons of thyme products, mainly in the form of dried leaves, which generate more than 8 million Dirhams [2]. Thyme has a wide range of biological and pharmacological properties, including antifungal, antioxidant and antimicrobial activities [3, 4]. One of these endemic species, *Thymus satureioides* Cosson constitutes a very important population in Southwest (SW) Morocco [5]. Wood has many qualities and uses (construction, furniture, …) but, because of its perishable nature, it remains subjected to many environmental factors and biodeterioration agents, mainly boring insects and decaying fungi that can cause significant damage to woods [6]. The common wood-decaying fungi, *Coniophora puteana*, *Gloeophyllum trabeum*, *Oligoporus placenta* and *Trametes versicolor*, cause important damages to woods and should always be included in laboratory tests to assess natural durability of woods and effectiveness of wood preservatives [7]. To limit this biological deterioration of wood, preventive or curative treatments with fungicides substances are often applied. However, most of the chemicals used are unfortunately harmful to human health and the environment [8]. Research for natural less toxic molecules such as essential oils, was then undertaken [9-11].

Essential oils (EOs) of *T. satureioides* were subjected to several tests that have proved their antioxidant, antibacterial and anti-inflammatory activities [4, 12, 13]. Antifungal activity of EOs of five other thyme species was recently successfully experimented as wood preservation agents [14]. However, evaluation of bioactivity against wood-destroying fungi of *T. satureioides* essential oil has not been experimented yet.

The aim of the present work is to study the chemical composition of EOs of four populations of *T. satureioides* growing at different altitudes of SW Morocco, and then to evaluate the antifungal activity of these oils against four wood-decaying fungi species.

2. Experimental details

2.1. *Plant material*

Ten samples of the aerial parts of *T. satureioides* were collected, at full flowering phase during April 2014, from each collection site in Southwest Morocco (Figure 1). Four populations were then prospected following an altitudinal gradient at these locations: Timoulay Aksri (altitude: 850 m), Aoulouz (altitude: 1020 m), Amskroud-East (altitude: 1050 m) and Oulad Berhil (altitude: 1240 m).

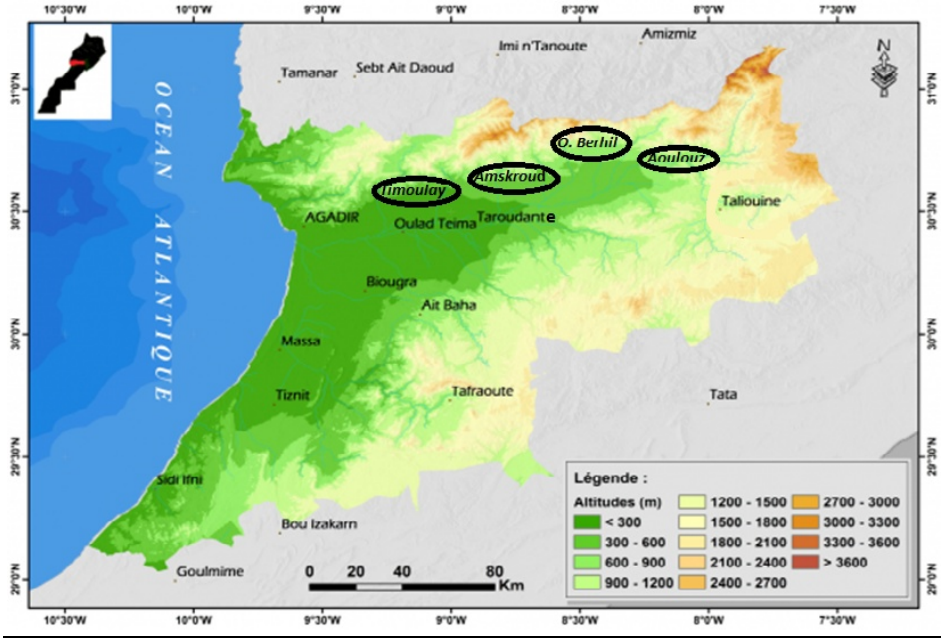

Figure 1: Studied populations of *Thymus satureioides* originated from Southwest Morocco

2.2 *Fungal strains*

Three brown-rot fungi strains used in this study were *Gloeophyllum trabeum* BAM Ebw.109, *Oligoporus placenta* FPRL. 280, and *Coniophora puteana* BAM Ebw. 15, in addition to one white-rot fungus, *Trametes versicolor* CTB 863 A. Fungi strains were maintained in the mycological collection of the Laboratory of Botany, Mycology and Environment, Faculty of Sciences, Rabat, Morocco. They were chosen for the significant damages that they cause to wood and wood-based products.

2.3 *Essential oil extraction*

The extraction of EOs was carried out by steam distillation in a Clevenger-type apparatus [14]. Three extraction assays, from each location sample were carried out by boiling for 2 h, 200 g of fresh leaf material into a one-liter flask containing water. The yield of the obtained essential oil was expressed in mL/100 g calculated on the basis of dry matter. Biomass humidity was measured from three samples of 30 g dried for 72 hours in an oven at 60 °C. The essential oil obtained was stored in a small dark glass bottle at 4 °C until use.

2.4. *Chemical composition of T. satureioides essential oils analysis*

Chemical analysis and components identification were performed for the first time by an electronically controlled pressure gas chromatograph (GC). The GC is a Hewlett-Packard (6890) system, equipped with a capillary column HP-5 (30 m x 0.25 mm x 0.25 µm film thickness) and a FID detector maintained at 250°C. The carrier gas is N_2 with 1.5 mL/min. The column temperature was programmed from 50 to 250 °C by step of 4 °C/min. 1µL of essential oil diluted in *n*-hexane was injected in split-splitless injector heated at 250 °C. A standard solution of *n*-alkanes (C_8-C_{26}) was also used to obtain the retention indices.

The GC-MS analysis were performed on a Hewlett-Packard (HP 6890) coupled with a Mass Spectrometer (HP 5973). Fragmentation is done by electron impact at 70 eV. The column is a capillary HP-5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column temperature is programmed from 50 to 200 °C for 10 minutes by step of 4 °C/min. The carrier gas was helium with a flow rate set at 1.5 mL/min. The device is connected to a computer system managing a mass spectrum library NIST 98. Compounds identification was achieved on the basis of retention indices (RI) and by comparison of their mass spectra (MS) with those reported in the literature especially in the Adams Registry of Mass Spectral Data [15].

2.5. *Antifungal activity of essential oils of* T. satureioides

The antifungal activity of *T. satureioides* EOs was determined by direct contact on agar medium according to the method reported by Remmal *et al*. (1993) [16]. In order to give the oil a homogeneous distribution in the medium, it was first emulsified in a sterile solution of water-agar at 0.2% (SA). To test tubes containing 13.5 mL of malt-agar medium (20 g/L malt extract and 15 g/L agar), sterilized in an autoclave and kept at 45°C in a water bath, were added aseptically 1.5 mL of different dilutions prepared so as to obtain final dilutions of EOs in the culture medium of respectively 1/100, 1/250, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/1750 v/v. The tubes were shaken vigorously and poured into Petri dishes. Similarly, control plates containing 13.5 mL of culture medium and SA solution alone were prepared. Petri dishes were inoculated by depositing two square fragments of 0.5 cm^2, taken from the margins of 10-day-old cultures on malt-agar medium. Three replicates for each treatment and fungus were prepared and incubated in the dark for 7 days at temperature of 22 °C. This biotest allows us also to determine the minimum inhibitory concentration (MIC) of EOs for each tested fungus. The MIC is defined as the lowest concentration for which no visual growth of the fungus was observed [17].

3. Results and discussion

3.1. Chemical composition of T. satureioides *essential oils*

Leaves of *T. satureioides* originated from SW Morocco gave yields of EOs varying between 1.35 and 2.32 % depending on the thyme population (Table 1). Oulad Berhil population gave the best yield while that of Timoulay Aksri yielded the lowest amount of EOs.

Table 1. Essential oils yields of *T. satureioides* and identified chemotypes

Collection location	Altitude (m)	Yield %	Chemotype
Oulad Berhil	1240	2.32	Borneol/α-Terpineol (B/Te)
Amskroud-East	1050	2.16	Borneol/Carvacrol/α-Terpineol (B/Ca/Te)
Aoulouz	1020	2.02	Borneol/Camphene/Thymol (B/C/T)
Timoulay Aksri	850	1.35	Borneol/α-Terpineol/Camphene (B/Te/C)

EOs yields obtained in this study are higher than those reported in previous works [4, 18]. They were positively correlated with an altitudinal range, in agreement with that reported by Zenasni *et al.* [19].

The chemical composition of EOs of *T. satureioides* is very different from those of other Moroccan thyme species. In fact, *T. algeriensis* EOs are dominated by camphor (27.70 %) [20]; those of *T. maroccanus* and *T. capitatus* are dominated by carvacrol, 72.61 % and 70.92 % respectively; those of *T. munbyanus*, *T. ciliatus*, *T. vulgaris* and *T. zygis* are dominated by thymol which contents reach 70.42 %, 44.20 %, 41.39 % and 33.32 % respectively [21, 22].

About fifty constituents were identified in EOs of *T. satureioides* leaves originated from SW Morocco, including thirteen dominant components, with borneol as major compound (33.03 to 41.80 %). Other compounds having contents of less than 5 %, were also found in these EOs, such as (E)-caryophyllene (3.30 to 4.36 %), terpinen-4-ol (2.04 to 2.94 %) and linalool (1.71 to 3.10 %) (Table 2). This composition is dominated by oxygenated monoterpenes group, which reached amounts of 62 to 77 % depending on thyme population (Table 2). This chemical identification allowed us to distinguish four following chemotypes: borneol/α-terpineol (B/Te), borneol/camphene/thymol (B/C/T), borneol/carvacrol/α-terpineol (B/Ca/Te) and borneol/α-terpineol/camphene (B/Te/C) (Table 1).

Qualitative and quantitative analysis of *T. satureioides* EOs showed great chemical variability and several chemotypes were distinguished for this thyme species in Morocco Atlas Mountains. In addition of the four chemotypes described in this study, six other chemotypes were already reported by previous works [19, 23, 24]. According to Jaafari *et al.* (2007) [24], two chemotypes were distinguished in Asni-My Brahim region (Marrakech region), which are borneol/thymol and borneol/carvacrol. According to the same study, particular chemotype, dominated by borneol (60%), was identified at the Tiznit region (Anti Atlas). In addition, two other chemotypes were identified in Agoundis valley (Central High Atlas), which are borneol/camphene/carvacrol and borneol/camphene/α-pinene [19]. The last chemotype, borneol/γ-terpinene/carvacrol, was identified in 2013 by Höferl *et al*. in Idni region (Central High Atlas).

The differences observed in chemical composition of *T. satureioides* chemotypes can be attributed to exogenous factors such as altitude where the plant grows, temperature, nature and soil composition, or even to endogenous factors such as genetic characteristics [19, 25-27].

Table 2. Chemical composition of EOs of *T. satureioides* populations

Compound	*IR*[a]	*Content in %*[b]			
		Aoulouz	*Timoulay Aksri*	*Amskroud-East*	*Oulad Berhil*
Tricyclene	926	0.54	0.33	0.18	0.18
α-Thujene	931	0.62	0.17	0.15	0.21
α-Pinene	938	6.65	4.24	2.43	2.58
Camphene	**952**	**11.67**	**8.78**	**5.10**	**5.13**
β-Pinene	980	1.67	1.03	0.70	0.93
Myrcene	995	0.78	0.25	0.29	0.38
α-Phellandrene	1008	0.04	0.02	0.02	0.03
δ-3-Carene	1013	0.04	0.02	0.02	0.02
α-Terpinene	1019	0.32	0.15	0.17	0.20
p-Cymene	1026	5.05	2.64	1.79	3.77
β-Phellandrene	1030	1.02	0.96	0.63	1.01
1,8-Cineole	1034	0.04	0.03	0.02	0.03
(Z)-βOcimene	1039	-	0.01	0.01	0.01
γ-Terpinene	1059	0.81	0.59	0.74	1.47
cis-Sabinene hydrate	1069	0.03	-	0.04	0.02
Terpinolene	1087	0.19	0.02	0.14	0.22
Linalool	1099	3.10	1.71	2.48	3.01
cis-Thujone	1104	0.06	0.08	0.10	0.08
Terpin-1-ol	1133	-	0.02	0.01	0.02
trans-Sabinol	1137	0.17	0.24	0.20	0.23
Camphor	1143	0.78	0.42	0.22	0.33
Isoborneol	1157	0.07	0.05	0.05	0.03
Borneol	**1166**	**33.03**	**41.80**	**37.74**	**40.11**
Terpinen-4-ol	1175	2.22	2.33	2.04	2.94
α-Terpineol	**1189**	**5.12**	**14.14**	**15.54**	**15.74**
cis-Dihydrocarvone	1194	0.49	0.51	0.40	0.47
trans-Dihydrocarvone	1201	0.11	0.07	0.07	0.08
Isobornyl formate	1224	0.46	0.82	0.63	0.79
Thymol methyl ether	1239	5.53	0.50	0.72	0.12
Bornyl acetate	1283	1.14	2.33	1.12	1.93
Thymol	**1289**	**8.64**	**1.08**	**0.35**	**3.38**
Carvacrol	**1297**	**1.05**	**3.24**	**15.23**	**4.17**
Thymol acetate	1350	-	0.07	0.05	0.03
α-Copaene	1373	0.22	0.08	0.08	0.14
(*Z*)-Caryophyllene	1404	0.06	0.06	0.05	0.09
(*E*)-Caryophyllene	1418	3.30	3.97	4.03	4.36
Aromadendrene	1438	0.45	0.27	0.20	0.25
α-Humulene	1453	0.17	0.25	0.28	0.27
Alloaromadendrene	1463	0.09	0.16	0.13	0.15
γ-Murolene	1479	0.07	0.02	0.02	0.12
α-Murolene	1499	0.08	0.04	0.04	0.18
γ-Cadinene	1514	0.34	0.32	0.31	0.30
δ-Cadinene	1525	0.49	0.28	0.24	0.33
α-Cadinene	1536	0.05	0.03	0.03	0.04
Caryophyllene oxide	1581	0.60	1.12	1.18	0.90
β-Oplopenone	1605	0.04	0.10	0.10	0.11
Murollol	1638	0.33	0.76	0.56	0.38
α-Cadinol	1654	0.08	0.21	0.27	0.10
Hydrocarbonated monoterpens		29.4	29.4	19.21	12.37
Oxygenated monoterpens		62.04	62.04	69.44	77.01
Hydrocarbonated sesqueterpens		5.32	5.32	5.48	5.41
Oxygenated sesqueterpens		1.05	1.05	2.19	2.11
Total		**98.01**	**98.01**	**96.57**	**97.57**
Number of compounds		**45**	**47**	**48**	**48**

[a] Retention Indice measured using capillary column HP-5
[b] Ratio of each peak area to the total area of the GC chromatogram (in %)

3.2 Antifungal activity of T. satureioides *essential oils against wood-decaying fungi*

According to the biotest conducted with *T. satureioides* EOs, a significant inhibitory activity on the four wood-decaying fungi was obtained. Therefore, 1/500 concentration of all EOs was sufficient to inhibit the growth of all tested fungal strains. Thus, *G. trabeum*, was the most sensitive fungus since it was inhibited by the minimum concentration of 1/1500 v/v of (B/C/T) chemotype. Concentration of 1/1250 of (B/C/T) and (B/Ca/Te) chemotypes was sufficient to stop the growth of *C. puteana*, while *T. versicolor* and *O. placenta* fungal strains were inhibited by only concentrations more than 1/500 of all EOs (Table 3).

Table 3. Minimal inhibitory concentrations (v/v) of the four chemotypes of *T. satureioides*

Fungal strains	Chemotypes			
	B/C/T	B/Ca/Te	B/Te	B/Te/C
G. trabeum	1/1500	1/1250	1/750	1/500
C. puteana	1/1250	1/1250	1/750	1/500
T. versicolor	1/500	1/500	1/500	1/500
O. placenta	1/500	1/500	1/500	1/500

B, Borneol; C, Carvacrol; Ca, Camphene; T, Thymol; Te, α-Terpineol

Antifungal activity of EOs of other thyme species was tested against the same wood-destroying fungi, like those of *T. bleicherianus*, which inhibited the growth of three fungi: *G. trabeum*, *C. puteana* and *O. placenta* at concentration of 1/3000 v/v, while growth of *T. versicolor* was inhibited by concentration up to 1/2000 v/v [14]. Other studies conducted by our team showed that these wood-decaying fungi are sensitive to the inhibitory activity of EOs of *Cedrus atlantica* [28].

In this investigation, EOs from Aoulouz population showed the higher antifungal activity. It is followed by those of Amskroud-East, Oulad Berhil, then Timoulay-Aksri, in relation probably with their contents of phenols. Therefore, EOs extracted from leaves of *T. satureioides* originated from Aoulouz and Amskroud-East contained high levels of thymol (8.64 %) and carvacrol (15.23 %) respectively, which conferred the species significant bioactivity. These two phenolic compounds are known for their antimicrobial properties [14, 29]. Among various pure EOs compounds tested on some wood-destroying fungi, thymol and carvacrol were the most active compounds against these fungi [30]. In addition, among a large number of pure EOs components tested against different kinds of bacteria, thymol showed the widest antibacterial activity spectrum followed by carvacrol and α-terpineol [31]. Furthermore, difference in effectiveness of antimicrobial activities of thymol and carvacrol was then explained by hydroxyl group position on their phenolic ring. Effectively, EOs of *T. bleicherianus*, *T. ciliatus*, and *T. zygis* showed fungicidal activity slightly higher than that of *T. capitatus*, probably in relation with their richness in thymol, while *T. capitatus* is dominated by carvacrol [14, 20]. Action of phenolic compounds on fungi is primarily based on the inhibition of fungal enzymes containing SH group in their active site [32, 33]. The synergistic effect between different compounds of EOs could also be involved in the observed antifungal activity [34].

Conclusions

Leaves of *T. satureioides* originated from SW Morocco gave yields of EOs varying between 1.35 and 2.32 %. Chemical analysis by GC-MS of these oils has identified fifty constituents dominated by borneol. Four chemotypes are thus distinguished, which are borneol/α-terpineol/camphene (Timoulay Aksri population), borneol/camphene/thymol (Aoulouz population), borneol/carvacrol/α-terpineol (Amskroud-East population), and borneol/α-terpineol (Oulad Berhil population).

All identified chemotypes were active against wood-destroying fungi tested, but that of Aoulouz location showed the greatest antifungal activity probably in relation with thymol richness, followed by that of Amskroud-east, which is rich in carvacrol. Environmental factors, such as altitude or temperature certainly influence the chemical composition of these oils and therefore their antifungal activity.

Consequently, the richness of *T. satureioides* EOs in phenolic terpenes gives it a high antifungal activity. This suggests the use of this oil for several uses, as wood preservative against wood-decaying fungi, as biopesticide agent against post harvest insects, and as bioacaricide against the parasite of bees, *Varroa destructor*.

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