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Chemical Composition of *Thymus serrulatus* Hochst. ex Benth. Essential Oils from Ethiopia: a Statistical Approach

Bruno Tirillini[a*], Roberto Maria Pellegrino[b], Mario Chessa[c] and Giorgio Pintore[c]

[a]*Institute of Botany, University of Urbino, via Bramante 28, 61029 Urbino, Italy*

[b]*Department of Chemistry, Laboratory of Organic Chemistry, University of Perugia, via Elce di Sotto 8, 06123 Perugia, Italy*

[c]*Dip. Farmaco-Chimico-Tossicologico, University of Sassari, Via Muroni 23, I-07100 Sassari, Italy*

bruno.tirillini@uniurb.it

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From the essential oil (EO) obtained from the aerial parts of *T. serrulatus* collected in Ethiopia, fifty-three compounds were identified by GC/MS, accounting for more than 99% of the total volatile fraction. Thymol and carvacrol were the main compounds, ranging from 10.0 ± 0.9 to 43.8 ± 3.8% and 4.5 ± 0.4 to 39.1 ± 3.8%, respectively, of the total. *o*-Cymene, γ-terpinene, and linalool were the most representative compounds in all the EOs.

Keywords: *Thymus serrulatus* Hochst. ex Benth., Lamiaceae, essential oil, thymol, carvacrol, statistics.

The genus *Thymus* (Lamiaceae) includes about 350 species worldwide and is widely distributed in temperate areas [1]. *T. vulgaris* contains 0.8-2.6% (usually ca. 1%) volatile oil, consisting of highly variable amounts of phenols, monoterpene hydrocarbons, and alcohols. There are several reports on the chemical composition of thyme oils; many indicate either thymol or carvacrol as the major compounds in the oils [1-23].

The leaves of *T. vulgaris* are used as an herb in food preparations, while the essential oil from the leaves is used in the alimentary, cosmetic and pharmaceutical industries. Thyme oil is used as an antispasmodic, carminative, antiseptic, anthelmintic, expectorant, antimicrobial (broad-spectrum antibacterial, antifungal and antiviral activity), antirheumatic, antioxidative, and natural food preservative [10,24-44]. The strong antimicrobial activity of thyme oil is ascribed mainly to the high content of phenolic constituents, such as thymol and carvacrol [24]. The essential oil of *T. vulgaris* has potent repellent activity against *Culex pipiens pallens* [27].

Two species, *T. schimperi* Ronninger and *T. serrulatus* Hochst. ex Benth., are indigenous to Ethiopia [16], while *T. vulgaris* has been recently introduced.

Chemical polymorphism concerning the essential oils of the genus *Thymus* is a widespread phenomenon. For example, the two Finnish species, *T. serpyllum* var. *serpyllum* and *T. serpyllum* var. *tanaenis*, turned out to form 4 different chemotypes each, with hedycaryol, germacra-1(10),5-dien-4-ol, germacra-1(10),4-dien-6-ol, linalool, and linalyl acetate as type-characterizing compounds. *T. schimperi* and *T. serrulatus* belong to the thymol-carvacrol chemotypes [16]. The leaves of *T. serrulatus* are used in Ethiopia as spices to flavor food, as well as for medicines. People in Bale harvest *T. serrulatus* for making a tea [16].

The aim of this study was to evaluate the essential oils of wild plants of *T. serrulatus*, collected from seven different areas of the Ethiopian plateaus and to acquire information on the thyme population by statistical methods. Table 1 reports the mean chemical analysis of five populations and includes all compounds found in the EOs from leaves and flowers. Fifty-three compounds were identified, which accounted for more than 99% of the total

volatile fraction from plants of *T. serrulatus*. Either thymol or carvacrol were the main compounds, with average amounts ranging from 10.0 ± 0.9 to 43.8 ± 3.8% and from 4.5 ± 0.4 to 39.1 ± 3.8%, respectively. *O*-cymene, γ-terpinene, and linalool were the most representative compounds in all the EOs.

Cluster analysis of the database, including a compound selection, put in evidence the presence of four "natural" groups: area 3 is joined with area 6 and 7, area 2 with area 4, while area 5 and area 1 remain ungrouped (Figure 1). The analysis shows that areas 3-6-7 join at a distance cluster (ds) = 38.88, while areas 2 and 4 join at a ds = 36.66. The first clusters were formed at a distance cluster (da) = 1.03.

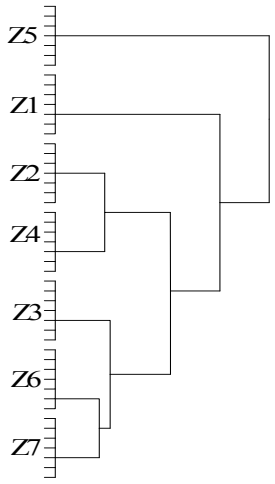

Figure 1: Dendrogram using Average Linkage (between groups)

Five major compounds listed in Table 2, which accounted for more than 75% of the essential oils, were selected to discuss the chemical variability due to areas of origin. Each area could be characterized by the chemical composition trend of the five major compounds. In Z1, *o*-cymene and carvacrol have the same percent value, in Z2 carvacrol is comparable to linalool and achieves its minimum value, in Z3 linalool achieves the minimum value, in Z4 carvacrol is comparable with γ-terpinene, in Z5 carvacrol is comparable to *o*-cymene, in Z6 linalool and γ-terpinene are comparable, and in Z7 no one compound is comparable to another. These remarks were supported by the ANOVA applied to a database in which the cases were the values of the five compounds, while the variables were the areas (Z1-Z7).

According to many authors [1-16], there are two chemotypes of *T. serrulatus*. The first one comprises the oils with a high thymol concentration and was in accord with the plants collected in Z2, Z4, and Z5 areas. The second one, with a high carvacrol content, corresponds to the plants collected in Z1, Z6, and Z7 areas. There is a question that might be of interest for these data: do the measurements of the main compounds discriminate between the two assumed groups of oils and can they be used to produce a useful rule for classifying other oils, such as those from Z3 areas or other oils that might become available? The Fisher's linear discriminant function analysis (FLDFA) works with data that are already classified into groups to derive rules for classifying new (and as yet unclassified) individuals on the basis of their observed variable values. The within-group covariance matrices suggest that the sample values differ to some extent, but according to Box's test for equality of covariances, these differences are not statistically significant. The canonical correlation value is 0.988 so that 97.6% of the variance in the discriminant function scores can be explained by group differences. In the Wilk's Lambda test, the lambda coefficient was 2.5%, and is the proportion of the total variance in the discriminant scores not explained by differences among the groups. The Fisher's linear discriminant function is:

z = -4.92 x meas1 – 20.84 x meas2 – 15.39 x meas3 + 79.21 x meas4 –74.99 x meas5.

The threshold against which an oil discriminant score is evaluated is –5.00. Thus, new oils with discriminant scores above –5.00 would be assigned to the thymol type; otherwise, they would be classified as carvacrol type. Following the derived classification rule, the oils from Z3 areas would be assigned to the carvacrol type.

In conclusion, if a representative lot of a plant population was hydrodistilled, a statistical approach may be useful for population inference. The evaluation of essential oils from wild plants living in different geographic areas could be helpful in the chemotype classification; the analyzed oils might be divided into two types according to the relative amounts of thymol or carvacrol. The "natural" group of oil did not correspond to the geographical areas as arbitrary chosen, but it might be possible redefine the areas in accordance with the cluster analysis. When the thyme population is distributed in an heterogeneous way across a large area, it might be possible to correlate the percent oil composition to the living sample area.

Table 1: Chemical composition (area percent ± SD) of *T. serrulatus*[a].

Compounds [b]	RI [c]	Z1 %	±SD	Z2 %	± SD	Z3 %	± SD	Z4 %	± SD	Z5 %	± SD	Z6 %	± SD	Z7 %	± SD
Methyl-2-methyl-butyrate	778	0.07	0.01	0.1	0.02	0.1	0.01	0.1	0.01	Tr	-	Tr	-	0.06	0.01
α-Thujene	931	2.3	0.2	2.8	0.26	3.1	0.3	1.7	0.2	0.8	0.07	1.7	0.1	2.0	0.2
α-Pinene	939	1.0	0.08	0.4	0.03	0.4	0.03	0.2	0.02	0.09	0.01	0.3	0.02	0.4	0.04
Camphene	954	0.07	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	0.04	0.01
2,4(10)-Thujadien	960	0.07	0.01	0.08	0.01	Tr	-	0.04	0.01	Tr	-	Tr	-	Tr	-
Sabinene	975	0.06	0.01	0.2	0.02	0.2	0.02	0.06	0.01	Tr	-	0.1	0.01	0.2	0.01
β-Pinene	980	0.2	0.02	0.09	0.01	0.09	0.01	0.06	0.01	Tr	-	0.08	0.01	0.1	0.01
1-octen-3-ol	982	0.1	0.01	0.9	0.06	0.7	0.07	0.5	0.05	0.09	0.01	0.1	0.01	0.2	0.02
3-Octanone	985	3.6	0.3	1.6	0.13	1.8	0.2	1.0	0.09	0.4	0.04	2.7	0.2	3.4	0.3
Myrcene	991	1.7	0.2	1.7	0.15	1.3	0.09	1.0	0.1	0.6	0.05	0.9	0.09	0.8	0.08
3-Octanol	992	1.2	0.1	0.7	0.05	0.6	0.06	0.4	0.04	0.1	0.01	0.8	0.07	0.9	0.08
α-Phellandrene	1003	0.4	0.04	0.4	0.04	0.3	0.03	0.3	0.02	Tr	-	0.1	0.01	0.2	0.02
α-Terpinene	1016	2.6	0.3	3.7	0.27	3.0	0.3	2.4	0.2	1.6	0.1	1.7	0.1	1.9	0.2
o-Cymene	1025	22.5	1.9	28.4	2.36	37.2	2.7	18.7	1.5	16.4	1.5	27.1	2.1	26.0	2.5
Limonene	1031	0.4	0.04	0.5	0.05	0.5	0.05	0.3	0.03	Tr	-	0.3	0.02	0.3	0.02
β-Phellandrene	1032	0.3	0.03	0.3	0.03	0.3	0.03	0.2	0.02	Tr	-	0.2	0.02	0.3	0.02
1,8-Cineole	1033	0.4	0.04	0.1	0.01	Tr	-	0.1	0.01	Tr	-	Tr	-	0.06	0.01
(*Z*)-β-Ocimene	1036	0.4	0.02	0.2	0.02	0.1	0.02	0.1	0.01	Tr	-	0.2	0.02	0.2	0.02
(*E*)-β-Ocimene	1051	0.1	0.02	0.1	0.01	Tr	-	0.09	0.01	Tr	-	Tr	-	Tr	-
γ-Terpinene	1061	15.6	1.3	22.8	2.06	8.0	0.7	13.7	1.3	12.0	1.1	4.1	0.3	3.1	0.2
cis-Sabinene hydrate	1069	0.9	0.07	1.0	0.1	1.4	0.1	0.8	0.08	0.8	0.06	1.5	0.1	1.8	0.2
cis-Linalool oxide	1087	0.07	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	0.04	0.01
Terpinolene	1089	0.1	0.01	0.2	0.02	Tr	-	0.1	0.01	Tr	-	Tr	-	0.03	0.01
Linalool	1098	6.2	0.6	5.0	0.5	1.7	0.1	3.3	0.3	3.3	0.2	3.6	0.3	4.8	0.4
(3*Z*)-Hexenyl isobutanoate	1147	0.2	0.02	Tr	-	Tr	-	0.2	0.02	Tr	-	Tr	-	Tr	-
Borneol	1170	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	0.03	0.01
Terpinen-4-ol	1177	1.04	0.08	0.7	0.05	0.6	0.05	0.2	0.02	0.4	0.03	0.2	0.02	0.1	0.01
p-Cymen-8-ol	1183	Tr	-	Tr	-	Tr	-	0.06	0.01	Tr	-	Tr	-	Tr	-
α-Terpineol	1189	0.7	0.07	0.5	0.05	0.6	0.05	0.6	0.05	0.4	0.02	0.2	0.02	0.4	0.04
cis-Dihydrocarvone	1193	0.1	0.01	Tr	-	Tr	-	0.04	0.01	Tr	-	Tr	-	0.07	0.01
Thymol. methyl ether	1235	0.2	0.02	0.2	0.02	0.2	0.02	0.1	0.02	Tr	-	0.3	0.02	0.2	0.02
Linalyl acetate	1257	0.06	0.01	4.0	0.36	Tr	-	2.0	0.1	2.3	0.2	Tr	-	Tr	-
Thymol	1290	10.1	0.8	16.3	1.3	14.9	1.4	34.7	3.1	43.8	3.8	11.5	1.0	10.0	0.9
Carvacrol	1299	20.6	1.8	4.5	0.37	19.1	1.6	13.9	1.0	15.0	1.2	39.1	3.8	38.0	3.3
Thymol acetate	1352	0.3	0.03	0.3	0.03	Tr	-	0.4	0.04	0.	0.01	Tr	-	0.06	0.01
Carvacrol acetate	1373	2.3	0.2	Tr	-	Tr	-	0.1	0.01	Tr	-	0.1	0.02	0.2	0.02
β-Bourbonene	1388	0.05	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-
α-Gurjunene	1410	0.07	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-
(*E*)-Caryophyllene	1419	1.3	0.1	1.3	0.11	1.7	0.1	1.2	0.1	1.3	0.1	1.2	0.1	1.5	0.1
α-*trans*-Bergamotene	1435	Tr	-	0.1	0.01	0.3	0.02	0.1	0.01	Tr	-	0.2	0.02	0.3	0.03
Aromadendrene	1441	0.9	0.07	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-
α-Humulene	1455	Tr	-	Tr	-	0.1	0.01	Tr	-	Tr	-	Tr	-	0.08	0.01
allo-Aromadendrene	1460	0.1	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	0.05	0.01
γ-Muurolene	1480	0.06	0.01	Tr	-	Tr	-	Tr	-	Tr	-	0.2	0.02	0.0	0.01
Germacrene D	1485	0.07	0.01	0.1	0.01	0.3	0.03	0.04	0.01	Tr	-	Tr	-	0.2	0.02
Viridiflorene	1497	0.07	0.01	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-
Bicyclogermacrene	1500	Tr	-	Tr	-	0.1	0.01	0.06	0.01	Tr	-	0.1	0.01	0.2	0.02
β-Bisabolene	1506	0.1	0.01	Tr	-	0.08	0.01	0.1	0.01	Tr	-	0.1	0.02	0.2	0.02
γ-Cadinene	1512	0.09	0.01	Tr	-	Tr	-	0.08	0.01	Tr	-	Tr	-	Tr	-
Δ-amorphene	1513	0.1	0.01	Tr	-	0.1	0.01	0.1	0.01	Tr	-	Tr	-	0.04	0.01
β-Sesquiphellandrene	1525	0.4	0.04	0.06	0.01	0.3	0.03	0.3	0.02	Tr	-	0.3	0.03	0.4	0.04
Spathulenol	1577	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	Tr	-	0.06	0.01
Caryophyllene oxide	1585	0.1	0.01	Tr	-	Tr	-	0.1	0.01	Tr	-	0.2	0.02	0.2	0.02
Total		99.96		99.68		99.59		99.99		99.79		99.52		99.48	

[a] essential oils of plants collected from the Z1-Z7 areas. [b] Compounds were listed in order of their elution from a DB-5MS column. [c] RI, retention indices as determined on DB-5MS column using homologous series of *n*-alkanes. Tr= trace (< 0.01%).

Table 2: Chemical variability of the major compounds of *T. serrulatus* essential oil from Z1-Z7 areas. Values within a row for each compound having different letters are significantly different from each other using Tukey's LSD test (P<0.05).

Compounds	Z1	Z2	Z3	Z4	Z5	Z6	Z7
o-Cymene	3.11c	3.30 d	3.59 e	2.96 b	2.79 a	3.26 cd	3.24 cd
γ-Terpinene	2.75 e	3.07 f	2.01 c	2.56 d	2.49 d	1.45 b	1.1 a
Linalool	1.80 d	1.60 c	0.51 a	1.21 b	1.16 b	1.22 b	1.56 c
thymol	2.30 a	2.80 c	2.73 c	3.50 d	3.75 e	2.46 b	2.26 a
Carvacrol	3.02 c	1.48 a	2.93 c	2.63 b	2.65 b	3.60 d	3.63 d

Experimental

Plant materials: Leaves and flowers of *T. serrulatus* growing in different area of Ethiopia were collected in May-June 2007 and dried at ambient temperature. A large number of plants (>5 kg) were randomly collected over the same area. The plants were collected in seven areas (Z1-Z7). Voucher specimens were deposited in the Herbarium of the CAMS – Univ. of Perugia (IPO-E1-05-07).

Extraction of oil: The plants were subjected to hydrodistillation using a Clevenger-type apparatus for 3 h yielding 0.8 ± 0.1% (mean value) of a yellowish oil. The oil was dried over anhydrous sodium sulfate and stored in sealed vials under refrigeration prior to analysis.

GC and GC-MS analysis: The GC analyses were carried out using an Agilent 6890N instrument equipped with a FID and an HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 μm), working from 60°C (3 min) to 210°C (15 min) at 4°C/min or an DB-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 μm) working from 60°C (3 min) to 300°C (15 min) at 4°C/min; injector and detector temperatures, 250°C; carrier gas, helium (1 mL/min); split ratio, 1 : 10.

GC-MS analyses were carried out using an Agilent 5975 GC-MS system operating in the EI mode at 70 eV, using the two above mentioned columns. The operating conditions were analogous to those reported in the GC analyses section. Injector and transfer line temperatures were 220°C and 280°C, respectively. Helium was used as the carrier gas, flow rate 1 mL/min. Split ratio, 1 : 10.

Identification of the components: The identification of the components was made by matching their spectra with those from mass spectral libraries and the identity of each component was confirmed by comparing their retention indices, for both columns, relative to the C6-C22 *n*-alkanes with those from the literature. When reported, co-elution gas chromatography with reference compounds was used for an additional confirmation of the compound identity. The percentage composition of the essential oil was obtained by the normalization method from the GC peak areas, without using correction factors.

Experimental design: The plants collected in each area were hydrodistilled separately and the ghost effect was minimized. The handling data was the percent concentration of the identified compounds. If we suppose that hydrodistillation of 7 batches of the same lot gives for each compound values ±10% across the mean, from each percent composition of the 7 hydrodistilled oils, we might obtain 7 parent percent oil compositions. A 49% composition (7 oils for 7 areas (Z1-Z7)) for 53 oil compounds were the basic data file.

Data file handling: Many problems arose from this type of database in a statistical analysis. The presence of compounds above 0.01% (tr) required a valuation: the addition to the database of 0.01, gave a statistical improvement and removed the null value. Compounds scarcely present in the database were removed. The independence of the data were checked using a linear or logaritmic coefficient of correlation between each pair of compounds: one of the two compounds with R >0.98 was removed (between limonene and β-phellandrene there was a high logarithmic correlation and β-phellandrene was removed). The great data dishomogeneity suggests the use of the Box-Cox method for the choice of most useful data transformation. The log-likelihood function suggests the use of natural logarithmic data transformation. According to Kolmogorov-Smirnov tests, the database in each group follows a normal distribution and according to the Levene test the variance of the database is the same in the groups (homoschedasticity assumption).

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