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Phytochemical Characterization of the Essential Oils Obtained from Mediterranean *Thymus spp.* (Lamiacea) Harvested at Different Stages of Growth

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Abstract: To prove more information on the chemical composition of the essential oils of *Thymus* taxa collected in different Algeria areas, and to determine the effect of harvesting periods on the composition of these essential oils: three *Thymus* species (*T. numidicus*, *T.ciliatus* and *T. algeriensis*) were collected during two periods of development, the EOs (essential oils) obtained by steam distillation of the aerial parts were analyzed by GC/MS (gas chromatography coupled with mass spectrometry). During the pre-flowering stage, the *RP*% (relative percentage) of Eos varied between 1.00% and 1.34% for all the species. The content of EOs increased during the flowering stage and reached particularly 2.10% and 2.85% for *T. numidicus* (from Berrahal) and *T. numidicus* (from Tacha), respectively. GC/MS analysis showed that these oils are very rich in oxygenated monoterpenes group (76.96%~82.11%) when the plants were harvested during the flowering stage of growth. Thymol was the major compound in the EOs contained in *T. numidicus* (Berrahal) and *T. ciliatus* with more than 39% and 54%, respectively. *Thymus numidicus* (Tacha) and *T. algeriensis* EOs were characterized by high content in p-cimene-7-ol (78.06% and 26.98%, respectively). These two chemotypes (thymol and p-cimene-7-ol) are considered as antioxidant and antimicrobial agents providing the basis for many applications in processed food preservation and pharmaceutical products.

Key words: Bioactive molecule, chemotype, *T. algeriensis*, *T. ciliates*, *T. numidicus* Benth.

1. Introduction

Thyme, a Lamiaceae aromatic plant, is a perennial medicinal species belonging to Europe, Africa and Asia including about approximately 110 species [1, 2]. The different species are distributed along the cost and even in the intern and arid areas. In the North Africa countries (Algeria, Tunisia and Morocco), thyme leaves extracts, despite their frequent use as a spice and infusions, are used in traditional medicine as astringent, expectorant, antiseptic, antirheumatic, diuretic, analgesic and cicatrizing agents, as reported in several studies [1, 3-5]. Thyme can also be used as a veterinary product (antispasmodic, antiseptic and digestive); it is applied as feed additives and for treating diseases for pets and farm animals [6].

EOs (essential oils) contained in plants constitute a potential source of bioactive molecules [7, 8]. In the opposite of lipids (fixed oils), EOs are volatile and odorous substances also called chemotypes because of the difference in quantity and quality of their compounds present in the same genus; this difference is related to genome or gene expression differences [9] and to some abiotic parameters, such as geographical origin, harvest time, temperature, storage conditions

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and drying time [10, 11]. Particularly, EOs extracted from *Thymus* species are considered as the most active oils because of their high concentration in phenolic compounds (mainly thymol and carvacrol). Their antioxidant and antimicrobial properties provide the basis for many applications in raw and processed food preservation and pharmaceutical products [12]. Essential oils have been investigated in several studies where the scope was the chemical analysis of these compounds and their biological activities against several bacteria, yeast and fungi [7, 13, 14]. Research interests must continue to identify EO bioactive compounds present in *Thymus* species native to different parts of the world. Since plant EOs are generally affected by the geographical location and the phase of the plant development, the aim of the present work was to provide more information on (1) the chemical composition of EOs extracted from some *Thymus* species originated from different Algerian areas and (2) the effect of harvesting period on the composition of these EOs. For this purpose, three species largely abundant in these regions, *Thymus numidicus*, *Thymus ciliatus* and *Thymus algeriensis* were selected. They were collected before and during flowering stage of growth in four regions of Eastern Algeria.

2. Materials and Methods

2.1 Plant Material and Chemical Reagents

Three species of Thyme, *T. numidicus*, *T. ciliatus* and *T. algeriensis* were collected from different areas of Eastern Algeria. The species were identified by experts from Botany Laboratory, Badji Mokhtar University-Annaba. Plant material consisted of leaves and flowering tops was air-dried and kept under dark until constant weight. Table 1 summarizes the areas and dates of collection of thyme species with some geographical coordinates of the different regions. The dates of collection were selected according to pre-flowering (March or April) and flowering stages (May or June) of plant growth.

2.2 Essential Oil Analysis

The essential oils were obtained by hydrodistillation using a Clevenger-type apparatus according to the standard procedure. Approximately 50 g of dried plant were placed in a flask with 500 mL distilled water for 3 h. Following distillation, the oils collected from the condenser were stocked in closed tubes and placed at 4 °C to ovoid oil alteration and oxidation.

The distillation was followed by determining the *RP*% (relative percentage) of EOs defined as the ratio between the EO mass (M_{EO}) and the dry weight of the thyme material (M_T); it was evaluated by the following relationship:

$$RP\% = \frac{M_{EO}}{M_T} \times 100 \qquad (1)$$

Oils analysis was carried out by GC-MS (gas chromatography coupled to mass spectrometry). The apparatus type SHIMAZU QP2010 with a FID detector was equipped with a capillary column (25 cm long, 0.25 mm in diameter). The analysis conditions were as follows: helium at 1.7 mL/min as gas carrier,

Table 1 Origin of *Thymus* species under study (Eastern Algeria).

Species name	Area of collection	Geographical Coordinates	Date of collection
T. numidicus-B	Berrahal-Annaba	36°76′52″ N 7°44′90″ E	March 2010 May 2010
T. numidicus-T	Tacha-Annaba	36°88′97″ N 7°37′79″ E	March 2010 May 2010
T. ciliatus	Ain Makhlouf-Guelma	36°19′33″ N 7°26′49″ E	April 2010 June 2010
T. algeriensis	Selaoua Anouna-Guelma	36°36′66″ N 7°31′66″ E	April 2010 June 2010

the injection volume was 0.1 mL and the ionization potential was 70 eV. The column temperature was fixed at 60 °C during 1 min then increased until 200 °C with a rate of 3 °C/min and finally maintained at this temperature during 16 min.

This technique is based on the measure of retention time which is specific to each compound present in oil. The compounds were identified by comparison of their mass spectra with those contained in the NIST bookshop.

3. Results and Discussion

3.1 Thymus Species Content in EOs

The different EOs obtained from the four *Thymus* species had a light yellow color. The *RP*% of these EOs were measured and reported in Table 2 which shows that during the pre-flowering stage, the *RP*% varied between 1.00% and 1.34% for all the species. The content of EOs increased during the flowering stage and reached 2.10% and 2.85% for *T. numidicus*-B and *T. numidicus*-T, respectively, showing that this thyme species is richer in EOs than the two others. Conversely in a similar study, Amrouni et al. [7] reported a slight higher relative percentage (3%) of EOs present in the oils of *T. ciliatus* collected from another Eastern region not far from Berrahal and Tacha (Djebel El Edough-Annaba). However, Hadef et al. [15] found lower percentages of EOs in *T. numidicus* originated from Lakhdaria, a region in center Algeria. A same relative percentage of EOs present in *T. algeriensis* (from Algeria) was obtained by Dob et al. [13] with 1.13%. Generally, the results found in our present study are very similar to those reported in literature with some few exceptions probably dues to some ecological and climatic conditions (temperature, location, soil type and harvest time) and some laboratory operating conditions (drying time, extraction and analytical techniques) [3].

3.2 Chemical Composition of EOs

GC-MS analysis of the four plants EOs provided the different compounds present in these oils. *Thymus* species are very variable in the essential oil composition. The quantity of each compound is also variable and this is related to various environmental and genetic factors [2]. The four extracted oils contained 43, 12, 52 and 19 compounds in the EOs of *T. numidicus*-B, *T. numidicus*-T, *T. ciliatus* and *T. algeriensis*, respectively (Table 3). The two species *T. numidicus*-B and *T. ciliatus* are very rich in thymol, carvacrol and terpinene which are considered as antimicrobial agents [16]. Thymol was the major compound of the EOs contained in *T. numidicus*-B and *T. ciliatus* with more than 39% and 54%, respectively. According to the chemical characterization of the same species (from the same regions), Giordani et al. [17] reported 66.31% and 60.52% of thymol in the extracted oils. The difference with our results may be due to some abiotic parameters not reported in the study, such as temperature and drying time. Several studies on the chemical characterization of Thyme species originated from different regions of the world (Algeria, Iran, Morocco, Turkey) have been conducted and have shown that thymol was the predominant compound in the EOs of plants [15, 18-21]. According to Nezhadali et al. [22], the high content of medicinal plants in thymol means that these plants could be used as flavoring agents in food, medicinal and perfume

Table 2 Relative percentage of EOs contained in *Thymus* species under study.

Species	*RP*%	
	Pre-flowering stage	Flowering stage
T. numidicus-B	1.28	2.85
T. numidicus-T	1.34	2.10
T. ciliatus	1.00	1.79
T. algeriensis	1.16	1.77

Table 3 Chemical composition (%) of *Thymus* EOs (flowering stage).

Compounds *	*T. numidicus*-B	*T. numidicus*-T	*T. ciliatus*	*T. algeriensis*
Monoterpene hydrocarbons				
α-thujene	0.18	-	0.14	-
α-pinene	0.57	1.93	0.46	3.08
Camphene	0.04	-	-	-
Myrcene	1.28	0.99	1.55	-
α-phyllandrene	0.26	-	-	-
β –cymene	6.54	5.73	5.96	7.74
Limonene	0.10	0.43	0.48	-
O-cimene	-	-	0.04	-
Trans β-ocimene	0.26	-	0.41	-
Cis β-cymene	0.40	-	0.65	-
γ-terpinene	6.12	6.88	4.75	5.64
Terpinolene	0.17	-	0.20	-
2-carene	0.65	-	0.60	-
4-carene	0.01	0.98	0.00	-
Dimethyl styrene	0.32	-	0.28	-
Oxygenated monoterpenes				
Eucalyptol	1.88	-	2.34	2.01
Linalool	7.47	0.73	8.55	2.60
Pinocarveol	0.05	-	-	2.67
Camphor	1.29	-	3.28	3.64
Borneol	0.94	-	0.75	-
Isoborneol	-	-	-	2.28
Terpinen-4-ol	1.90	0.57	0.96	-
γ-terpineol	0.10	-	0.23	-
Thymol	39.66	-	54.04	-
Carvacrol	23.52	-	7.09	-
Pinen-10-ol	-	-	0.23	-
Cis-pinen-3-ol	-	-	0.58	-
Trans-3-carene-2-ol	-	-	0.42	-
Pinen-3-one	-	-	0.14	-
Verbenone	0.05	-	0.13	13.18
Thujenal	-	-	0.14	-
Isothujol	-	-	0.09	1.08
Carveol	-	-	0.14	-
Thymol-methyl-ether	-	-	1.00	-
Caprique aldehyde	-	-	-	0,96
Trans-linalool oxyde	0.07	-	-	-
Cis-linalool oxyde	-	-	0.19	-
γ-terpineol acetate	-	-	0.05	-
P-cimene-7-ol	-	78.06	-	26.98
Vinyl format	-	0.16	-	-
Bergamol	-	-	-	1.7
Carveol dihydro	-	-	-	0.83
Methyl ter buthy ether	-	2.59	-	19.63
Caproique aldehyde	-	-	0.02	-
Caprylic alcohol	0.03	-	-	-

(Table 3 continued)

Compounds *	*T. numidicus*-B	*T. numidicus*-T	*T. ciliatus*	*T. algeriensis*
Sesquiterpene hydrocarbons				
α-cubebene	0.02	-	-	-
y-langene	0.05	-	-	-
Copaene	0.13	-	0.04	-
Caryophyllene	0.26	-	0.38	-
Humulene	0.17	-	-	-
γ-murolene	0.43	-	0.11	-
D-germacrene	0.21	-	0.19	-
α-farnesene	-	-	0.08	-
β-farnesene	-	-	0.34	-
γ-cadinene	0.65	-	0.26	-
Bisabolene	0.16	-	-	-
α-amorphene	0.02	-	-	-
Bourbonene	0.15	-	0.06	-
Aristolene	0.14	-	-	-
Fenchene	-	-	0.12	-
Aromadendrene	0.05	-	-	-
Bergamotene	-	-	0.02	-
Oxygenated sesquiterpenes				
Mertenyl acetate	0.04	-	0.04	2.59
α-trans-bisabolene epoxyde	0.03	-	0.05	1.44
α-tetradecine	-	-	0.06	-
α-dodecylene	-	-	0.07	-
Vinyl amyl carbinol	3.37	0.95	2.03	1.12
Citroviol	0.26	-	-	-
Isovaleral	-	-	-	0.83
Longipinene epoxyde	-	-	0.03	-
Ascaridol epoxyde	-	-	0.07	-
Mercenyl acetate	-	-	0.03	-
3-decene-2-ol	-	-	0.02	-
Eugol acetate	-	-	0.11	-

"-": Compound not identified.

industries. In addition, this monoterpene phenol possesses different biological and pharmacological properties, such as antimutagenic, antitumor, antioxidant and anti-inflammatory [16].

The major constituent in the oils of *T. numidicus*-T and *T. algeriensis* was p-cimene-7-ol; it was concentrated at more than 78% and 26.98%, respectively. The predominant compound in thyme plants differ from species to another and from an area to another. Ballester-Costa et al. [12] characterized four Mediterranean *Thymus* species (*T. vulgaris*, *T. zygis*, *T. mastichina* and *T. capitatus*) originated from Spain; the authors found that the major compound in the different oils was respectively 1,8-cineole (51.94%), thymol (48.59%), carvacrol (69.83%) and linaool (44.00%). A Moroccan *Thymus* species (*T. maroccanus*) was analyzed for its EOs at different developmental stages; the analysis by GC/MS revealed the presence of 28 components with 14.1%~77.6% of carvacrol as major compound [4].

Essential oils extracted from plants consist of chemical compounds groups known as monoterpenes, sesquiterpenes and their oxygenated and hydrocarbons derivatives. These compounds have the ability to easily diffuse across cell membrane to induce biological reactions [23]. The most represented chemical groups in the four species selected in the present study are oxygenated monoterpenes (phenolics compounds) with 76.96%~82.11%; the monoterpene hydrocarbons were present with approximately 26% in the four species. The two other groups (sesquiterpene hydrocarbons and oxygenated sesquiterpenes) were present at very low percentage (Fig. 1). It has been demonstrated that the anti-microbial activity of most essential oils is related to their phenolic monoterpenes [24-26].

3.3 Effect of Growth Stage on the Chemical Composition of Thymus EOs

Identified compounds in the *Thymus* EOs were different in number and percentage of the chemical groups during flowering and pre-flowering stages of plant growth (Table 4). Studies on some *Thymus* species indicated that in the plant's life cycle, the oil production is usually at its highest level during the flowering period [3]. This is in agreement with our results which show that plants harvested during the flowering stage contained more concentrated compounds. Particularly, the *RP*% of oxygenated monoterpens (and oxygenated sesquiterpenes) was higher than that of the pre-flowering stage and attained (76.96%~82.11%) (0.95%~5.98%) for the four species. Consequently, the monoterpenes hydrocarbons and sesquiterpene hydrocarbons concentration decreased. This may be due to an increasing in photosynthetic activity which induces a high rate of biosynthesis of volatile compounds (mainly the phenolic compounds) at full flowering stage [4].

Some monoterpenes constituents (cymene, γ-terpinene, carvacrol and p-cimene-7-ol) were absent (or present at very low concentrations) in the different EOs when the plants were harvested before flowering (Table 5). Their concentration increased significantly during the flowering stage of growth mainly

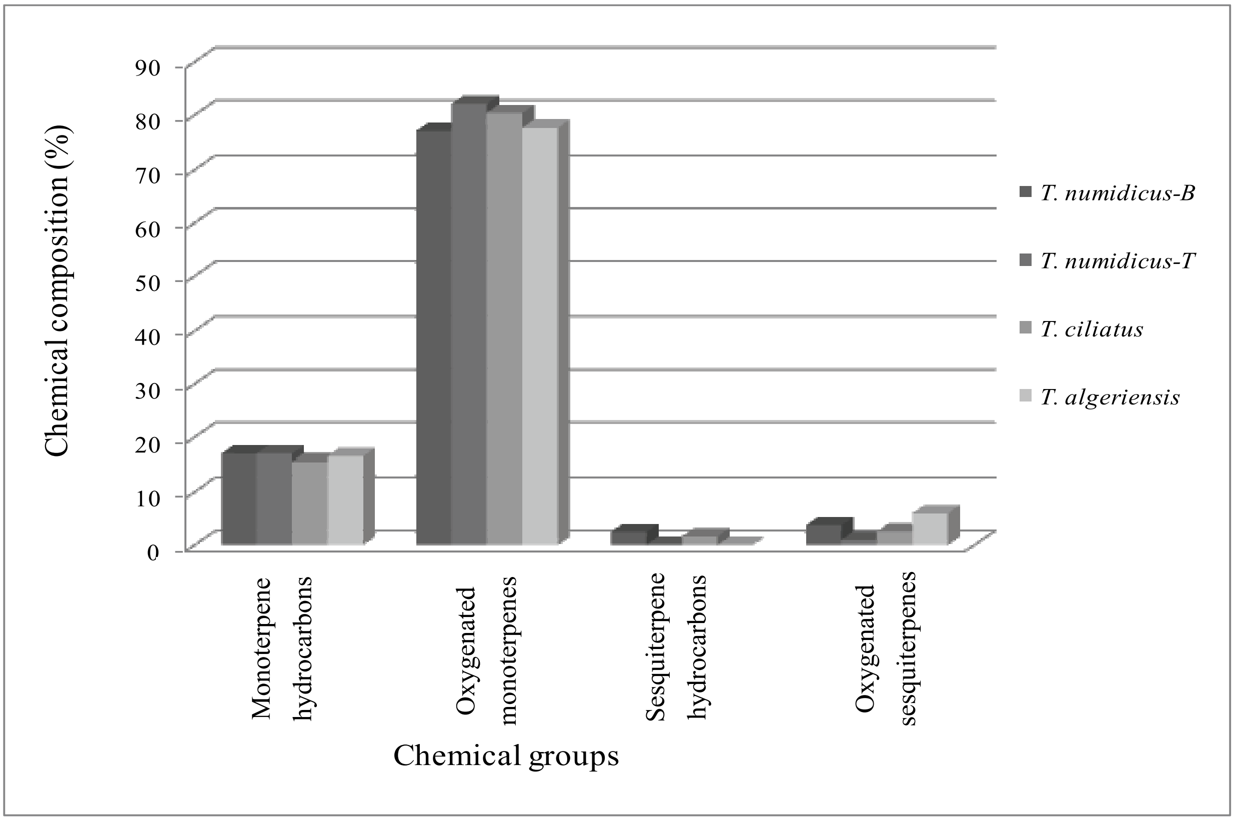

Fig. 1 Chemical composition of the different chemical groups present in *Thymus* EOs.

Table 4 Number of compounds and relative percentage of EOs (%) contained in *Thymus* species harvested during flowering stage and pre-flowering stage of plant growth.

Species		Number of Compounds	Monoterpene Hydrocarbons	Oxygenated monoterpenes	Sesquiterpene Hydrocarbons	Oxygenated sesquiterpenes
T. numidicus-B	Pre-flowering stage	24	38.23	54.48	6.09	1.20
	Flowering stage	43	16.9	76.96	2.44	3.70
T. numidicus-T	Pre-flowering stage	14	31.18	67.20	1.13	0.49
	Flowering stage	12	16.94	82.11	0.00	0.95
T. ciliatus	Pre-flowering stage	13	35.8	54.07	10.13	0.00
	Flowering stage	52	15.52	80.37	1.6	2.51
T. algeriensis	Pre-flowering stage	30	25.36	61.86	10.68	2.10
	Flowering stage	19	16.46	77.56	0.00	5.98

Table 5 Chemical composition (%) of *Thymus* EOs extracted from plants during flowering stage (in bold) and pre-flowering stage (in normal) of growth.

Compounds*		*T. numidicus*-B	*T. numidicus*-T	*T. ciliatus*	*T. algeriensis*
β-cymene	Pre-flowering stage	-	-	-	-
	Flowering stage	6.54	5.73	5.96	7.74
Limonene	Pre-flowering stage	-	-	-	5.13
	Flowering stage	0.10	0.43	0.48	-
Isolimonene	Pre-flowering stage	-	-	-	5.5
	Flowering stage	-	-	-	-
(E) β-ocimene	Pre-flowering stage	8.80	-	-	-
	Flowering stage	0.26	-	0.41	-
O-cymene	Pre-flowering stage	22.35	23.14	11.88	6.36
	Flowering stage	-	-	-	-
γ-terpinene	Pre-flowering stage	0.16	5.06	22.34	-
	Flowering stage	6.12	6.88	4.75	5.64
Terpinolene	Pre-flowering stage	4.33	-	-	-
	Flowering stage	0.17	-	0.20	-
Eucalyptol	Pre-flowering stage	-	-	-	5.31
	Flowering stage	1.88	-	2.34	2.01
Linalool	Pre-flowering stage	31.14	13.01	23.58	4.68
	Flowering stage	7.47	0.73	8.55	2.6
Camphor	Pre-flowering stage	-	-	-	33.30
	Flowering stage	1.29	-	3.28	3.64
Thymol	Pre-flowering stage	20.28	51.20	25.08	-
	Flowering stage	39.66	-	54.04	-
Carvacrol	Pre-flowering stage	-	-	-	-
	Flowering stage	23.52	-	7.09	-
Verbenone	Pre-flowering stage	-	-	-	-
	Flowering stage	0.05	-	0.13	13.18
Thymol methyl ether	Pre-flowering stage	-	-	5.41	-
	Flowering stage	-	-	1.00	-
p-cimene-7-ol	Pre-flowering stage	-	-	-	-
	Flowering stage	-	78.06	-	26.98
Methyl ter buthyl ether	Pre-flowering stage	-	-	-	-
	Flowering stage	-	2.59	-	19.63

* The reported compounds are those with composition ≥ 5%.

for *T. numidicus*-T whose oil was very rich in p-cimene-7-ol (78.06%). The flowering period can be profitable for extracting plant EOs and isolating these chemotypes for their use in food, cosmetic and perfumery industries [27]. For thymol, the concentration was variable in *T. numidicus*-B and *T. ciliatus* at the two different stages of growth. The values decreased from 39.66% and 54.04% (during flowering stage) to respectively 20.28% and 25.08% in EOs when the plants were harvested during the pre-flowering stage. Only O-cimene (and linalool) was present in plants during pre-flowering stage and was not detected (was detected at low concentrations) in *Thymus* species when harvested in their second stage of growth. A camphor-rich chemotype distinguished the oil of *T. algeriensis* from all other oils. The high content of *T. algeriensis* oil (33.30%) suggests great potentialities of this species for industrial exploration, mainly as an antifungal and anti-inflammatory agent [28]. Additionally, in their study on the antioxidant capacity of some Lamiaceae species, Khled Khoudja et al. [29] confirmed that *T. algeriensis* containing high phenolic compounds showed a strong ability to act as an antioxidant agent.

4. Conclusions and Perspectives

The highest essential oil content was observed at flowering stage of growth in the four *Thymus* species originated from Eastern Algeria. The high thymol content of *T. numidicus*-B, *T. numidicus*-T and *T. ciliatus* and the high concentration of camphor in *T. algeriensis* make these four species promising bioressources for use in pharmaceutical, cosmetic and food industrial applications.

This first part of our work encourages further investigation for testing the extracted EOs from the four *Thymus* species for a bioactivity evaluation such as antimicrobial and antioxidant activities. Research may then continue to investigate a microbiological study in order to suggest new therapeutic applications with these Mediterranean plants.

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