

Chemical composition and Antioxidant Activities of *Thymus pallidus* Essential Oils

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Abstract

The analysis of essential oil of *Thymus pallidus* (*T. pallidus* oil) by GC led to the identification of 36 components accounting for (98.4%) of the total oil. The main compounds of essential oils of *T. pallidus* were thymol (44.5%), p-cymene (18.5%), γ -terpinene (14.7%), linalol (3.7%), carvacrol (3.5%), borneol (3%) and trans caryophyllene (1.9%). The antioxidant activities of the essential oil of *T. pallidus* were evaluated by using three methods: free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity, the Ferric Reducing Power (FRAP), and β -Carotene. The antioxidant assays revealed that the essential oil tested from *T. pallidus* showed strong activities. On the basis of these results, it can be concluded that this plant species represents prominent new herbal raw material that can be used in food, pharmaceutical and cosmetic industries.

Keywords

Essential oil; *Thymus pallidus*; Antioxidant activities; GC/MS.

1. Introduction

The thyme is an aromatic plant of the Mediterranean flora, commonly used as spices and as traditional medicine remedies. Close to 100 species have been identified in the kind of *Thymus* across the world [1]. They are reported to possess some biological effects such as antispasmodic [2], antibacterial [3-4], antifungal [5], anti-tabagism [6], giardicidal [7] and antioxidant activities [8]. In Morocco, the thyme is represented by many species of which certain are endemic. The flowered stem contains essentially flavonoids (derived of apigenol and luteolol), acids phenols (in particular, caffeic and rosmarinic), tannins, resin and especially essential oil rich in chemical compounds which are responsible for the majority of its pharmacological effects [9]. The thyme has been used, in Moroccan traditional

medicine, in the treatment of diarrhoea, fever, cough, infected areas and wounds. It was also used as a tonic and stimulant [10-11] and, generally, for its anti-inflammatory properties after topical or oral administration [12-13-14]. Considering the popular use of this plant to relieve some pains, we focused in this report on investigating the chemical composition and the antioxidant Activities of the essential oils, as well as various extracts of different thyme species.

Essential oils (EOs) are subtle, aromatic, and volatile liquids extracted from various plant parts as secondary metabolites [15]. Due to their antioxidant and antimicrobial effects, EOs has a large potential as food preservatives reducing the oxidative reactions and microbial contaminations during food handling, processing and storage [16].

The aim of this study was to examine the bioactivity of the essential oils of *T. pallidus* in relation to their constituents analysed by gas chromatography and gas chromatography-mass spectrometry.

2. Materials and methods

2.1. Plant material and preparation of essential oils

The aerial parts of *T. pallidus*, were collected during the full flowering period of the plants (May 2016) in Amizmiz of Morocco. Voucher specimen was deposited in the herbarium of Faculty of Sciences of Marrakech (Morocco). The plant materials were dried at ambient temperature for essential oil isolation. The sampled plants were treated separately for extracting essential oils. 100g of dried vegetal material were water-distillated (3h) using a Clevenger-type apparatus [17]. The extracted essential oils were dried over anhydrous sodium sulfate, filtered and stored at -4°C until analysis.

2.2. GC analysis (GC-FID analysis)

Analysis was carried out using a Perkin-Elmer Autosystem XL GC apparatus equipped with dual flame ionization detection (FID) system and fused-silica capillary columns (60m x 0.22 mm I.D., film thickness 0.25µm), Rtx-1(polydimethylsiloxane) and Rtx-wax (polyethyleneglycol). The oven temperature was programmed from 60°C to 230°C at 2°C/min and then held isothermally at 230°C for 35min. Injector and detector temperature was maintained at 280°C. Samples were injected in the split mode (1/50), using helium as carrier gas (1mL/min); the injection volume was 0.2µL of pure oil. Retention indices (RI) of compounds were determined relative to the retention times of series of n-alkanes (C5-C30) with linear interpolation, using the Van den Dool and Kratz et al. [18] equation and software from Perkin-Elmer. Component relative concentrations were calculated based on GC peak areas without using correction factors.

2.3. GC-MS analysis

Samples were also analysed using a Perkin-Elmer Turbo mass detector (quadrupole), coupled to a Perkin-Elmer 88 Autosystem XL, equipped with fused-silica capillary columns Rtx-1 and Rtx-Wax. Carrier gas: helium (1mL/min), ion source temperature: 150°C, oven temperature programmed from 60°C to 230°C at 2°C/min and then held isothermally at

230°C (35 min), injector temperature: 280°C, energy ionization: 70 eV, electron ionization mass spectra were acquired over the mass range 35-350 uma, split: 1/80, injection volume: 0.2µL of pure oil.

3. Antioxidant activities

3.1. DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) test was carried out as described before [19]. Different concentrations of the sample were prepared in pure methanol, after that, 50µL of various dilutions of the essential oil and its main active compounds were mixed with 5mL of a 0.4 mmol/L (v/v) DPPH solution. After a 30 min incubation in the dark, the absorbance of the mixture was determined at 517nm using Uviling 9400 (SECOMAM) spectrophotometer. Butylated hydroxytoluene (BHT) and ascorbic acid were used as positive controls. The ability to scavenge the DPPH radical was calculated using the following formula (1):

$$\text{DPPH Scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where A_0 is the absorbance of the control after 30 min, and A_1 is the absorbance of the sample after 30 min. Tests were carried out in triplicate.

3.2. Reducing power determination (FRAP)

Reductive ability was investigated by the Fe^{3+} to Fe^{2+} transformation in the presence of the oils, using the method of Oyaizu (1986) [20]. Samples (EOs and control substances) were serially diluted with pure ethanol to produce a test range of 200µg/mL to 2000µg/mL for EOs, and from 5µg/mL to 100µg/mL for control substances. The different sample concentrations were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (2.5mL, 1%). The mixture was then incubated at 50 °C for 20 min. 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. Finally, the upper layer of solution (2.5mL) was mixed with distilled water (2.5 mL) and FeCl_3 (0.5mL, 0.1%), and the UV absorbance was measured at 700nm in a spectrophotometer.

The oil concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance at 700nm against the corresponding oil concentration. BHT and Ascorbic Acid were used as reference compounds. The test was carried out in triplicate, and IC_{50} values were reported as means \pm SD.

3.3. -Carotene bleaching test

The -carotene bleaching method is based on the loss of the yellow color of -carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of -carotene bleaching can be slowed down in the presence of antioxidants [21]. A modification of the method described by Koleva et al. [22] was employed. -carotene (2 mg) was dissolved in 10 mL of chloroform and linoleic acid (20 mg) and Tween 40 (200 mg) were added to 1mL of this solution. Chloroform was evaporated under vacuum at 40 °C and 100

mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. Reference compound (BHT) and ascorbic acid, samples (EOs and control substances) used in pure ethanol. The emulsion (5mL) was added to a tube containing 0.2mL of different concentrations of essential oils (200, 400, 600, 800 and 1200µg/mL). The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 °C for 120min, when the absorbance was measured again. BHT and ascorbic acid were used as the positive control. In the negative control, the extract was substituted by an equal volume of pure ethanol. The antioxidant activity (%) of the *Thymus pallidus* essential oil was evaluated in terms of the bleaching of the α -carotene using the following formula (2):

$$I \% = (A_{\alpha\text{-carotene after 2 h assay}} / (A_{\text{initial } \alpha\text{-carotene}} - A_{\alpha\text{-carotene after 2 h assay}})) \times 100 \quad (2)$$

Where $A_{\alpha\text{-carotene after 2 h assay}}$ is the absorbance values of α -carotene after 2h assay remaining in the samples, and $A_{\text{initial } \alpha\text{-carotene}}$ is the absorbance value of α -carotene at the beginning of the experiment. All tests were carried out in triplicate, and oil concentration providing 50% inhibition (IC_{50}) was obtained plotting inhibition percentage versus oil solutions concentrations.

3.4. Statistical analysis

The data was statistically analyzed by SPSS (20.0) software, using a completely randomized design. Means of the traits were compared by Duncan's multiple range test at $p < 0.05$ level. Analytical data for hierarchical cluster analysis were treated by means of the SPSS statistical software.

4. Results and Discussion

4.1. Chemical composition of essential oil

The average yield of *Thymus pallidus* essential oil was calculated based on the dry plant material obtained from the aerial parts of the plant (Table 1). The qualitative and quantitative analytical results were shown in Table 1. Oxygenated monoterpenes were the major constituents (57.5%) followed by hydrocarbon monoterpenes (37.6%) which were the main groups of constituents in *Thymus pallidus* oil.

The average yield of essential oils extracted from the *Thymus pallidus* (TP oil) collected in Amizmiz was 2.2%, it was important than that obtained from *Thymus pallidus* from Ourika valley which is 1.60% [23].

The chemical composition of the essential oil isolated from *Thymus pallidus* (TP oil) shows 36 identified compounds, representing (98.4%) of the total oil. Thymol is the main component (44.7%) in the essential oils followed by p-cymene (18.3%), α -terpinene (14.7%). Besides other components with relatively low concentrations were identified, especially linalool (3.7%), carvacrol (3.5%), borneol (3%) and trans caryophyllene (1.9%). In order to compare our obtained results with those of Boufekran (Meknes), three main components of TP oil were identified: α -terpinene (42.21%), thymol (23.95%) and p-cymene (8.3%) [24].

Qualitative and quantitative variations encountered in the chemical composition of essential oils of the aerial parts of *Thymus pallidus* may be due to the used part of plant [25], the vegetative stage [26-27] or even to genetic factors [28].

4.2. Antioxidant activities

The antioxidant activity ascribe to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts [29]. Thus it is important that for evaluating the effectiveness of antioxidants, several analytical methods and different substrates are used. The methods chosen are the most commonly used for the determination of antioxidant activities of essential oils.

The essential oils of *Thymus pallidus* were subjected to screening for their possible antioxidant activity by three test systems, namely DPPH free radical scavenging, the ferric ion reduction assay and β -Carotene (Table 2).

The DPPH assay showed an IC_{50} of $1245.23 \pm 3.75 \mu\text{g/mL}$, which was less potent than the pure compounds used as positive controls, namely the synthetic antioxidant BHT ($IC_{50} = 534.96 \pm 3.78 \mu\text{g/mL}$) and the ascorbic acid ($IC_{50} = 9.45 \pm 0.85 \mu\text{g/mL}$). Similarly, the ferric reductive capacity resided with the oils obtained from the *Thymus pallidus* ($IC_{50} = 556.45 \pm 2.24 \mu\text{g/mL}$) compared with ascorbic acid ($IC_{50} = 75.80 \pm 2.12 \mu\text{g/mL}$) and BHT ($IC_{50} = 143.56 \pm 1.75 \mu\text{g/mL}$) (Table 2).

Table 1. Chemical composition of essential oil from *Thymus pallidus* aerial parts

N ^a	Components	^b Ir j	^c Ir a	^d Ir p	TP ^e
1	α -Thujene	932	923	1026	0.5
2	α -Pinene	936	931	1026	0.5
3	Camphene	950	944	1072	1
4	Oct-1-en-3-ol	963	961	1448	0.4
5	Octan-3-one	964	966	1254	0.2
6	β -Pinene	978	971	1114	0.1
7	Octan-3-ol	981	981	1391	0.1
8	Myrcene	987	981	1163	1
9	α -Phellandrene	1002	1001	1168	0.1
10	α -Terpinene	1013	1011	1184	1.4
11	p-Cymene	1015	1013	1275	18.3
12	Limonene	1025	1020	1204	0.3
13	β -Phellandrene	1023	1020	1216	0.1
14	γ -Terpinene	1051	1049	1245	14.7
15	E-Hydrate sabinene	1053	1055	1462	0.2
16	Nonen-3-ol	1058	1065	1522	0.1
17	Z Linalol oxyde THF	1072	1074	1441	0.1
18	p-Cymenene	1075	1076	1436	0.1
19	Terpinolene	1082	1082	1286	0.1
20	Linalol	1086	1086	1547	3.7
21	Camphre	1123	1121	1506	0.2

22	Borneol	1150	1152	1700	3
23	Terpinen-4-ol	1164	1164	1600	0.7
24	α -Terpineol	1176	1174	1694	0.1
25	Carvacrol methylether	1226	1225	1603	0.1
26	Linalylacetate	1239	1242	1557	-
27	Thymol	1267	1278	2180	44.5
28	Carvacrol	1278	1284	2207	3.5
29	α -Terpinylacetate	1335	1336	1695	-
30	Geranylacetate	1362	1363	1756	
31	Trans caryophyllene	1421	1416	1600	1.9
32	Aromadendrene	1443	1440	1609	-
33	Ledene	1491	1494	1700	-
34	γ -Cadinene	1507	1508	1754	0.2
35	Calamenene	1517	1511	1830	0.1
36	δ -Cadinene	1520	1517	1754	0.1
37	E-Nerolidol	1553	1552	2041	0.1
38	Spathulenol	1572	1565	2120	0.1
39	Caryophyllene oxyde	1578	1570	1980	0.8
Oxygenated monoterpenes					57.5
Hydrocarbon monoterpenes					37.6
Oxygenated sesquiterpenes					0.9
Hydrocarbon sesquiterpenes					2.4
Total identified (%)					98.4
Oil yield (%)					2.2

Notes: ^aOrder of elution are given on apolar column (Rtx-1); ^bIr j = retention indices on the Joulain; ^cIr a = retention indices on the apolar column (Rtx-1); dIr p = retention indices on the polar column (Rtx-Wax); ^eRelative percentages of components (%) are calculated on GC peak areas on the apolar column (Rtx-1) except for components with identical RI a (concentration are given on the polar column). - : not detected.

Table 2. Antioxidative capacities (IC₅₀ values) of the aerial parts of *Thymus pallidus* essential oils, Ascorbic acid and BHT.

	DPPH (IC ₅₀ μg/mL)	Reducing power (IC ₅₀ μg/mL)	-Carotene bleaching test
<i>Thymus pallidus</i> oil	1245.23±3.75	556.45 ±2.24	461.75±3.86
Ascorbic Acid	9.45 ± 0.85	75.80 ± 2.12	60.45. ± 1.12
BHT	534.96 ± 3.78	143.56±1.75	86.48±2.15

For the -Carotene assay, the IC₅₀ of 461.75±3.86 μg/ mL, was less potent than the pure compounds used as positive controls, namely the synthetic antioxidant BHT (IC₅₀= 86.48±2.15μg/mL) and the ascorbic acid (IC₅₀= 60.45 ± 1.12μg/mL).

The *in vitro* antioxidant activity of the essential oils of several *Thymus* species has been reported previously [8], and this activity has been mainly attributed to their content of phenolic components, especially thymol and carvacrol [8], and the strong radical scavenging activity of these compounds was well established [30-31]. The plants were classified into one major chemotype including thymol/p-cymene, is also characterized by strong antioxidant activity which may be higher than that of phenols. The presence of strongly activated phenolic compounds in this oil is probably the reason for this property [8-31]. According to Ruberto et al. [32] and Tepe et al. [8], they have reported that the presence of major components: camphene, -pinene, -terpinene and trans caryophyllene, have slight or no antioxidant activity [8-31]. In general, the antioxidant activity of essential oils is the product of additive, synergistic and/or antagonistic effects, as they are complex mixtures of several classes of compounds

5. CONCLUSIONS

The essential oil of different parts of *Thymus pallidus* obtained by hydrodistillation was analysed by GC-FID and GC/MS allowed the identification of 36 main components which accounted (98.4%) of the total amount. The essential oil was dominated by oxygenated monoterpenes (57.5%) and Hydrocarbon monoterpenes (37.6%).

The antioxidant activities of the essential oil of *Thymus pallidus* were evaluated by using three methods: free radical scavenging activity DPPH (2,2-diphenyl-1-picrylhydrazyl), the Ferric Reducing Power (FRAP), and -Carotene. The obtained results show that the essential oil of *Thymus pallidus* has a low activity than that of ascorbic acid and BHT.

Moreover, thymol, p-cymene, -terpinene and carvacrol as the main components of the EOs of the *Thymus* species studied may be considered as valuable natural sources of antioxidants that are useful for both food and pharmaceutical purposes. Future study may be directed toward the investigation of the enzymes or genes underlying the biosynthetic pathways in the different thyme species to provide new insights into the possibilities for increasing the major compounds in this genus.

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