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Variation in the essential oils of *Thymbra spicata* L. growing wild in Lebanon according to the date of harvest

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Thymbra spicata, an aromatic shrub belonging to Lamiaceae family and growing wild in Lebanon, was collected from Nahr Ibrahim in ten different harvest dates (from 11 October 2011 until 11 May 2012) and the composition of the essential oils (EOs) of air-dried aerial parts extracted by Clevenger-type hydrodistillation was determined by gas chromatography coupled with mass spectrometry (GC–MS). The amount and nature of the chemical compounds varied considerably from one sample to another depending on the harvest date. Oxygenated monoterpenes showed majority over monoterpene hydrocarbons only during the flowering stage (in May). In general, twenty-seven compounds of the EOs, which made up 89.4–98.7%, were identified in the oil of *Thymbra spicata* and the yield ranged between 3.5% and 6.6%. The main components were: carvacrol (16.1–62.9%), α-thujene (1.7–4.8%), myrcene (1.1–5.1%), γ-terpinene (11.4–24.1%) and *p*-cymene (8.1–46.8%).

Keywords: *Thymbra spicata*; essential oil; chemical composition; hydrodistillation; GC–MS

Introduction

Essential oils (EOs) are the most important raw materials of the fragrance and aroma industry. They are also used in the food and pharmaceutical industries due to their therapeutic, antimicrobial and antioxidant activities. Nevertheless, they have biological activities that make them able to be used as herbicides, pesticides and anticancer compounds (1).

The EO production does not depend only on plant genetics or developmental stage. The environment and its changes can influence in a significant way biochemical pathways and physiological processes that alter plant metabolism and, therefore, the EO biosynthesis (2).

Thymbra spicata L., from the Lamiaceae family, has a wide distribution in Lebanon and it is of economic importance because of its constituents especially the phenolic compounds carvacrol and thymol, which have been shown to be effective against soil-borne pathogens, food storage fungi, mycotoxic species, phytopathogens and human pathogens (3, 4). *Thymbra spicata* L. is used in the traditional medicinal system of Turks, Greeks, Egyptians and Romans to treat asthma and bronchitis as well as being used in the food industry for flavor, aroma and preservation (5). The leaves have recently gained much popularity as a remedy to combat hypercholesterolemia (6). Besides, the dried plant, softened in boiled water used to be applied to wounds as a drug (7).

Taking into consideration these studies, we followed the variations of the EO concentrations and their main constituents in the leaves of *T. spicata* from early autumn to middle spring. The purpose of this study was – on the basis of the known antifungal and antibacterial activity of a number of constituents of the EOs – to determine the variations of the EO compositions throughout the period investigated and to estimate the best time of sampling for their use as antifungal and antibacterial agents. To our best knowledge, no articles dealing with the variation in the oil composition of the Lebanese *T. spicata* have been published to date.

Experimental

Plant species and sample collection

The aromatic perennial shrub, *T. spicata* L., was harvested before and during the flowering period in ten different harvest time starting from October 11, 2011 until May 11, 2012 from a meadow in Nahr Brahim at 200 m altitude. Only the aerial parts were collected in the early morning so that the plant material is as fresh as possible.

The plant samples were systematically identified by Dr Marc El Beyrouthy according to the New Flora of Lebanon and Syria (8) and Voucher specimens numbered (MNV191a–j) are deposited in the Herbarium of the Faculty of Agricultural and Food Sciences at USEK University Lebanon.

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Hydrodistillation by Clevenger

From each harvest, 50 g of fresh aerial parts were air-dried for seven days before extraction, and then oil was extracted. Each sample was mixed with distilled water in a glass bulb connected to a Clevenger-type apparatus and subjected to hydrodistillation for 3 hours according to the method described in the *European Pharmacopoeia* (1997). The condensed oils were collected and dried using anhydrous sodium sulfate and after filtration stored at 4°C until analysis by gas chromatography coupled with mass spectrometry (GC–MS).

Yield evaluation

Yields were evaluated by measuring the weight of the oil extracted after drying (w of oil/w of dry) 50 g of the fresh aerial parts.

Essential oil analysis

GC analysis

Analytical GC was carried out on a Thermo Electron Corporation gas chromatograph fitted with a HP-5 MS capillary column (30 m × 0.25 mm), 0.25 μm film thickness.

Helium was the carrier gas (0.8 mL/minute). Column temperature was initially kept at 40°C for 5 minutes, then gradually increased to 250°C at 2°C/minute rate, held for 15 minutes and finally raised to 310°C at 10°C/minute. Diluted samples (1/100, v/v; in pentane) of 1 μL were injected at 250°C, manually and in the splitless mode. Flame ionization detection (FID) was performed at 280°C.

GC–MS analysis

GC–MS was performed using a Varian gas chromatograph CP3800 coupled with mass detector 1200 MS/MS. The split/splitless injector model 1177 was at 280°C in split 1:100. The CP 8400 auto sampler injected 1 μL of oil sample each time. GC–MS analysis was carried out using a fused silica capillary column Factor HP-5 MS, measuring 30 m × 0.25 mm internal diameter, film thickener of 0.25 μm; the oven temperature program adopted was 40°C (5 minutes) with an increase of 5°C/minute until 310°C (1 minute). Mass spectra were recorded at 70 eV, manifold 40°C, ion source temperature 280°C, transfer line 320°C, acquisition: full scan 40–800 amu. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m × 0.20 mm), 0.20 μm film thickness. In both cases, helium was used as carrier gas.

Qualitative and quantitative analyses

Most constituents were identified by gas chromatography by comparison of their retention indices (RI) with those of the literature (9, 10) or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{24}) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in NIST and Wiley 275 Libraries and our home-made library or with mass spectra from literature (9, 11). Standards of some EOs of known composition (such as the EO of *Rosmarinus officinalis* L.; Phytosun' Aroms, Plelo, France) have been injected in similar conditions to check the retention times and the mass spectra. Component relative concentrations were calculated based on GC peak areas without using correction factors.

Statistical analysis

Analysis of variance (ANOVA; SPSS 16.0 software) was performed to assess whether there was a significant variation in the three major components of the oil of *T. spicata* (carvacrol, *p*-cymene and γ-terpinene), according to date of harvest. The results were expressed as means±standard deviation and considered significantly different at the 0.05 level.

Results and discussion

Chemical composition of the essential oils of Thymbra spicata

Twenty-seven components were identified in the Lebanese *T. spicata* EO samples analyzed (Figure 1), amounting 89.4–98.7% of the total oil (Table 1).

Results showed that monoterpene hydrocarbons were the major portion of all samples before May (before the flowering stage) with the lowest value (29.3%) when extracted in May (during the flowering stage), and the highest value (80.4%) when extracted in April (before flowering). They were followed by the oxygenated monoterpenes that were less abundant, with the lowest value (17.4%) when extracted in April (before flowering), and reaching the major amount (63.7%) in May (during the flowering stage). Sesquiterpenes were in minor amounts of all samples ranging between 0.9% and 4.9%

The main components of the oils were: carvacrol (16.1–62.9%), γ-terpinene (11.4–22%) and *p*-cymene (8.1–46.8%). Also abundant were α-thujene (1.7–4.8%), myrcene (1.1–5.1%), α-terpinene (0.6–4%), caryophyllene oxide (0.1–2.8%), α-pinene (0–1.9%), β-pinene (0–1.3%) and thymol (0.2–1.1%), in addition to other components, which were of less abundance (Table 1).

The highest content of carvacrol (62.9%) was observed during the flowering stage in May, while the lowest amount (16.1%) was noted in April and before flowering. Also, *p*-cymene (46.8%) was the most

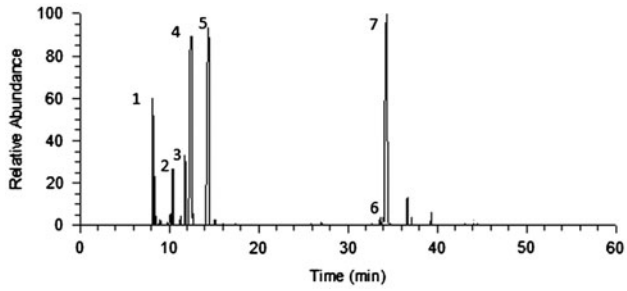

Figure 1. Chromatogram of *Thymbra spicata* L. obtained from extraction of essential oil from air-dried aerial parts by Clevenger-type hydrodistillation in October 11, 2011. Peak 1, α-thujene (4.8%); 2, myrecene (3.3%); 3, α-terpinene (3.4%); 4, *p*-cymene (27.2%); 5, γ-terpinene (24.1%); 6, thymol (0.3%); 7, carvacrol (29.8%).

abundant compound when extracted in April, with the lowest value (8.1%) obtained in May. In addition, dried aerial parts that were harvested in October contained the highest amount of γ-terpinene (24.1%), with the lowest value (11.4%) obtained in February. However, thymol was observed at low percentages all the time.

As a result, flowering period largely affected the amount of the major compounds by increasing and decreasing their values, and this was obvious comparing the amount of carvacrol (62.9%), that reached the maximum value, with the amount of *p*-cymene (8.1%) that decreased reaching the lowest value during this stage.

Influence of harvest date on the three major components and on essential oil yields of Thymbra spicata

The yield ranged between 3.5% and 6.6% for the dried aerial parts. The highest oil yield was obtained in October and then decreased with time reaching the lowest values in March and then started to increase from April to May (Figure 2).

The EO yield of a given species may be influenced by intrinsic parameters (such as growth stages) and extrinsic ones (such as pedoclimatic conditions and extraction methods) (12). In fact many previous works done on Lamiaceae reported that the full-flowering stage is characterized by the highest EO yield (13–16). For example, the yields of EO at different growth stages of *Artemisia annua* were in the order of pre-flowering < post-flowering < full-flowering (17). These results could be explained by the low rate of biosynthesis of volatile compounds during the vegetative stage that may be due to partial inactivation of enzymes necessary to the biosynthesis of certain compounds (12).

The ANOVA showed that the date of harvest has a large effect on the components studied. Carvacrol was constant compounds in all months and only showed a significant higher value in May (62.5±0.7). Also *p*-cymene results were significantly lowest in May compared with October in one side and March/April from the other side; also we note that the level *p*-cymene was significantly higher in March and April compared with other months. γ-terpinene values was significantly higher in October compared with December, January and February (Table 2).

The variation in the EO composition could be attributed to both interactions between genetic (biotic) and environmental (abiotic) factors (18). Regarding the evolution of these compounds through the vegetative cycle, it can be stated that the most volatile components (from α-thujene to γ-terpinene) show their maximum concentrations at the vegetative cycle. Other components such as carvacrol were detected at greatest concentration at the full flowering stage.

It is known that γ-terpinene is the precursor of *p*-cymene at the same time as carvacrol (19). According to the results showed in Table 1, the concentration of these components showed synchronized patterns of variation during the entire vegetative cycle. The phenological stages in which the conversion among components begins can be considered between April and May, beyond which *p*-cymene decreased its concentration and carvacrol increased its presence in the EO. Carvacrol, the phenolic component that defines the EO quality, showed its maximum concentration at the full flowering phenological stage being coincident with a decrease in *p*-cymene relative concentration. These statements agree with those published by others (20). For these authors, generally thyme EO from *T. vulgaris* was found to be rich in the active monoterpene phenols (thymol and carvacrol) and their corresponding terpenic hydrocarbons precursors (*p*-cymene and γ-terpinene), which collectively showed synchronized patterns of variation during the different collection periods and in different seasons.

Comparison of the main components of the Lebanese Thymbra spicata with other countries

The phenolic compound, carvacrol (16.1–62.9%), was the major component of the Lebanese EO of *T. spicata*,

Table 1. Variation in the essential oil composition and yield according to the date of harvest.

Date of harvest				Oct 11	Oct 25	Nov 11	Nov 25	Dec 11	Jan 11	Feb 11	Mar 11	Apr 11	May 11
Air-dried yield (%, w/w)				6.6	6	5.2	5.2	4.5	4.8	3.6	3.5	3.7	5.6
Compound ID	R_i[a]		R_i[b]										
Monoterpene hydrocarbons													
α-Thujene	929	1035	R_i, MS, CoGC	4.8	3.7	4.8	3.2	4.5	4.2	3	3.3	2.7	1.7
α-Pinene	938	1076	R_i, MS, CoGC	1.6	1.5	1.8	1.9	1.8	1.8	1.7	1.6	1.1	0.5
Camphene	953	1076	R_i, MS, CoGC	0.2	0.2	0.3	0.7	0.4	0.4	0.6	0.5	0.3	0.1
β-Pinene	980	1118	R_i, MS, CoGC	0.9		1	0.7	1.3	1.3		1.2	0.1	0.2
Myrcene	993	1174	R_i, MS, CoGC	3.3	4	2.9	5.1	2.8	2.7	3.6	1.8	2	1.1
α-Phellandrene	1004	1033		0.5	0.3	0.4	0.3	0.4	0.5	0.4	0.3	0.5	0.2
α-Terpinene	1013	1188	R_i, MS	3.4	2.1	2.3	0.6	1.9	2.09	1.5	2.2	4	
p-Cymene	1025	1280	R_i, MS, CoGC	27.2	28.4	34.9	28.5	37.6	35	36.2	42.1	46.8	8.1
β-Ocimene	1040	1225		0.06	0.05	0.04						0.1	0.1
γ-Terpinene	1057	1255	R_i, MS, CoGC	24.1	18.5	18.9	15.3	14.3	12.8	11.4	16.2	22	17.1
Total				66.3	59.1	67.8	56.7	65.2	60.9	58.5	69.5	80.3	29.2
Oxygenated monoterpenes													
Cis-Sabinene hydrate	1058	1556		0.1	0.1	0.1	0.1	0.2	0.2	0.6	0.2	0.2	
Terpinolene	1086	1265		0.1	0.1	0.1	0.5	0.1	0.1	0.1	0.1	0.1	
Linalool	1098	1553	R_i, MS	0.1	0.1	0.1		0.2	0.2		0.1	0.1	
p-Menth-2-en-1-ol	1129	1638							0.1	0.1	0.1	0.1	
Borneol	1167	1719	R_i, MS, CoGC	0.1	0.1	0.1	0.1	0.3	0.3	0.6	0.2	0.2	0.1
Terpinen-4-ol	1176	1611	R_i, MS	0.3	0.5	0.4	0.1	0.7	0.8	1	0.5	0.3	0.3
p-Cymen-8-ol	1182	1864	R_i, MS		0.1	1	1.1	0.2	0.2	0.6	0.1	0.1	
α-Terpineol	1189	1706			0.1					0.2		0.04	0.07
Isothymol methyl ether	1239	1607					0.2		0.1	0.1			
Cumin aldehyde	1240	1759		0.1			0.1	0.1	0.1	0.2	0.1	0.1	
Carvone	1241	1752				0.1		0.1	0.2	0.2	0.1		
Thymol	1293	2198	R_i, MS, CoGC	0.3	0.6	0.4	0.3	0.6	0.7	1.1	0.4	0.2	0.2
Carvacrol	1299	2239	R_i, MS, CoGC	29.8	35	28.2	25.3	28.1	30.8	28.5	25.3	16.1	62.9
Total				30.9	36.7	29.6	27.8	30.5	33.6	32.9	27	17.2	63.7
Sesquiterpenes													
Caryophyllene	1415	1612	R_i, MS, CoGC	0.9	0.7	0.4	1.9	0.7	0.8	0.9	0.5	0.3	1
Viridiflorene	1491	1695					0.1						
Bisabolene	1508	1741	R_i, MS		0.08								
Caryophyllene oxide	1577	2008	R_i, MS, CoGC	0.5	1.3	0.5	2.8	0.9	1.2	2.3	1	0.7	0.1
Total				1.4	2.1	0.9	4.9	1.7	2.1	3.3	1.5	1	1.1
Sub total				98.7	97.9	98.4	89.4	97.5	96.6	94.7	98	98.5	94.1

Notes: [a]Retention index on a HP-5MS column; [b]Retention index on an Innovax column; [c]Ri, retention index identical to bibliography; MS, identification based on comparison of mass spectra; Co-GC, retention time identical to authentic compounds. NI, not investigated; blanks mean not detected.

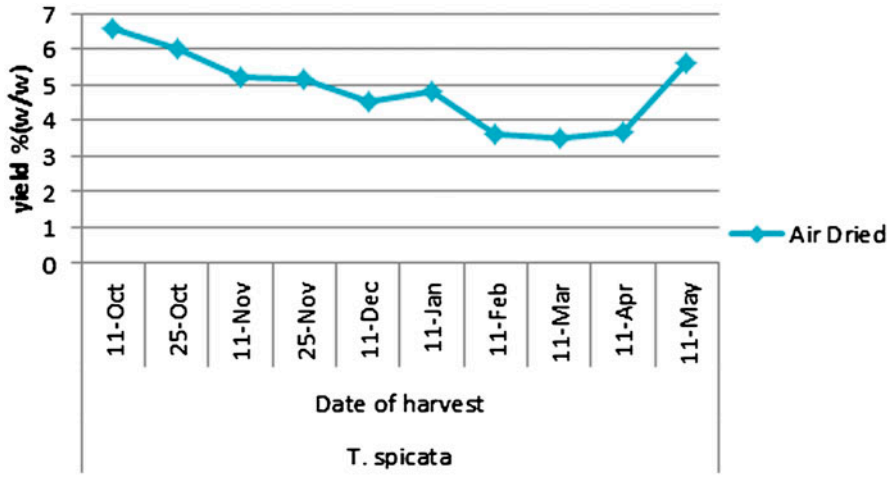

Figure 2. Variation in the yield of essential oil of *Thymbra spicata* according to the date of harvest.

Table 2. Variation in the major components of *Thymbra spicata* essential oil according to the date of harvest.

		Components mean±standard error		
Month	No.	*p*-Cymene	γ-Terpinene	Carvacrol
October	3	27.xx±0.7^{b}	22.9±1.1^{c}	31.2x±1.3^{a}
October	3	28.1±0.7x^{ab}	21.5±2.6^{c}	32.54±2.2x^{a}
November	3	31.6±4.6^{ab}	18.3±1.4^{abc}	33.42±8.1^{a}
November	3	31.7±4.2^{ab}	15.3±2.3x^{abc}	29.27±3.4^{a}
December	3	38.5±5.1^{ab}	11.5±5.8^{a}	33.16±7.5x^{a}
January	3	39.4±3.8x^{ab}	12.2±0.6^{ab}	28.09±2.4^{a}
February	2	36.6±0.5^{ab}	10±2.xx^{a}	30. xx±2.2^{a}
March	2	40.7±2^{c}	15.9±0.4^{abc}	27.86±3.6^{a}
April	2	40.7±8.7^{c}	20.6±2.7^{bc}	24.1x±11.3^{a}
May	2	7.5x±0.8^{a}	17.xx±0.1^{abc}	62.5x±0.7x^{b}
Total	26			

Note: No.: number of sample; ^{a,b}Means with different superscripts in a column differ significantly.

Table 3. Comparison of *Thymbra spicata* L. main components from Lebanon with data previously published from other geographic origins.

Origin	Lebanon	Greece	Turkey		
Reference	Our results	T. Markovic et al. (24)	H. Baydar et al. (23)	S. Hanci et al. (22)	M.Z. Ozel et al. (21)
Extraction method	Clevenger	Clevenger	Clevenger	Steam distillation	Subcritical water extraction
Main components	%				
p-Cymene	8.1–46.8	5.6	9.2	14.1	0.6–2.9
γ-Terpinene	11.4–24.1	8.1	11.6	19.4	0.4–1.9
Thymol	0.2–1.1	–	0.1	0.3	0.9–3.7
Carvacrol	16.1–62.9	74	75.5	52.8	79.5–86.2

which is in concordance with the carvacrol chemotype previously reported growing in Turkey and Greece. Moreover, the percentage of γ-terpinene (11.4–24.1%) and thymol (0.2–1.1%) from Lebanese *T. spicata* oil are nearly similar to the ones from Turkey (21–24). It is important to note that *p*-cymene (8.1–46.8%) showed the majority only in the Lebanese plant compared with the same species from other regions (Table 3).

It is important to compare and explore the variants of E.Os from different provenances, since this will most probably affect their potential biological activities, jeopardizing their use either in food industries or for medical purposes.

Conclusion

The aim of this study was to investigate the effect of harvest date on the EO content and to estimate the best time of sampling. An important variation was observed during the flowering stage by which the oxygenated monoterpenes became predominant over the monoterpene hydrocarbons. The chemical composition of EOs from *T. spicata* extracted by hydrodistillation has revealed a strong predominance of carvacrol, and a strong predominance of *p*-cymene and γ-terpinene during different months investigated.

These different harvesting periods of the samples could partly be responsible for these differences of the oil yield and the proportions of the several constituents of the EO, which may vary greatly according to the developmental phase of the plant. Generally, a great variability and diversity is observed concerning the chemical composition of the EOs of different origin due to climatic and soil variation conditions, to the vegetative cycle, to seasonal variation etc. (25).

In future, further studies would benefit to investigate the practical implications and non-toxic dosage of these natural sources to be used for medicinal purposes as antifungal and antibacterial agents.

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