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Essential oil composition and variability in *Hyptis fruticosa*

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Abstract: The composition of six samples of essential oil (EO) extracted from leaves, flowers and seeds of several plants of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, was investigated by GC/MS and GC/FID. 1,8-Cineole, spathulenol, α-pinene, β-pinene were the major constituents. Ten constituents that have not been previously described in the composition of the oil of *H. fruticosa* were identified. Hydrocarbons sesquiterpenes represented the main group, followed by hydrocarbons monoterpenes. The results were submitted to Cluster Analysis which allowed three groups of EO to be distinguished with respect to the content of α-pinene/β-pinene, 1,8-cineole and spathulenol. Growth stages of the plants and geographical parameters seem to be important factors determining the variability of the oil. Sesquiterpenes were mainly produced in the seeds.

Introduction

The genus *Hyptis* Jacq. consists of approximately 400 species distributed from the South of the United States to Argentina (Bordignon, 1992), and exhibit a major morphological diversity in the Brazilian Cerrado (Harley, 1988). Plants of *Hyptis* spp. are of great economical and ethnopharmacological importance and they have been alleged to possess medicinal properties. Their use is recommended in folk medicine for the treatment of several diseases such as gastrointestinal disorders, skin infections, nasal congestion, fever, cramps, and pain (Pio-Corrêa, 1931; Rojas et al., 1992; Septímio, 1994). Furthermore, antifungal, antimicrobial, and insecticidal properties have been reported (Souza et al., 2003; De Oliveira et al., 2004).

The annual species *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, known as "alecrim-do-campo" in the state of Sergipe, Northeast Brazil, is an aromatic uncultivated shrub popularly used as an analgesic, with anticonvulsive activities. Antinociceptive effect and the acute toxicity of the essential oil of *H. fruticosa* was demonstrated (Menezes et al., 2007). Diterpenoid quinones have been isolated from the roots and presented antimicrobial and antitumoral activities (De Araújo et al., 1974; Marletti et al., 1976). Pharmacological studies correlating the effects of the oil to the cardiovascular system were carried out in rats (Santos et al., 2007). Moreover, the oil have been considered as an important alternative insecticide for the control of *Aedes aegypti* larvae, since it provides a rich source of bioactive compounds that are biodegradable and non toxic (Silva et al., 2008). Although relatively few works dealing with the essential oil of *H. fruticosa* have been performed, it appears from the data reported that only random samples have been analyzed or utilized, and obtained exclusively from the leaves.

The aim of this work was to evaluate the composition and variability of the essential oil of *H. fruticosa* extracted from the leaves during vegetative, flowering and fruiting stages, as well as from the flowers and the seeds. Considering the distribution of this plant, the essential oil from leaves (vegetative stage) collected in a different location with diverging environmental conditions, was also examined. Knowledge about important differences in amounts and incidence of the major constituents would be a probable contribution towards technical strategies for potential studies.

Material and Methods

Plant material

Aerial parts of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, were collected from plants at

different growth stages, distributed randomly in an area of 10000 m^2, near São Cristóvão at Sítio Tujubeba 10°56' LS and 37°11' LW, altitude 45 m (Coastal Tablelands). The leaves, in August 2008, vegetative growth stage (A); November 2008, flowering growth stage (B), and April 2009 fruiting growth stage (C). The flowers (D) in December 2008 during the peak of blossoming; the seeds (E) in April 2009. Moreover, in April 2009 leaves were collected from individuals of the same plant species, distributed randomly in a corresponding area during the vegetative growth stage (F), in a different location with diverging geographical parameters, at Parque Nacional da Serra de Itabaiana (PNSI) 10°45' LS and 37°20' LW, altitude 300 m. Analysis of the soil was performed by the Instituto Tecnológico e de Pesquisas do Estado de Sergipe (ITPS) according to MAQS-Embrapa (1999). The plants were identified by Dr. Ana Paula Prata and the voucher specimens of each location (10.922, Sítio Tujubeba; 13.640, PNSI) are deposited in the Herbário da Universidade Federal de Sergipe (UFS).

Extractions and analysis of essential oil

The oil was obtained from fresh leaves, flowers and seeds by hydrodestilation in a Clevenger-type apparatus until no more condensing oil could be seen (3 h). The analysis of the chemical composition was performed at the Laboratório de Cromatografia, Departamento de Química.

Gas Chromatography-Mass Spectrometry

Oil sample analysis was performed on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC/MS) instrument employing the following conditions: column J&W Scientific DB-5MS (Folsom, CA, USA) fused silica capillary column (30 cm x 0.25 mm i.d, composed of 5% phenylmethylpolysiloxane), operating in electron impact mode at 70 eV; Helium (99.999%) was used as carrier gas at a constant flow of 1.2 mL min-1 and an injection volume of 0.5 µL was employed (split ratio of 1:83) injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 50 °C (isothermal for 2 min), with an increase of 4 °C/min., to 200 °C, then 10 °C/min to 300 °C, ending with a 10 min isothermal at 300 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Gas-Chromatography (GC/FID)

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) equipment, under the following operational conditions: capillary ZB-5MS column (5% phenyl-arylene-95%-dimethylpolysiloxane) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA), under same conditions GC/MS. Quantification of each constituent was estimated by area normalization (%). Compounds concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Identification of essential oil constituents

Identification of individual constituents was performed by computerized matching of the acquired mass spectra with those stored in NIST21 and NIST107 mass spectral library of the GC/MS data system. A mixture of hydrocarbons (C_9H_{20}-$C_{19}H_{40}$) was injected under these same conditions and identification of constituents was then performed by comparing the spectra obtained with those of the equipment data bank and by the Kovats index, calculated for each constituent as previously described (Adams, 2007). Retention indices were obtained with equation proposed by Van Den Dool & Kratz (1963).

Statistical analysis

With the objective of verifying the difference in between samples from different locations, as well as from different sampling periods, a Normality Test was used to determine whether the data set was well-modeled by a normal distribution. Saphiro-Wilk (Royston, 1982) test was utilized and the results indicated that none of the variables had a normal distribution. The results made it impossible to use a parametric test to compare involved variables. The non-parametric test used for independent samples was Wilcoxon-Mann-Whitney (Siegel & Castellan, 2006). A *p*-value less than 0.05 rejects the null hypothesis of differences between samples (Table 1). Data was submitted to Cluster Analysis (Wichern & Johnson, 2007), in order to identify which constituent differentiated each sample. Cofenetic correlation coefficient was calculated (Sneath & Sokal, 1973). To perform the tests Saphiro-Wilk, Wilcoxon-Mann-Whitney and the Cluster Analysis, the software R Development Core Team (2009) was applied.

Table 1. Statistics and *p*-value for Shapiro-Wilk normality test.

	A	B	C	D	E	F
Statistics	0.48	0.46	0.49	0.49	0.40	0.78
p-value	8.07 E-13	5.27 E-13	1.03 E-12	1.14 E-12	8.27 E-14	7.61 E-08

Results and Discussion

Analysis performed by ITPS (MAQS-Embrapa, 1999), confirmed the low fertility of the soil (Table 2). Tujubeba soil is a Red-Yellow Dystrophic Sand-Argisoil (Tb A arenic). The climate is tropical sub-humid, annual average precipitation of 1.300 mm (Melo et al., 2004). The PNSI soil is a Litholic pre-Cambrian, Red-Podzolic Eutrophic, and Dystrophic Quartz Sand. The climate is semi-arid, annual average precipitation ranging from 700 to 900 mm (Santos & Andrade, 1992). The rainfall period for the whole region starts from April through to August, with a dry summer.

The EO from seeds E and flowers D collected at Sítio Tujubeba yield 0.11% and 0.25% respectively, and from leaves A, B, and C gave an average yield of 0.46%, SD=0.06. The EO from leaves F collected at PNSI yield 0.75%.

Table 3 shows the qualitative and quantitative analytical results. A total of 56 compounds were identified accounting for about 99% of the volatile constituents.

The EO sample from leaves A accounted for 29 volatile constituents corresponding to 99.03% of the identified composition; in leaves B, 38 constituents (98.96% identified); in leaves C, 31 constituents (94.35% identified); in flowers D, 35 constituents (94.30% identified); in seeds E, fourteen constituents (68.43% identified). In the EO from leaves F, 41 constituents were identified corresponding to 93.33%.

In leaves A, the EO contained mainly hydrocarbon monoterpenes (45.52%); in leaves B the oxygenated monoterpenes prevail (37.3%), as well as for leaves C (30.13%). The flowers D contained mostly hydrocarbon monoterpenes (46.69%), and in the seeds E, oxygenated sesquiterpenes was the main group of constituents (48.51%). The prevailing group in leaves F (PNSI) was the hydrocarbon sesquiterpenes (38.11%). Considering the individual amounts identified only in the leaves and flowers, important differences were found mainly in 1,8-cineole (6.10-25.55%) which predominated in B and C; α-pinene (4.52-21.21%) the major component in the A and D, respectively; β-pinene (4.76-14.25%) the second major constituent in D and the third major in A. Camphor (2.13-7.40%), with higher concentration in B. Other major constituents, for instance limonene (3.91-7.62%), β-caryophyllene (4.77-8.79%), and bicyclogermacrene (1.93-9.30%) had a significant variation throughout the samples.

In leaves F, none of the constituents reached 10%, although exclusive constituents such as *p*-menta-1(7),8-diene (0.34%), α-terpinene (0.23%), *cis*-muurola-4(14),5-diene (0.83%), *cis*-cadin-4-en-7-ol (0.36%), eudesm-7(11)-en-4-ol (2.10%) were identified.

In the EO from leaves A the concentrations of the major constituents are by far higher than in leaves F, for example α-pinene (21.21%). They are statistically different with p=0.09588 in average, significant for the geographic divergence. On the other hand, constituents identified in flowers D, showed a composition comparable qualitatively and quantitatively to the oil sample in leaves A (Figure 1). All the compounds were previously found in the essential oil or scent of other flowers species (Zoghbi et al., 2001; Jürgens et al., 2002). Two constituents, α-thujene (0.18%) and (*E*)-β-ocymene (0.38%) were identified only in D. Only fourteen constituents were identified in the EO from seeds E, with spathulenol (22.57%) as the major constituent. The

Table 2. Analysis of the soil fertility.

Essay	Results		Unities	MDL[a]	
	Tujubeba	PNSI			
pH in water	5.09	5.70			
Organic matter	14.50	24.10	gr/dm^3		
Calcium + Magnesium	1.63	1.66	cmolc/dm^3	0.30	0.30
Aluminum	0.45	0.28	cmolc/dm^3	0.05	0.05
Sodium	0.07	0.02	cmolc/dm^3		
Potassium	0.07	0.02	cmolc/dm^3		
Hidrogen + Aluminum	3.40	1.43	cmolc/dm^3		
Sodium	16.90	4.90	ppm	1.50	1.50
Potassium	26.30	9.10	ppm	0.70	0.70
Phosphorus	ND[b]	2.70	ppm	0.70	0.70
Iron (Fe)	233.17	6.41	ppm		0.044
Coper (Cu)	0.90	0.23	ppm		0.010
Manganese (Mn)	0.86	0.79	ppm		0.030
Zinc (Zn)	3.48	0.30	ppm		0.070

[a]MDL: Method Detection Limits; [b]ND: Not detected.

Table 3. Constituents of essential oil of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, obtained from the leaves collected at Sítio Tujubeba during vegetative stage (A), flowering stage (B), fruiting stage (C), and at PNSI during the vegetative stage (F). Constituents from flowers (D) and seeds (E).

KI[a]	Constituent	Leaves A	Leaves B	Leaves C	Flowers D	Seeds E	Leaves F
924	α-Thujene				0.18		
931	α-Pinene	21.21	5.37	10.53	20.51		4.52
947	Camphene	0.66	1.14	1.21	0.61		0.63
967	Sabinene	2.11	0.87	1.87	2.03		5.09
973	β-Pinene	14.25	4.76	7.66	13.64		5.78
974	Octen-3-ol		0.52				
988	Myrcene	0.89	0.74	0.76	0.90		0.59
1006	*p*-Menta-1(7),8-diene						0.34
1008	α-Phellandrene				0.27		0.50
1019	α-Terpinene						0.23
1023	*p*-Cymene	0.92	1.50	0.58	0.36		0.59
1027	Limonene	4.84	4.07	3.91	4.08		7.62
1029	β-Phellandrene				2.31		4.86
1031	1,8-Cineole	16.08	25.55	23.41	12.46	2.06	6.10
1046	(*E*)-β-Ocymene				0.38		
1056	γ-Terpinene	0.64	0.62	1.03	1.07		1.35
1087	Terpinolene			0.39	0.35		0.41
1089	*p*-Cimenene		0.54				
1099	Linalool		1.02	0.59	0.47		
1146	Camphor	2.45	7.40	4.62	2.13	2.97	4.00
1171	Borneol	2.05	2.67	1.51	1.73	1.83	0.74
1174	Terpinen-4-ol		0.36		0.21		0.45
1194	α-Terpineol		0.30				
1345	α-Cubebene	1.07	1.32	1.05	0.96		1.28
1374	α-Ylangene		2.22				
1376	α-Copaene	2.24		2.02	2.07		3.11
1382	β-Bourbonene		0.24	0.38			0.26
1386	β-Cubebene	0.89	0.90	0.84	0.79		0.88
1417	β-Caryophyllene	6.82	8.79	6.30	6.43	4.77	7.30
1448	*cis*-Muurola-3,5-diene		0.80		0.20		0.45
1453	*trans*-Muurola-3,5-diene	0.38		0.89			0.86
1454	α-Humulene	1.19	1.48	1.24	0.80		1.49
1460	*cis*-Cadina-1(16),4-diene		0.55				
1467	*cis*-Muurola-4(14),5-diene						0.83
1479	γ-Muurolene		3.39		2.52		
1486	Germacrene-D	2.29		2.13			2.53
1489	Viridiflorene	1.47	1.36	1.07	1.46		2.57
1494	Bicyclogermacrene	5.27	9.30	8.43	6.05	1.93	7.00
1496	α-Muurolene		0.84				
1512	δ-Amorphene		1.27				
1513	γ-Cadinene			1.22	0.62		1.10
1516	δ-Cadinene	1.18		0.51	0.60	1.61	1.56
1519	*trans*-Calamenene	1.71	0.68	1.01	1.01		1.19
1520	*cis*-Calamenene					2.06	
1531	*trans*-Cadina-1,4-diene	0.57	1.18	0.71	0.85		1.42

Table 3. (cont.)

1534	α-Cadinene	1.32	0.74	0.97	1.35	1.53	4.28
1575	Spathulenol	2.61	2.99	4.10	2.36	22.57	2.88
1580	Caryophyllene oxide	1.58	1.22		1.36	8.70	1.96
1586	β-Copaen-4-α-ol		0.29				
1592	Viridiflorol	1.25	1.05	2.03	1.18	5.86	2.09
1639	*cis*-Cadin-4-en-7-ol						0.36
1640	*epi*-α-Cadinol		0.32			2.78	
1663	α-Cadinol	0.58		1.38		8.60	1.59
1691	Shyobunol		0.60				
1700	Eudesm-7(11)-en-4-ol						2.10
1979	Manool oxide	0.51				1.16	0.44
	Aliphatic alcohol		0.52				
	Monoterpenes hydrocarbons	45.52	19.61	27.94	46.69		32.51
	Oxygenated monoterpenes	20.58	37.30	30.13	17.00	6.86	11.29
	Sesquiterpenes hydrocarbons	26.40	35.06	28.77	25.71	11.90	38.11
	Oxygenated sesquiterpenes	6.02	6.47	7.51	4.90	48.51	10.98
	Diterpenes	0.51				1.16	0.44
	Total identified	99.03%	98.96%	94.35%	94.30%	68.43%	93.33%

[a]KI - Kovats Indexes experimental.

higher concentration of manool oxide (1.16%) occurred in E (Table 3).

The constituents that have not been reported in the composition of the EO of *H. fruticosa* were the monoterpenes hydrocarbons: α-thujene, *p*-menta-1(7),8-diene, α-phellandrene, α-terpinene, (*E*)-β-ocymene, and terpinolene; a sesquiterpene hydrocarbon *trans*-muurola-3,5-diene; the oxygenated sesquiterpenes *cis*-cadin-4-en-7-ol and eudesm-7(11)-en-4-ol, plus the labdane-type diterpenoid manool oxide (Menezes et al., 2007; Silva et al., 2008).

The results showed considerable differences between the EO samples. In leaves F it is probably due to the edaphic-climatic parameters. However, the variations in composition for the other samples appear to be related to reproductive strategies and environmental interactions. Table 4 shows the *p*-values for the differences between samples. Chemical polymorphism in the genus *Hyptis* have been investigated by several authors (Azevedo et al., 2001; Arrigoni-Blank et al., 2005). Results showed that genetic variations within the same species, as well as the climate and soil types, could vary the chemical characteristics of the active constituents. Infraespecific variability within the essential oil of *H. suaveolens* was reported (Azevedo et al., 2002). Cluster analysis (Figure 1) demonstrated the similarity of the chemical composition of the EO amidst the six samples. The cofenetic correlation was elevated: 0.9478206, indicating that the dendrogram did not show distortion capable of interfering in the interpretation of the results. The analysis separated the main groups: hydrocarbon monoterpenes (41.57%, SD=7.87) in the Cluster I; oxygenated monoterpenes (33.71%, SD=5.06) in the Cluster II and oxygenated sesquiterpenes (48.51%, SD=48.51) in the Cluster III.

Consequently, three main types of oil were found: Cluster I (50% of the samples) was characterized by the substantial percentages of α-pinene (20.86%, SD=0.35), β-pinene (13.94%, SD=0.30); Cluster II (33.3% of the samples) had 1,8-cineole (24.48%, SD=1.07) as the main compound, bicyclogermacrene (8.86%, SD=0.43),β-caryophyllene (7.54%, SD=1.24), camphor (6.01%, SD=1,39); and Cluster III (16.6% of the samples) showed a substantial percentage of spathulenol (22.57%, SD=22.57), caryophyllene oxide (8.70%, SD=8.70), and α-cadinol (8.60%, SD=8.60). The mean chemical composition of each Cluster is presented in Figures 2-4.

The composition of the EO from Sítio Tujubeba collected in November 2002 (Silva et al., 2008) is not

Table 4. Statistics and *p*-value for comparison in between samplings.

	A-B	A-C	A-D	B-C	B-D	C-D	A-F
Statistics	1422	1545	1500	1681	1663	1530	1289
p-value	0.3813	0.8878	0.6824	0.5022	0.5762	0.8210	0.09588

similar to any of the samples observed in this study. In the same way, the oil analyzed in July 2003 (Menezes et al., 2007) presented a composition closer to leaves C (Cluster II), although, the main component 1,8-cineole showed a much lower percentage (16.68%). Santos et al. (2007) reported that EO of *H. fruticosa* induced hypotensive effect with the increase of heart rate in rats, and hypothesizes that these effects could be due to the presence of constituents such as α-pinene, caryophyllene, and 1,8-cineole, substances with potent physiological effects. Silva et al. (2008) tested selected compounds of EO with high larvicidal activity, such as γ-terpinene, caryophyllene oxide, found in *H. fruticosa* in lower percentages. Conversely, 1,8-cineole a major constituent is not the active principle in larvicidal bioassays. However, isolated or synergistical actions of these constituents obtained from several samples, have not been systematically tested.

On the other hand, these constituents have been tested elsewhere, and exert inhibition on the growth of plants and seed germination (Harborne, 1993). A number of terpenes can act as toxins, repellents or attractants to other organisms. It is assumed that they have ecological roles in antagonistic or mutualistic interactions (Gershenzon & Dudareva, 2007). Nishida et

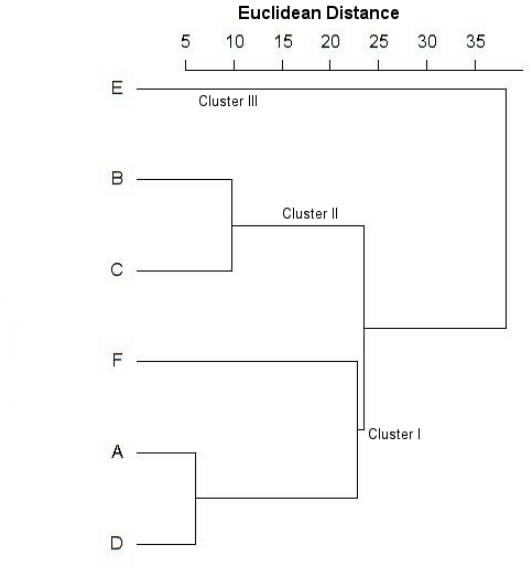

Figure 1. Two-dimentional dendrogram representing EO composition similarity relationships amidst six samples of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae. Samples are represented by letters corresponding to source, and are clustered using Euclidean Distance furthest neighbour complete linkage (Wichern, 2007). Cofenetic correlation coefficient r= 0.9478206.

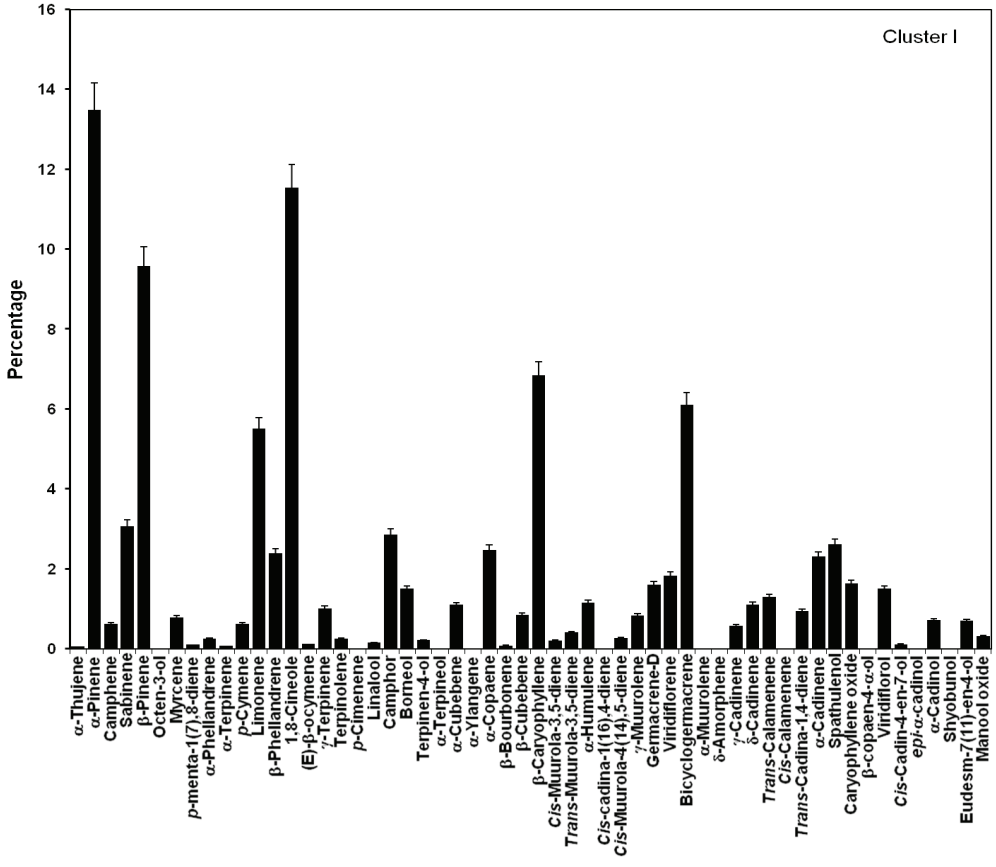

Figure 2. Mean chemical composition of the essential oil cluster I of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae. Vertical lines show ± standard deviations.

al. (2005) reported that *Salvia leucophylla* (Lamiaceae) produces several volatile monoterpenoids, for instance: camphor, 1,8-cineole, α-pinene, β-pinene, and camphene that potentially act as allelochemicals. In conclusion, considering the large number of important terpenes and the broad distribution of this species, little is known about the way they succeed. A full understanding of the function of the volatiles constituents of *H. fruticosa* in defense or attraction requires knowledge.

The results obtained in this work showed that oil samples from *H. fruticosa* can have a changeable composition, depending on sampling period, location, and parts of the plant. Experiments using the whole oil may have to take into consideration the above mentioned aspects. Further studies taking in account chemical polymorphism and infraespecific variability, are necessary.

Acknowledgments

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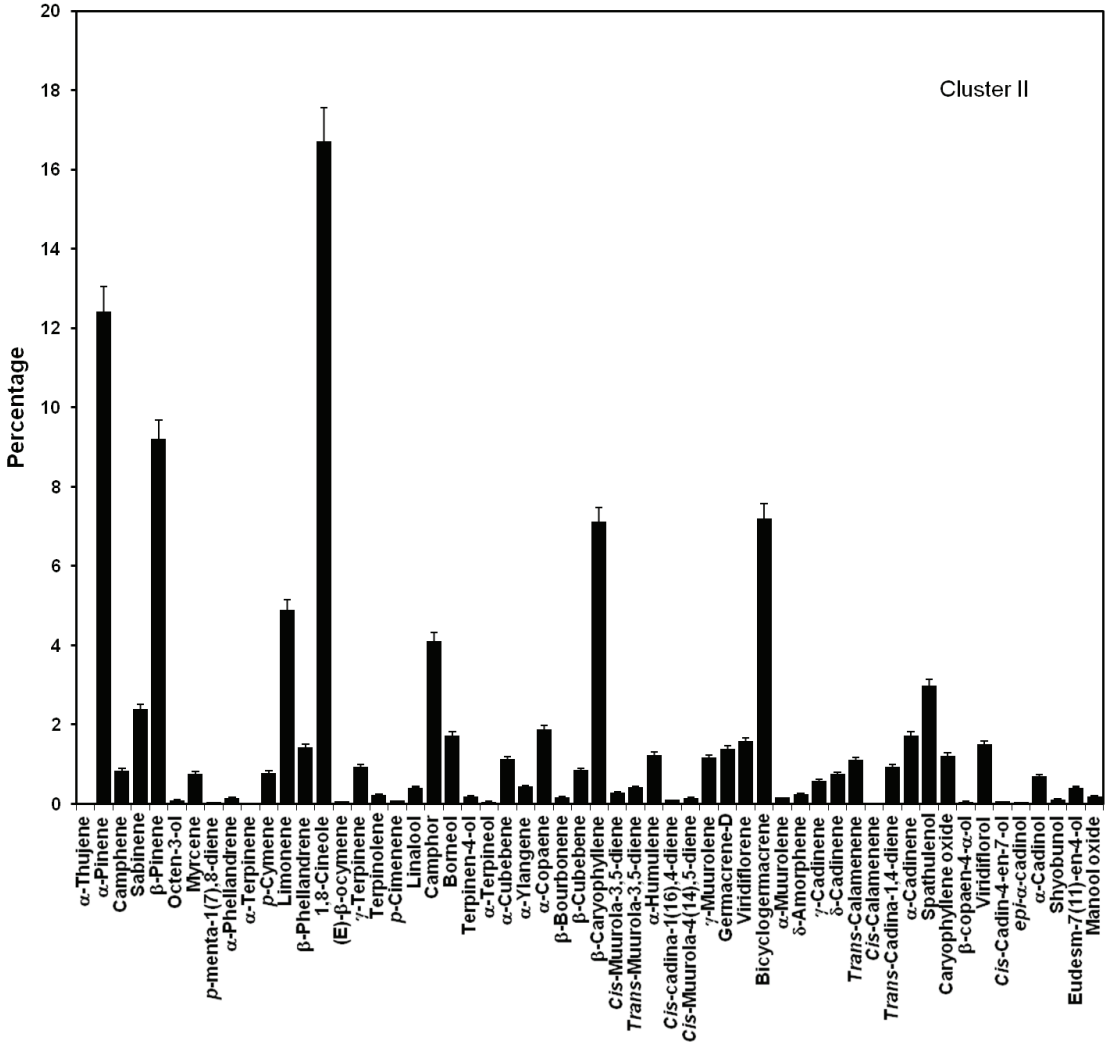

Figure 3. Mean chemical composition of the essential oil cluster II of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae. Vertical lines show ± standard deviations.

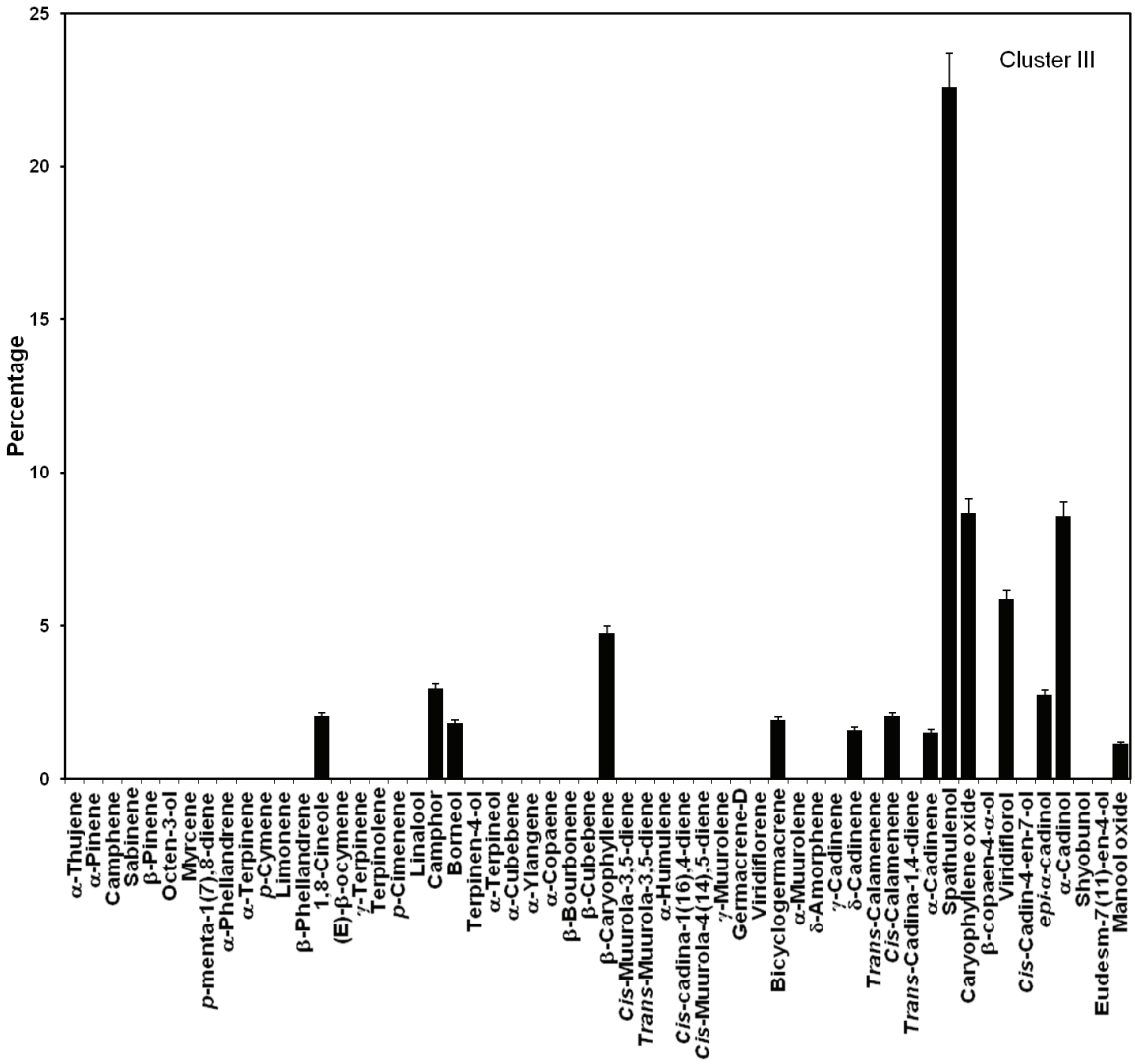

Figure 4. Mean chemical composition of the essential oil cluster III of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae. Vertical lines show ± standard deviations.

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