CHEMICAL COMPOSITION OF ESSENTIAL OILS AND SECRETORY HAIRS OF *THYMUS DACICUS* BORBÁS RELATED TO HARVESTING TIME

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We dedicate this work to Academician Cristofor I. Simionescu, a great scientist, who contributed to the development of chemical engineering in Romania and opened up new research directions

Thyme (*Thymus* spp.), a famous herb from the Lamiaceae family, is a medicinal and aromatic plant with important bio-economical, commercial and pharmaceutical potential. Among the species of this genus, *Thymus dacicus* is less studied in terms of both structure and chemical composition of secondary metabolites. In the present work, the authors investigated the effect of harvesting time on the composition of essential oils and on glandular hairs of *Thymus dacicus*. For this purpose, the vegetal material was collected in 3 different phenophases (vegetative, anthesis and fruiting), during 2 consecutive years, from Novaci, Gorj County, Romania. Regarding the chemical composition of essential oil, major qualitative and quantitative differences, depending on phenophase and harvest year, have been found. Following the studies conducted, we have found that the density of hairs tends to increase from spring to autumn, in both sides of the leaf lamina, with the highest values in the anthesis phase.

Keywords: thyme, phenophases, hair structure, terpenoids

INTRODUCTION

Among the medicinal and aromatic plants from the Lamiaceae family (plants with important aromatherapeutic properties),[1] the genus Thymus is noteworthy for the numerous species and varieties of wild-growing plants.[2] Many of these species are specific to the Mediterranean area.[3] The aromatic and medicinal properties of the genus Thymus made it one of the most popular plants all over the world. Among *Thymus* species, some are well known as regards their composition and activities, such as *Thymus vulgaris*, *Thymus pannonicus*, *Thymus pulegioides* and *Thymus zygioides*, which synthesize phenolic compounds and essential oils abundant in monoterpenes.[4] *Thymus* species are commonly used as herbal tea, flavoring agents and medicinal plants[3] due to their biologically active substances (such as thymol, carvacrol, geraniol, linalool and other compounds from their essential oils),[5-10] which present antimicrobial, antioxidant, anti-inflammatory, antiproliferative *etc*. activities. However, some species of the genus have been less studied, such as *Thymus dacicus*, for which relevant data concerning chemical composition and activities are scarce. Moreover, for such species, there is little information regarding the anatomy of various organs, including secretory structures.

In *Thymus* species, similar to other Lamiaceae taxa, the formation and the accumulation of the essential oils take place in glandular hairs, which are distributed on the surface of the epidermis of the aerial parts of the plant. The content of the

essential oil can depend on the origin, climate, harvest, as well as on drying and storage conditions.[11,12] The harvesting date, time of the day and weather conditions are very important for the quality and quantity of essential oils. Also, the accumulation of essential oils in plants depends directly on light. Li and colaborators[13] studied the essential oils production in thyme and sage, and their results showed that the highest yield of essential oils and percentage of thymol and myrcene in thyme occurred when the plants were harvested in full sunlight. In general, sunny days should be preferred, after morning dew disappears.[14]

The main objective of this paper was to highlight the possible effect of harvesting time on the composition of essential oils and on glandular hairs of *Thymus dacicus* Borbás. For this purpose, samples of this species were collected in vegetative, full flowering and fruiting phases.

EXPERIMENTAL

Plant material

The vegetal material was represented by *Thymus dacicus*, a species that grows in the wild Romanian flora. The species was collected in 3 different phenophases (vegetative, anthesis and fruiting), in June, August and September, during 2013 and 2014, from Novaci, Gorj County, Romania. *Thymus dacicus* is a perennial plant with initial vigorous recumbent stems, then ascendent, very branched ones. The leaves are elliptic or prolonged, green in color, both faces are covered with hairs, nervures are little prominent. The inflorescence is capitate. The calyx is 3-4 mm long; the corolla is lilac-red, 6-7 mm long.[15] The identification of taxa was made by Dr. Ioan Sârbu from the Botanical Garden "Anastasie Fătu", Iasi, using the following works: "Flora Europaea"[16] and "Illustrated Flora of Romania – Pteridophyta et Spermatophyta".[17] The collected material was registered and stored in "Alexandru Ioan Cuza" University's Herbarium from Iaşi.

Isolation and analysis of essential oils

The dried aerial parts of the plant (100 g) were subjected to hydro-distillation, for 3 hours, using a NeoClevenger apparatus, according to the method recommended by the European Pharmacopeia (1997).[18] The yield of essentials oils was 0.2% for vegetative phase, 0.8% for anthesis phase and 0.5% for fruiting phase. The obtained essential oils were stored at +4 °C until analysis. The chemical composition of the essential oil was established by GC-MS analysis with the help of an Agilent Technologies 6890N gas-chromatograph coupled to a mass detector (MSD) of the 5975 inert XL Mass Selective Detector type. The conditions for chromatography were the following: column HP 5MS, mobile phase discharge gas: helium, at a flow rate of 1 mL/min, injector temperature of 250 °C, detector temperature of 250 °C, temperature regime from initial 40 °C (10 degrees/min) to 280 degrees, injected volume of 0.1-0.3 µl, splitting ratio of 1:100. The DB5 chromatographic column has a length of 30 m, an interior diameter of 0.25 mm and a film diameter of 0.25 µm. The separated compounds were identified by means of the NIST spectrum database, and the peak position was confirmed by the Kovats retention index.

Structural and density analyses of secretory hairs

The histo-anatomical investigation was conducted on fresh leaves and stems using transmission electron microscopy analyses. The material was prefixed in 2.7% glutaraldehyde, dehydrated in successive, increasing in concentration, acetone solutions. The samples were embedded in Epon 812 epoxy resin and polymerized at 60 °C. The blocks were sectioned with a Leica Ultramicrotome, to obtain semi-thin and ultrathin sections, for analyses under the optical microscope and electron microscope. The obtained sections were contrasted with uranyl acetate and lead citrate and examined with a Jeol 1010 TEM. The density of secretory hairs was determined by counting visible hairs on peeled epidermis strips under the optical microscope, at a 10x magnification. Strips from both lower and upper epidermis were analyzed and results were expressed on area unit.

RESULTS AND DISCUSSION

Chemical composition of essential oils

A high chemical variability was observed in the volatile oils of the various species in the Thymus genus.[19,20] Regarding *Thymus dacicus*, a less studied species, following our analysis of essential oils, a total of 55 compounds were identified, representing between 84.228% and 99.128% of the total number of identified compounds (Table 1). The highest number of chemicals (38 compounds) was identified in the volatile oil derived from individuals collected in 2013, in the stage of fruiting. The lowest number of compounds (25) was identified in the volatile oil derived from plants collected in 2013 in the vegetative stage.

Analyzing the results, a number of qualitative and quantitative differences have been observed, depending on phenophase and harvest year. The main chemical components identified were geraniol, linalool, geranyl acetate, γ-cadinol, caryophyllene oxide and muurulol. Kisgyörgy and collaborators[21] reported that the main components of essential oils of *Thymus dacicus* collected from our country are as follows: carvacrol – 30%;

thymol – 16.8%, nerol, α-terpineol and linalyl acetate. In the present study, these compounds were also identified in the essential oils of this species, in different concentrations.

Table 1

Chemical composition of the essential oil of *Thymus dacicus*, collected in various phenophases in two consecutive years (2013-2014) from Novaci, jud. Gorj (% area)

Compound	RT, min	Vegetative stage		Anthesis stage		Fruiting stage	
		2013	2014	2013	2014	2013	2014
α-Pinene	13.640	-	0.53	-	-	0.809	-
Camphene	14.332	-	0.842	-	-	0.361	-
Octen-3-ol	15.863	-	0.395	-	0.445	0.808	0.577
Myrcene	16.435	-	2.276	-	-	-	-
β-Pinene	16.463	-	-	-	-	1.145	0.188
α-Terpinene	17.538	-	-	-	-	0.297	0.258
B-Cymene	17.875	-	0.378	-	-	0.897	-
p-Cymene	17.904	-	7.466	-	4.698	-	10.832
Limonene	18.109	-	0.328	-	0.25	0.236	-
Eucalyptol	18.207	0.661	0.527	-	0.492	-	0.623
cis-β-ocimene	19.012	-	0.372	-	-	-	-
γ-Terpinene	19.452	-	0.278	-	1.313	0.923	2.119
cis-Sabinene hydrate	19.795	2.022	1.824	-	-	2.057	-
Linalool	21.201	-	0.702	11.44	1.928	11.667	0.359
Linalyl acetate	21.157	-	-	1.822	-	-	-
Camphor	22.985	-	0.943	0.552	-	1.254	-
Borneol	23.876	0.842	2.632	2.636	0.598	1.95	1.043
Terpinen-4-ol	24.333	1.642	0.487	1.197	-	6.206	-
α-Terpineol	24.859	-	3.819	9.879	0.248	3.568	-
Nerol	26.271	2.568	2.628	1.305	1.489	1.051	1.893
Methyl thymol	26.717	2.14	5.407	-	3.057	0.401	4.78
Neral	26.860	0.811	0.349	0.665	0.456	1.213	1.015
Geraniol	27.254	5.696	18.376	2.724	33.025	1.39	28.81
Geranial	27.666	1.974	-	1.162	-	1.891	0.769
Citral	27.808	-	-	-	0.76	-	-
Bornyl acetate	27.837	1.798	7.894	1.741	-	5.106	-
Thymol	28.454	2.314	5.397	-	4.124	-	4.697
Carvacrol	28.934	6.025	0.365	-	12.477	-	16.045
α-Terpinyl acetate	30.660	-	-	-	7.904	-	0.174
Neril acetate	31.106	-	1.543	0.383	-	1.39	-
Lavandulol acetate	31.581	-	-	1.027	-	-	-
Geranyl acetate	31.763	1.589	18.489	-	12.683	1.126	11.044
α-Burbonene	31.809	0.728	-	-	-	0.301	0.486
β-Burbonene	31.998	-	0.324	-	0.517	9.7	1.799
β-Cariophyllene	33.186	1.299	5.333	2.518	2.49	-	1.788
Alloaromadendren	34.347	-	-	1.047	-	1.275	-
α-Cariophyllene	34.541	-	1.435	-	-	0.584	-
Germacrene D	35.141	-	2.166	0.808	3.065	1.125	-
γ-Elemene	35.678	-	1.546	0.537	0.586	3.355	0.729
β-Bisabolene	35.953	3.136	1.377	2.052	5.283	-	4.972
γ-Cadinene	36.010	1.05	0.968	-	0.449	1.017	-
β-Cadinene	36.204	-	-	0.879	-	-	0.625
τ-Cadinene	36.262	0.772	2.394	-	-	1.624	-
Elemol	37.045	0.85	0.388	0.875	-	0.362	-
Trans-Nerolidol	37.599	0.367	3.081	0.304	0.28	0.456	0.317
Spathulenol	37.919	-	-	9.079	-	9.572	0.32
Farnesene	38.148	0.811	0.33	-	-	-	-
Caryophyllene oxide	38.324	2.334	10.531	2.525	0.511	3.171	0.883
Leden	38.776	-	-	0.845	-	-	-
Cubenol	39.302	-	1.107	-	-	-	-
τ-Cadinol	39.845	-	-	2.563	-	2.261	-
τ-Muurulol	40.228	-	9.698	5.456	-	3.845	-
γ-Cadinol	41.342	5.23	-	18.807	-	7.834	-
Aromadendrene epoxyde	41.645	-	-	2.453	-	1.118	0.285
Total		84.228	87.879	87.281	99.128	93.346	97.145

An important aspect to note is the presence of large amounts, in some cases, or the complete absence, in other cases, of two phenolic monoterpenes (thymol and carvacrol) – these monoterpenes are specific to the species of the genus Thymus. It was noted that thymol and carvacrol were missing in the anthesis and fruiting stage of the samples harvested in 2013, and were present in small quantities in the vegetative stage.

Meanwhile, in the samples of 2014, the two phenolic monoterpenes were present in large quantities in all the three phenophases analyzed. Another monoterpene specific to *Thymus* plants, p-cymene, was present in large amounts, in all vegetative stages, but only in the plants harvested in 2014. This monoterpene was, however, missing in the essential oil of the plants harvested in 2013. Also, geraniol and geranyl-acetate were present in large amounts, in all the stages, in the essential oils of the plants collected in 2014; while in those harvested in 2013, these chemicals were present in small amounts.

Table 2
Variation of temperature and daily rainfall in June, August and September (2013, 2014)*

Parameter	June		August		September	
	2013	2014	2013	2014	2013	2014
Average minimum temperature (ºC)	13.5	10.33	14.6	13.26	7.2	9.66
Average maximum temperature (ºC)	24.5	23.66	28	27.06	19.8	23.84
Average daily rainfall (mm)	3.5	1.16	3.79	1.59	1.52	1.06

*Source http://md.freemeteo.com/[32]

For *Thymus dacicus*, there is little data[21] regarding the essential oil composition, and no available information concerning variations as a function of phenological stages or environmental factors. Therefore, the variations recorded in the present paper concerning the composition of volatile oils of *Thymus dacicus* may be attributed to differences in climatic conditions (precipitation, temperature, humidity) during the harvesting periods. The variations of temperature and rainfall, for the harvesting periods are presented in Table 2. As shown in the table, there were not significant differences in terms of temperature during the harvesting periods in the years 2013-2014. However, major differences were reported regarding the daily rainfall for June and August, in 2013 being recorded double amounts of rainfall, compared to 2014. This may explain the differences occurring in the chemical composition of volatile oils.

The composition of essential oils is known to be affected by a series of factors, among which intra- and interspecific variations, intraindividual variations, climate conditions and cultivation and harvesting measures are the most prominent. Within climatic factors, the yield and constituents of volatile oils are most influenced by the length of the day, the irradiance, temperatures, water supply, as well as by soil properties.[22] Significant modifications of the yield and in the composition of essential oils were reported for different *Thymus* species, such as *Thymus vulgaris*, *Thymus carnosus* and *Thymus caespititius*.[23] For *Thymus vulgaris*, a yield between 4.6 and 2.7% was recorded for northern populations and for middle and southern populations, respectively, in plants from the flora of Jordan.[24] Such changes can be explained by different environmental factors on the sites – a hypothesis that refers to varying climate parameters on the same site, during different harvesting periods. As an example, an essential oil more abundant in low molecular weight compounds was obtained from *Thymus zygis* ssp. *gracilis* when grown under more intense irrigation, while the greatest thymol concentrations in the same oil were obtained under lower levels of irrigation.[25] The altitude and amount of available water were also correlated with the presence of metabolites, such as p-cymene, γ-terpinene or carvacrol in *Thymus piperella*.[26] From a genetic point of view, it is considered that environmental factors may alter the dominance of some allele genes, which are responsible for the synthesis of essential oils, as has been proved for *Thymus pulegioides*.[27] The phenological stage of the plants also plays an important part in the synthesis of volatile oils in *Thymus* species. Variations in the amounts of many constituents of the volatile oils were reported as a function of the developmental stages, as in the case of *Thymus serpylloides* with the amount of thymol ranging between 0.8 and

15.8%,[28] *Thymus kotschyanus* with carvacrol amounts in the range of 40-61%,[29] or *Thymus caramanicus* with p-cymene in the range of 3-8.9%.[30]

Glandular hairs

Previous studies on the secretory structures of essential oils from the *Lamiaceae* family showed that this family presents two types of secretory hairs (peltate and capitate), located in the aerial organs, particularly in the leaf lamina.[31] Generally, the secretory hair (peltate or capitate) consists of a basal region, composed of one or more cells, a foot and a uni- or multicellular gland, comprising one or more secretory cells.[32] In addition to the above structures, some authors consider that the epidermal cells arranged radially around the base portion of the secretory hair are part of the hair structure.[4] It is believed that the basal cells do not function as typical epidermal cells, but as an accessory to the glandular hair with a role in the volatile oil secretion. The size of these cells, their shape, arrangement, vacuolation and plasmodesmata density at the level of periclinal walls probably contribute to the collection of photosynthesis products from the mesophyll level and also to their transport to the level of basal cells of the glandular hairs. Subsequently, these products will arrive through pedicel cells to the gland of the secretory hair, where they have a role in developing the essential oil under the action of "the enzymatic machinery" controlled by secretory cells.[32]

Using microscopy techniques, two morphological types of secretory hairs were identified in *Thymus dacicus* located at the surface of the organs: peltate and capitate. The peltate hairs consist of a unicellular base, a pedicel (foot) and a gland formed by more than 2 cells. The capitate hairs are formed by a unicellular base, a uni- or bicellular pedicel and a gland composed of 1 or 2 cells. Depending on the number of cells that form the gland, the secretory hair can be divided into three categories: 1) hairs with a unicellular gland (present in all aerial organs – generally, these hairs have a unicellular pedicel and the glandular cell produces volatile oil, which extends towards the thin cellulosic wall and bulges the cuticle that covers the gland (Fig. 1 a, b, c; Fig. 2)); 2) hairs with a bicellular gland (observed more rarely, in particular in the vegetative stage – the cuticle that covers the gland is cut off from the cell wall); 3) hairs with a multicellular gland (present in all aerial organs, in all stages of vegetation – the gland can be formed by 4, 8 or 12 secretory cells (Fig. 1 d)).

In cross section, the glandular hairs are present on both sides of the limb, in very deep depressions, sometimes opposite, therefore in those places the mesophyll is extremely thin.

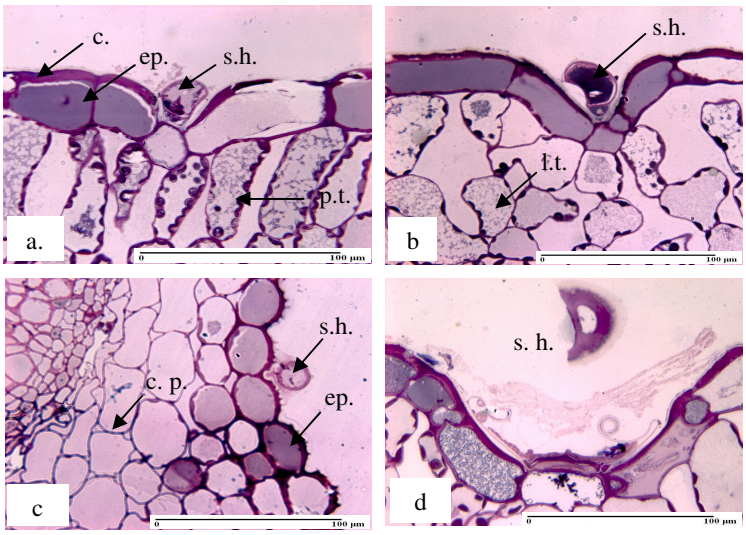


Figure 1: Semi-thin sections through the leaf and stem of *Thymus dacicus* – a. Secretory hair with unicellular gland from the upper epidermis of foliar blade; b. Secretory hair with unicellular gland from the lower epidermis of foliar blade; c. Secretory hair with unicellular gland from the stem (median level); d. Secretory hair with multicellular gland from the upper epidermis of foliar blade (abbreviations: c. cuticle; ep. epidermis; s.h. secretory hair; p.t. palisade tissue; l.t. lacunar tissue; c.p. cortical parenchyma)

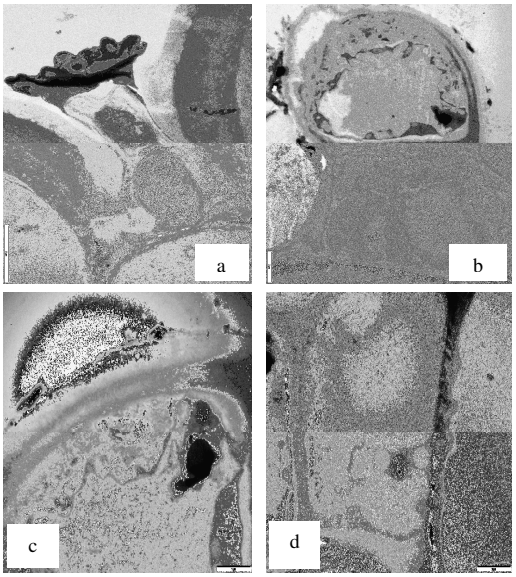
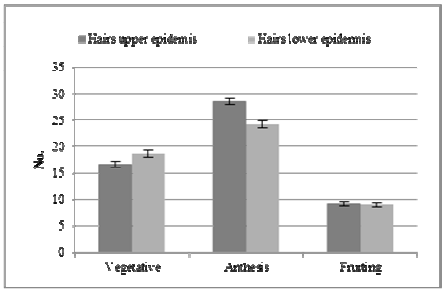
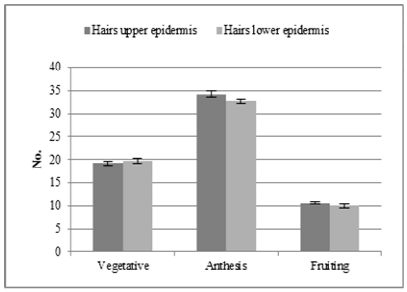


Figure 2: Ultrathin sections through glandular hairs of *Thymus dacicus* – a. Secretory hair with bicellular pedicel from foliar blade; b. Secretory hair with unicellular pedicel and gland from stem; c. Detail – secreting cell and covering cuticle; d. Detail – pedicel of unicellular glandular hair



a.



b.

Figure 3: Variation of secretory hair density as a function of phenophases
(a. year 2013; b. year 2014)

In general, the hairs in the secretory phase are easily distinguishable as the cuticle in the upper region of the secreting cells is 2-3 times thicker, compared with the cuticle covering the sidewalls of the gland. When the hairs reach maturity, the secretory cells begin to produce volatile oil, which diffuses through the external wall, and forms a bulge.

Concerning the density of hairs, their number tends to increase from spring to autumn, in both sides of the leaf lamina (Fig. 3). The highest density was recorded in the anthesis stage, followed by the vegetative phase and the fruiting phase. In the anthesis stage, for both harvesting years, the number of hairs on the upper epidermis was higher than the number on the lower epidemis. Slight variations of hair density were recorded between the two analyzed years, with higher density in 2014.

CONCLUSION

The variations in the composition of *Thymus dacicus* essential oils parallel differences in the harvesting period and climatic conditions. Also, the harvesting time can influence the secretory hair density, with a tendency to increase from spring to autumn. Such results offer a basis for further studies aimed at establishing the optimum conditions and harvesting times for *Thymus dacicus* as a source of essential oils with significant bioactivities.

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