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Research Article

Temporal Variation on Chemical Composition, Anti-inflammatory and Antioxidant Activities of the Essential Oils of *Thymus sibthorpii* Benth. (Lamiaceae) Growing Wild in Kilkis (Northern Greece)

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ABSTRACT
This study is aimed at assessing the essential oil composition**,** anti-inflammatory and antioxidant activities of the essential oil of *Thymus sibthorpii* collected from Neo Gynaikokastro, Kilkis in Central Macedonia, northern Greece (during the main flowering period in May 2014, as well as at four consecutive months of 2016 (March-June).The chemical composition of the essential oils was studied by GC-MS. Furthermore, the oils were evaluated *in vitro* for their:(i) antioxidant activity, using the DPPH interaction and inhibition of linoleic acid lipid peroxidation induced by the dihydrochloric acid of 2,2-azobis-2-amidinepropane (AAPH), and (ii) anti-inflammatory activity, using the soybean lipoxygenase assay. In total, 44 compounds were identified. The oils of flowering samples were mainly composed of phenolic compounds, and belonged to the linalool chemotype, while those of pre-flowering stage to the thymol chemotype. Their antioxidant and anti-inflammatory potentials scored average in comparison to reference compounds and were relatively higher in pre-flowering stages or at the end of flowering. The results reported here may serve the complex chemotaxonomy of taxa of the genus *Thymus* and the investigation of chemotypes of *T. sibthorpii* in temporal and/or geographical scales. The essential oils of *T. sibthorpii* due to their biological activities could be used in many cases as natural preservatives, food additives, functional food ingredients, nutraceuticals, pharmaceuticals and cosmeceuticals.
Keywords*:* medicinal-aromatic plants, nutraceuticals, volatile constituents, flowering stages

INTRODUCTION

The essential oils of *Thymus* taxa (species and subspecies) show a high chemical variability due to different intrinsic (genetic diversity) and extrinsic (ecological and environmental) factors[1]. Environmental factors such as temperature, radiation, photoperiod, water availability as well as soil fertility (nutrients) play an important role in the quantity and quality of the essential oils[2]. Seasonal variation of essential oils have been studied to date mainly in the most commercialized thymes (mainly cultivated) i.e. *Thymus zygis* L.[3], *T. vulgaris* L.[4,5], *T. serpyllum*[6] and *T. longicaulis* C. Presl[7,8].

Thymus sibthorpii Benth. (Lamiaceae) is a wild perennial sub-shrub which is a range-restricted species native only to the Balkan Peninsula and Anatolia in Turkey[1] with medicinal-aromatic properties and conservation concern[9,10]. In Greece, it is wild growing in the phytogeographical regions of the mainland and the North and West Aegean Islands[11]. To date, DNA barcoding and species-specific propagation protocols have been produced for *ex situ* conservation, allowing also its further sustainable cultivation at large scale as a commercial crop[12]. Natural extracts of *T. sibthorpii* were found to present antimicrobial, antioxidant, and anticancer activities[13, 14] and limited studies exist to date regarding its essential oils and biological properties[15, 16, 17, 14]. In this framework, the aim of the present study was to determine the chemical composition, free radical scavenging and anti-inflammatory activities of the essential oils from the aerial parts of wild-growing *T. sibthorpii* individuals, during different seasons (spring and summer), examining plant material collected progressively at the beginning or pre-flowering stage (March) till the end of flowering (June).

MATERIALS AND METHODS

Plant material

Plant material (*T. sibthorpii* individuals) was collected (almost 30gr/month) repeatedly at different times of the year and was taxonomically identified by Dr. Nikos Krigas. The first plant collection was done during in May 2014 including aerial parts of plants at the end of flowering, i.e. >50% of the individuals in fruiting, with desiccated flowers (TS-1). Later collections were done during 2016 in March (TS-2, pre-flowering stage: no individual in flowering), April (TS-3, beginning of

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flowering: <25% of the individuals in flower), May (TS-4, full flowering: >50% of the individuals in flowering) and June (TS-5, end of flowering). All plants were harvested from wild populations at the same geographical location i.e. Neo Gynaikokastro, a village located in Kilkis Prefecture, Central Macedonia, northern Greece.

A voucher specimen has been deposited at the Herbarium of the Balkan Botanic Garden of Kroussia (acronym BBGK according to Index Herbarium) and a duplicate was kept at the School of Pharmacy, Aristotle University of Thessaloniki (Greece) under No. Krigas N. & Lazari D. 8019.

Essential oil Isolation

Almost 25gr from the air-dry aerial of the each samples (all the amount of each monthly collections) was submitted in hydrodistilation, using a Clevenger apparatus according to standard procedures as described in European Pharmacopoeia (2005)[18]. The volatiles were trapped in 5 mL GC grade n-pentane, according to a standard procedure (European Pharmacopoeia, 2005), dried over anhydrous sodium sulfate, and kept in closed, air-tight Pyrex containers at -4ºC. Essential oil yield was expressed per mL per 100 g d.w.

Analysis of the oil samples

Gas chromatography-mass spectrometry

Essential oil analyses were performed using the method described by Hodaj-Çeliku et al. (2015)[19]. Essential oil analyses were performed on a Shimadzu GC-2010-GCMS-QP2010 system operating at 70eV. This was equipped with a split/splitless injector (230ºC) and a fused silica HP-5 MS capillary column (30 m x 0.25 mm i.d., film thickness 0.25 μm). The temperature program was from 50ºC to 290ºC, at a rate of 4ºC/min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Injection volume of each sample was 1μL. Retention indices for all compounds were determined according to Van den Dool and Kratz[20], using n-alkanes as standards. The identification of the components was based on comparison of their mass spectra with those of NIST21 and NIST107[21] and by comparison of their retention indices with literature data[22]. Component relative concentrations were calculated based on GC peak areas without using correction factors.

Interaction with DPPH

The antioxidant activity of the essential oils was determined in terms of hydrogen-donating or radical-scavenging ability, using the stable radical 1.1-diphenyl-2-picrylhydrazyl (DPPH), according to the method of Hadjipavlou and Pontiki (2015)[23]. To a solution of DPPH (0.1 mM in methanol) were added (10μL) the test samples dissolved in DMSO (20mg/mL stock solution). The antioxidant activity of each sample was recorded at 517nm after 20min and 60min. The percentage of reducing activity was calculated and compared with the reference compound nor-dihydroguaiaretic acid (NDGA).

Inhibition of linoleic acid lipid peroxidation

The method of Hadjipavlou and Pontiki (2015)[23] was followed, in the same way described by Hodaj-Çelikuet al. (2015)[19]. Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234nm in the presence of 2,2'- azobis(2-amidinopropane) dihydrochloride (AAPH) of 50μL of 40mM AAPH solution as a free radical initiator in 0.05 M phosphate buffer, pH 7.4. Oxidation was carried out in the presence of the tested essential oils (10 μL/10 mg/mL stock solution). The rate of oxidation at room temperature was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. Trolox was used as a reference drug.

Soybean lipoxygenase inhibition study *in vitro*

The tested samples (10 μL) were dissolved in DMSO (10mg/mL stock solution) and the analysis performed was according to the assay developed by Hadjipavlou and Pontiki (2015)[23]. Each of the samples was incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution in *tris*-buffer pH 9. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm and the percentage of inhibition resulted from essential oil was compared with the appropriate standard inhibitor nordihydroguaretic acid (NDGA).

RESULTS AND DICUSSIONS

Composition of the essential oils studied

In all cases, the extracted oil was yellowish in color and had a strong aromatic thyme-like odor. The yields of the volatile fractions obtained from *T. sibthorpii* aerial parts were on dry weight basis 2.50% (TS-1), 1.42% (TS-2), 1.38% (TS-3), 1.29% (TS-4) and 1.00% (TS-5) (v/w), respectively. Oil yields were richer either before flowering (TS-2) or during flowering (TS-1), although noticeable variation may be attributed to climatic differences between different years (compare TS-1 with TS-3 both collected in May). Table 1 presents the percentage amounts of identified compounds of essential oils as well as the percentage amounts of some group compounds in the same samples. Similar total yields of *T. sibthorpii* essential oils have been also reported from other studies[15, 14, 17] reported yields from 0.2 to 3.5% and concluded that the total essential oil content of individual wild populations of *T. sibthorpii* is also probably related to the habitat type in which they thrive. According to Koureas (2012)[17], *T. sibthorpii* plants collected during flowering from *Quercus ilex* forests in Greece (Habitat type 9340 according to the EU Directive 92/43/EEC) were characterized by the highest average yield (1.9%), whereas plants from thermophilous oak woods of the Eastern Mediterranean and the Balkans (Habitat type 924A) and semi-natural reforested areas (Habitat type 1031) by the lowest average yields (1.0%). In support to this trend, the plant populations examined in our study were wild growing in pseudomaquis formations (Habitat type 5350) that are characterized by intermediate (1.6%) average yields according to Koureas (2012)[17]; this was also confirmed in our study.

The different components (n=44) of the essential oils of the studied samples of wild *T. sibthorpii* from Kilkis area, Northern Greece are listed in Table 1 according to their increasing retention times. The more complex essential oils were those obtained at the

end of flowering (TS-5) with 37 compounds, followed by TS-2, TS-3 and TS-4 with 36, 35 and 34 compounds, respectively. The sample TS-1 obtained during full flowering two years earlier was the less complex in compounds (n=19); there is no apparent reason for this and perhaps can be attributed to climatic differences between 2014 and 2016. Other studies have revealed more complex essential oils for *T. sibthorpii* plants collected during flowering including 60[15] material from Northen Greece or 50 compounds[16] material from Anatolia, Turkey) or simpler ones including only 20 compounds[14], material from Northern Greece.

Table 1: Composition of the essential oils of *Thymus sibthoprii* collected from Neo Gynaikokastro, Kilkis in Central Macedonia, Northern Greece during full-flowering (May 2014, TS-1) and during four consecutive months in 2016 before flowering in March (TS-2) and while flowering in April (TS-3) May (TS-4) and June (TS-5).

Year of collection				**2014**	**2016**				**[d]ID**
Flowering stage				**Full**	**Before**	**Beginning**	**Full**	**End**	
Total yield				**2.50%**	**1.42%**	**1.38%**	**1.29%**	**1.00%**	
	[a]Compounds	**[b]RI$_{exp}$**	**[c]RI$_{bibl}$**	**TS-1**	**TS-2**	**TS-3**	**TS-4**	**TS-5**	
1	α-Thujene (MH)	**925**	**924**	nd	0.1	tr	0.2	0.4	RI, MS
2	α-Pinene (MH)	**930**	**932**	nd	0.1	tr	0.2	0.6	RI, MS,Co-
3	Camphene (MH)	**944**	**946**	nd	0.1	tr	0.1	0.4	RI, MS
4	Sabinene (MH)	**971**	**969**	nd	nd	nd	0.1	0.4	RI, MS
5	β-Pinene (MH)	**972**	**974**	nd	0.1	0.1	nd	nd	RI, MS, Co-GC
6	1-Octen-3-ol	**981**	**974**	nd	1.4	1.3	0.8	0.9	RI, MS
7	3-Octanone	**988**	**979**	nd	0.3	0.6	0.2	0.8	RI, MS
8	Myrcene (MH)	**990**	**988**	nd	0.9	0.6	0.4	0.9	RI, MS, Co-GC
9	3-Octanol	**997**	**988**	0.1	1.0	1.0	0.5	0.6	RI, MS
10	α-Phellandrene (MH)	**1001**	**1002**	nd	nd	nd	0.1	0.2	RI, MS
11	α-Terpinene (MH)	**1013**	**1014**	nd	1.0	0.7	0.2	1.0	RI, MS, Co-GC
12	p-Cymene (MH)	**1021**	**1020**	tr	**2.7**	**1.7**	0.8	**5.2**	RI, MS, Co-GC
13	Limonene (MH)	**1025**	**1024**	nd	0.4	0.5	0.2	**5.8**	RI, MS, Co-GC
14	1,8-Cineole (OM)	**1027**	**1026**	0.2	1.6	1.0	0.4	nd	RI, MS, Co-GC
15	γ-Terpinene (MH)	**1057**	**1054**	nd	**11.9**	**8.9**	1.0	**4.8**	RI, MS, Co-GC
16	*cis*-Sabinenehydrate (OM)	**1064**	**1065**	0.1	1.0	0.5	0.4	**3.0**	RI, MS
17	*cis*-Linalooloxide (furanoid) (OM)	**1074**	**1067**	nd	nd	nd	tr	0.1	RI, MS
18	Terpinolene (OM)	**1085**	**1086**	nd	0.1	0.1	tr	0.2	RI, MS, Co-GC
19	*trans*-Linalooloxide (furanoid)	**1087**	**1084**	nd	nd	nd	tr	0.1	RI, MS
20	*trans*-Sabinenehydrate (OM)	**1096**	**1098**	nd	0.1	0.1	0.1	0.2	RI, MS
21	Linalool (OM)	**1099**	**1095**	**73.3**	**0.6**	**37.1**	**70.6**	**39.4**	RI, MS, Co-GC
22	Borneol (OM)	**1160**	**1165**	**1.3**	**1.7**	**1.4**	**0.8**	**2.2**	RI, MS, Co-GC
23	δ-Terpineol (OM)	**1166**	**1162**	nd	nd	nd	nd	0.1	RI, MS, Co-GC
24	Terpinen-4-ol (OM)	**1176**	**1174**	0.1	0.8	0.5	0.3	**3.0**	RI, MS, Co-GC
25	α-Terpineol (OM)	**1190**	**1186**	nd	0.2	0.2	0.1	0.6	RI, MS, Co-GC
26	*cis*-Dihydrocarvone (OM)	**1197**	**1191**	nd	nd	nd	nd	0.2	RI, MS
27	*trans*-Dihydrocarvone (OM)	**1204**	**1200**	nd	nd	nd	nd	0.1	RI, MS
28	Hexylisovalerate	**1241**	**1241**	0.1	tr	tr	0.1	0.2	RI, MS
29	Carvacrol, methylether	**1246**	**1241**	0.1	nd	nd	tr	0.8	RI, MS
30	Bornylacetate	**1285**	**1287**	nd	tr	0.1	nd	tr	RI MS

31	Thymol (OM)	**1292**	**1289**	**13.9**	**38.6**	**29.1**	**14.9**	**20.4**	RI, MS, Co-GC
32	Carvacrol (OM)	**1303**	**1298**	nd	2.4	2.0	nd	nd	RI, MS
33	Thymolacetate	**1354**	**1349**	nd	0.2	nd	nd	nd	RI, MS
34	β-Elemene (SH)	**1376**	**1389**	0.4	0.1	0.3	0.3	0.6	RI, MS
35	β-Bourbonene (SH)	**1378**	**1387**	nd	0.1	0.1	nd	nd	RI, MS
36	β-Caryophyllene (SH)	**1411**	**1417**	**5.7**	**12.2**	**5.4**	**3.9**	**2.8**	RI, MS, Co-GC
37	β-Copaene (SH)	**1423**	**1430**	tr	0.1	0.1	tr	0.1	RI, MS
38	α-*trans*-Bergamotene (SH)	**1431**	**1432**	nd	0.1	tr	nd	nd	RI, MS
39	α-Humulene (SH)	**1446**	**1452**	0.2	0.3	0.2	0.1	0.1	RI, MS, Co-GC
40	Germacrene D (SH)	**1473**	**1484**	**2.7**	**4.5**	**2.3**	**2.4**	**0.9**	RI, MS, Co-GC
41	Bicyclogermacrene (SH)	**1490**	**1500**	0.3	0.1	0.2	0.3	0.3	RI, MS
42	β-Bisabolene (SH)	**1508**	**1505**	0.6	**13.5**	**3.1**	**0.4**	**0.7**	RI, MS
43	δ-Cadinene (SH)	**1518**	**1522**	0.3	0.1	0.4	nd	nd	RI MS
44	Caryophylleneoxide (OS)	**1578**	**1582**	0.6	0.7	0.3	0.1	1.0	RI, MS, Co-GC
Total %				**100**	**99.1**	**99.9**	**100**	**99.1**	
Monoterpene Hydrocarbons (MH)				tr	17.3	12.8	3.1	19.9	
Oxygenated Monoterpenes (OM)				88.9	46.5	71.7	80.7	69.5	
Sesquiterpene Hydrocarbons (SH)				10.2	31.1	12.1	7.4	5.4	
Oxygenated Sesquiterpenes (OS)				0.6	0.7	0.3	0.1	1.0	

[a]The compounds detected are listed in order of elution from an HP-5 MS capillary column. [b]RI_{exp}: Retention Index indices as determined on a HP-5 MS capillary column using a homologous series of n-alkanes (C9-C25); [c]RI_{bibl}: Retention Index indices as determined in bibliography; [d]ID (Identification method): RI = Retention Index, MS = Mass Spectrum, Co-GC: co-injection with authentic compound;.nd=not detected. Concentrations below 0.05% are marked as tr (traces); MH= mototerpene hydrocarbon, OM= oxygenated monoterpene, SH= sesquiterpene hydrocarbon, OS= oxygenated sesquiterpene.

Table 2: Reducing ability (RA, percentage%) expressed as interaction of essential oils of *Thymus sibthorpii* from Neo Gynaikokastro (Kilkis, Northern Greece) with DPPH (percentage, %), inhibition of lipid peroxidation (LP percentage, %) and inhibitory activity on soybean LOX (percentage, %).TS-1: Full-flowering sample (May 2014); TS-2: Sample before flowering (March) and samples during flowering in 2016 (TS-3: April; TS-4: May and TS-5: June).

Parameters and samples	**RA (%)**		**Inhibition of LOX (%)**	**LP (%)**
Time	20min	60min		
Concentration	20μl	20μl	10μl	10μl
TS-1	27	53	na	1
TS-2	40	52	50	58
TS-3	43	64	21	7.0
TS-4	26	68	7	na
TS-5	57	42	40	3
NDGA	81	93	92	
TROLOX				88

na: no activity was found under the experimental conditions. The results are the mean of 3-6 measurements and the standard deviation (SD) was less than 10%.

Linalool was the main constituent in each essential oil of *T. sibthorpii* from Kilkis area, Northern Greece collected during its flowering period from April to June (TS-1, TS-3, TS-4, TS-5: 37.1-73.3%). However, this was not the case detected at its pre-flowering period (TS-2), during which linalool was as less as 0.6% (Figure 1). According Özek et al., 2011[24], only (-) linalool isomer was found in the essential oil yield from this *Thymus* species. Seasonal differences are known to occur in some only medicinal-aromatic plants such as Greek oregano *Origanum vulgare* subsp. L. *hirtum* (link) Iestw[25], but also in thyme plans such as *T. vulgaris*[5] and *T. longicaulis*[7,8]. All studied essential oils of *T. sibthorpii* were characterized by high percentages of oxygenated monoterpenes and thus can be classified as oils belonging to the linalool chemotype, except from the one corresponding to the pre-flowering

TS-2 sample which showed high thymol content (38.6%) and therefore should be classified to the thymol chemotype.

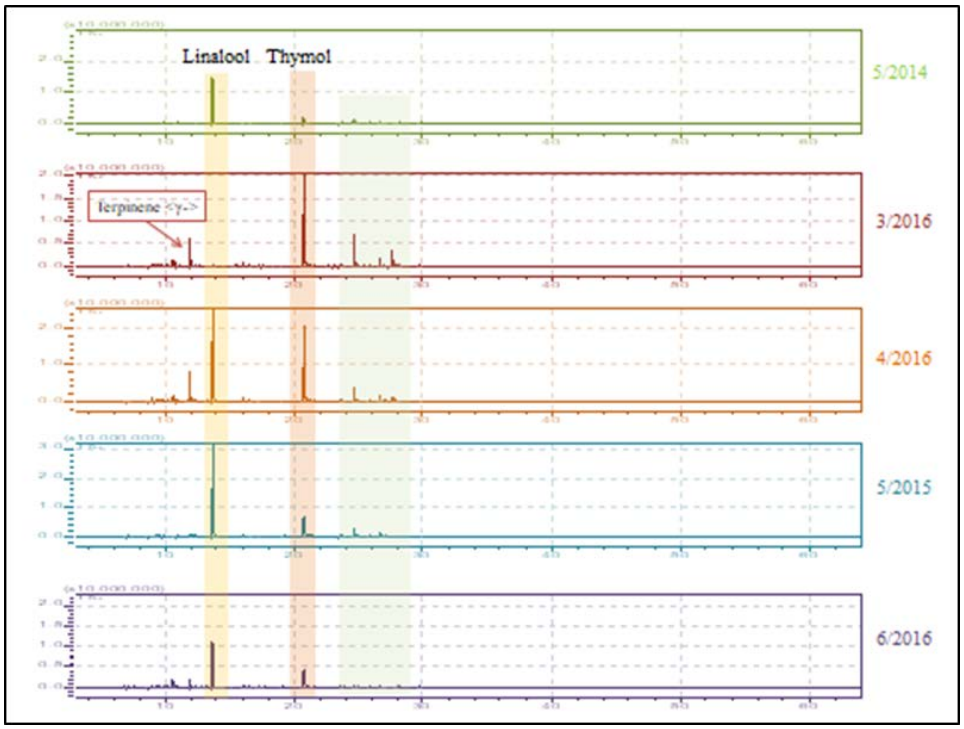

Figure 1: GC from each sample and the differences between their composition

It is known that the essential oil content of individual wild populations of *T. sibthorpii* is closely related to the habitat type in which they thrive[17]. In the present study, all the examined samples were collected from the same habitat type (pseudomaquis: habitat type 5350 according to the EU Directive 92/43/EEC) resulting in almost all essential oils to be assigned to the linalool chemotype. The presence of a different chemotype with high thymol content in the same geographical area (Gynaikokastro, Kilkis) is not astonishing, since different populations with distinct phytochemical profiles (or different chemotypes of *T. sibthorpii*) may co-exist in small geographic regions (cf. Koureas 2012[17]. Such results are further in accordance with those obtained by Katsiotis *et al.* (1990)[15] who reported a geraniol chemotype(subtypes geraniol-thymol-p-cymene and geraniol-linalool-citronellyl acetate) for the essential oils obtained from the same species collected during flowering from nearby region (Mt Chortiatis, Central Macedonia, Northern Greece) and similar habitat type with those obtained by Kontogiorgis *et al.* (2016)[14] who reported the essential oil of the same species collected in flower from similar habitat types of Mt. Chortiatis (June) as α-terpinyl acetate-a-terpineol chemotype. On the other hand, Baser*et al.* (1992)[16] reported that the essential oil of the same species collected in similar habitat types during the flowering period (June) from Tekirdag (Hayrabolu at Fidanlik, European Turkey) was classified to the thymol chemotype. The above mentioned chemotypes of *T. sibthorpii* seem to co-exist in a rather small geographic region of similar latitudes (ca. 800 km in straight line), all corresponding to material collected during flowering (June) from similar lowland (<700m) habitat types (pseuodmaquis).

It is known that wild populations of *Thymus* taxa (species and subspecies) show increased chemical polymorphism and their phytochemical composition may vary noticeably during the plants' phenological cycle, with pre-flowering and full flowering dominated by high levels of bioactive phenolic compounds[26]. In our study, the percentage of oxygenated sesquiterpenes was low in every examined sample and it seems that this was not related to the month of the original collection. The percentage of sesquiterpenes hydrocarbons was higher in the pre-flowering stage (31.1%, TS-2) in comparison to the samples examined during flowering. The percentage of monoterpene hydrocarbons was higher in the pre-flowering and at the end of flowering (TS-2and TS-5, respectively), intermediate during the beginning of flowering (TS-3) and lower during the full flowering period (TS-1 and TS-4).

There were no major seasonal variations in the composition of the examined essential oils of *T. sibthorpii*. Higher contents of the main components such as Thymol (38.1%), γ-terpinene (11.9%) and β-bisabolene (13.5%) were observed at the beginning of spring (March) in respect to the flowering period (April-June). In addition, other noticeable quantitative differences in essential oil composition between the pre-flowering (TS-2) and the flowering stages (TS-3, TS-4,TS-5) were apparent. Such differences occurred in the contents of limonene, γ-terpinene, linalool, terpinen-4-ol, thymol, β-caryophyllene, germacrene D and β-bisabolene which were increased in the samples examined during the

flowering stages. The qualitative differences above described between the studied samples are indicated with bold numbers in Table 1.

Biological activities

All the essential oils interacted with the stable free radical DPPH and the results are given in Table 2. In all cases the reducing ability of the essentials oils was time dependent, with the exception of TS-5 (end of flowering sample). Thus, it seems that the antioxidant activity is increased after an hour of interaction.

None of the examined samples was found to be better inhibitor than the NDGA (nordihydroguaiaretic acid), which was used as reference compound. This experiment expresses the anti-inflammatory activity of the essential oils studied. TS-2 (pre-flowering stage) exhibited the higher inhibition (50%) followed byTS-5 (end of flowering) with 40%.Among the examined oils, TS-2 (the sample of the pre-flowering stage) presented the stronger anti-lipid peroxidation with 58% compared to TROLOX used as reference. Sample TS-4 did not exhibit any inhibition, whereas TS-1, TS-3 and TS-5 showed very low inhibition. These results are probable due to the temporal variations of the major compounds of the essential oils, i.e. linalool, thymol, β-caryophyllene and germacrene D.

CONCLUSION

The results reported here may serve the complex chemotaxonomy of the taxa in genus *Thymus* and the investigation of chemotypes of *T. sibthorpii* in temporal and/or geographical scales. Almost all of the studied samples of *T. sibthorpii* belong to the linalool chemotype, except for the very early collected sample (TS-2) characterized by high thymol content. The main compounds were linalool, thymol, β-caryophyllene and germacrene D. Oxygenated monoterpenes were the major constituents in all samples. Interestingly, two of investigated samples (TS-1 and TS-4) collected in the same month but at different year, have approximately the same chemical composition (Figure 1). The essential oils of *T. sibthorpii* due to their biological activities could be used in many cases as natural preservatives, food additives, functional food ingredients, nutraceuticals, pharmaceuticals and cosmeceuticals.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. It is to specifically state that “No Competing interests are at stake and there is “No Conflict of Interest” with other people or organizations that could inappropriately influence or bias the content of the paper

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