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CAPILLARY GC-MS ANALYSIS OF VOLATILE AND SEMI-VOLATILE COMPOUNDS OF *THYMUS GLABRESCENS* WILLD.

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This work deals with the chemical composition of the aerial part of *Thymus glabrescens* essential oil isolated by steam distillation using a Clevenger apparatus. The chemical content of volatile and semi-volatile compounds of the plant infusion and tincture is presented. The volatile compounds from infusion and tincture were isolated by Solid Phase Extraction (C_{18} cartridge) and elution with dichloromethane. Essential oil diluted in dichloromethane and the infusion and tincture dichloromethanic extracts were analysed by gas chromatography coupled with mass spectrometry (GC-MS). The main constituents of volatile oil were mono- and sesquiterpenic hydrocarbons (5.68% and 11.95%), monoterpenic alcohols (16.33%), sesquiterpenic alcohols (44.8%). The content in main volatile compounds of the infusion and tincture was estimated quantitatively using linalyl acetate as internal standard. The analysis of volatile compounds from *Thymus glabrescens* shows that through infusion and maceration most of the mono- and sesquiterpenic hydrocarbons are lost.

INTRODUCTION

The use of plant extracts, as well as other alternative forms of medical treatment, has enjoyed great popularity in the last 15 – 20 years. Many species from the spontaneous flora become an increasingly important source for the pharmaceutical industry.

Thyme (*Thymus*) is a genus of about 350 species of aromatic perennial herbs from the family *Lamiaceae*. They are native to Europe, North Africa and Asia. Some *Thymus* species grow in Romania, on the mountainous pastures, at altitudes ranging between 400 and 900m. Various species of Thymus have been traditionally used as medicinal plants, as antiseptics, antispasmodics, sedatives, stimulants and tonics. These activities are due mainly to their volatile and semivolatile content. Wild *Thymus* species (such as *Thymus serpyllum*) are an important source plant for honey bees.

The chemical composition of the essential oils proved to be very different from species to species, [1-12] varying also with soil and climatic conditions [13-15] and vegetation stage at the moment of harvest. [16, 17] For instance, in *Thymus vulgaris*, which due to its flavour is mainly used as culinary herb, the main compound is thymol, reaching up to 80% of the total oil. [9] Previous research [18] concerning the species *Thymus alpestris* showed that the predominant compound in the volatile oil is carvacrol (47.74%), another phenol well-known for its antimicrobial activity.

The scientific literature is rich in information concerning the chemical composition of some species of Thymus (wild or cultivated), but others, such as *Thymus glabrescens*, have been studied less. [19, 20] Knowing the composition in volatiles of this species is important, especially since the aerial part of *Thymus glabrescens* Willd. is a component of *Serpylli herba*,[21, 22] which is used in therapeutics.[23]

The aim of the present study is to elucidate the chemical composition of essential oil from the aerial part of *Thymus glabrescens* Willd., growing wild in Roumania.

Many species of Thymus are used as infusions or tinctures, which are more easily obtained, and therefore it is important to know the content in volatile compounds of these products as well. This study tries to obtain also a quantitative estimation of the volatile compounds from infusion and tincture of *Thymus glabrescens*.

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EXPERIMENTAL PART

1. Reagents and solvents

All solvents and reagents were purchased from Merck, Darmstadt, Germany: dichloromethane, methanol were SupraSolv for gas chromatography; ethanol 96% reag. Ph. Eur; cartridges for solid-phase extraction were LiChrolut RP-18 columns, 500mg; anhydrous Na_2SO_4 granulated for organic trace analysis was used; authentic standards were 1.8-cineol (eucalyptol), (1S)-(+)-borneol, linalyl acetate, bornyl acetate, (+)-camphor, borneol, farnesol, all of them reag. Ph. Eur.

The $C_8 - C_{20}$ n-alkanes used for the determination of the Kovats retention indexes were also from Merck.

2. Plant material

The aerial plant material of *Thymus glabrescens* Willd. was harvested during its flowering stage, from mountainous pastures with altitudes between 400 and 800m, in the Apuseni Mountains, Roumania.

3. Essential oil extraction

The 50 grams of air dried vegetal product were hydro-distilled with 400mL water in a Clevenger-type apparatus for 3h. The essential oil was dried over anhydrous Na_2SO_4, stored in a dark glass bottle and kept at 4°C until analysis. The oil sample was diluted in dichloromethane (1/200) for GC analysis and 2µL were injected.

4. Infusion sample preparation

100mL of boiling water were poured over samples of 5g of *Thymus glabrescens* plant. After approximately 30min the cold extracts were filtered on glass fiber filter. The residue from the filter was rinsed three to four times with distilled water, and the final filtrated infusion was adjusted to 100mL volume.

5. Tincture sample preparation

The tinctures were prepared according to the working model from the Romanian Pharmacopoeia.[24]

A. Preparation of the tincture through maceration

Ten grams of finely cut plant were maintained in a bottle in darkness with 100g (70%) ethanol, for 10 days, and stirring 3-4 times a day. After that the solution was filtered and adjusted to 100g with 70% ethanol. The tincture was stored at 4°C.

B. Preparation of the tincture through percolation

Ten grams of air dried plant were moistened in 5g of solvent (70% ethanol) for 3h in a closed recipient. The content of the recipient was then transferred in the percolator where it was kept for 24h for maceration. After this time, the apparatus was set so that 1.5g of tincture were obtained for 1g of sample per day. After seven days the obtained tincture was adjusted to 100g, using the same solvent.

6. Solid-phase extraction of infusion and tincture pharmaceutical forms

A 500mg C_{18} solid-phase extraction (SPE) cartridge was conditioned by eluting it with 2 x 4mL dichloromethane, followed by 2 x 4mL of methanol. The cartridge was allowed to dry after each flush. Then 2 x 4mL of distilled water were passed through the cartridge (the cartridge should not be allowed to dry before sample application). By applying a slight vacuum, the obtained infusion was allowed to pass through the cartridge at a flow of 1 – 2 mL/min. The cartridge was then dried for 5 – 10 min with nitrogen. The compounds retained on C_{18} cartridge were eluted with 2 x 1mL dichloromethane. For the quantitative analysis, the RP-18 column was eluted with 2 x 1mL solution of the internal standard (linalyl acetate), 200ng/µL concentration.

For solid-phase extraction of tincture pharmaceutical form, 10mL tincture were diluted with distilled water to 1000mL. 500 mL of the water solution were allowed to pass through the C_{18} cartridge, and after that the same mode of operation used for the infusion was applied.

7. Gas chromatography – mass spectrometry

GC–MS analyses were carried out with a Fisons Instruments GC 8000, equipped with an electron impact quadrupole, MD 800 mass spectrometer detector. The electron ionization energy was 70eV, ion-source temperature 200°C and the interface temperature 280°C. A split–splitless injection (split ratio 1:30) at 280°C was employed.

A fused silica column 5% phenylpoly (dimethylsiloxane) (DB-5MS 30m x 0.32mm i.d. and 0.25µm film thickness, J&W Scientific) was used. The oven temperature was programmed as follows: from 40°C (3 min hold) raised at 5°C/min to 120°C (2 min hold), then at 10°C/min to 250°C and finally hold at 250°C for 5 min. The carrier gas (helium) flow rate was 2mL/min. Two microliter of sample were injected. These conditions were applied for essential oil, infusion and tincture samples. Data acquisition was performed with MassLab software for the mass range 30–600u with a scan speed of 1 scan/s.

The identification of compounds was performed by comparing their mass spectra with data from Adams,[25] US National Institute of Standards and Technology (NIST, USA), WILEY 1996 Ed. mass spectra library and a personal library of 600 spectra. The identification of compounds was also based on the Kovats retention indices.

The Kovats retention indices were calculated by using n-alkanes $C_8 - C_{20}$ and the experimental values were compared with those reported in the literature. [26, 27]

8. Quantitative analysis

In order to determine quantitatively the content of the main compounds from tincture and infusion, the sample elution from the C_{18} cartridge was done with a solution of linalyl acetate (used as internal standard), 200ng/µL concentration.

RESULTS AND DISCUSSION

The average content in volatile oil of *Thymus glabrescens* samples (5 determinations) was rather small, 4.0mL/kg, but above the minimum value of 3.0 mL/kg specified by the European Pharmacopoeia for the aerial part of *Thymus serpyllum*. [23] This value is comparable with previous results obtained on the same species of Thymus, harvested in Romania. [28]

In fig. 1 are shown the chromatograms of essential oil (a), tincture (b) and infusion (c). Table 1 shows the relative content of volatile compounds from essential oil, expressed as percentage from total area.

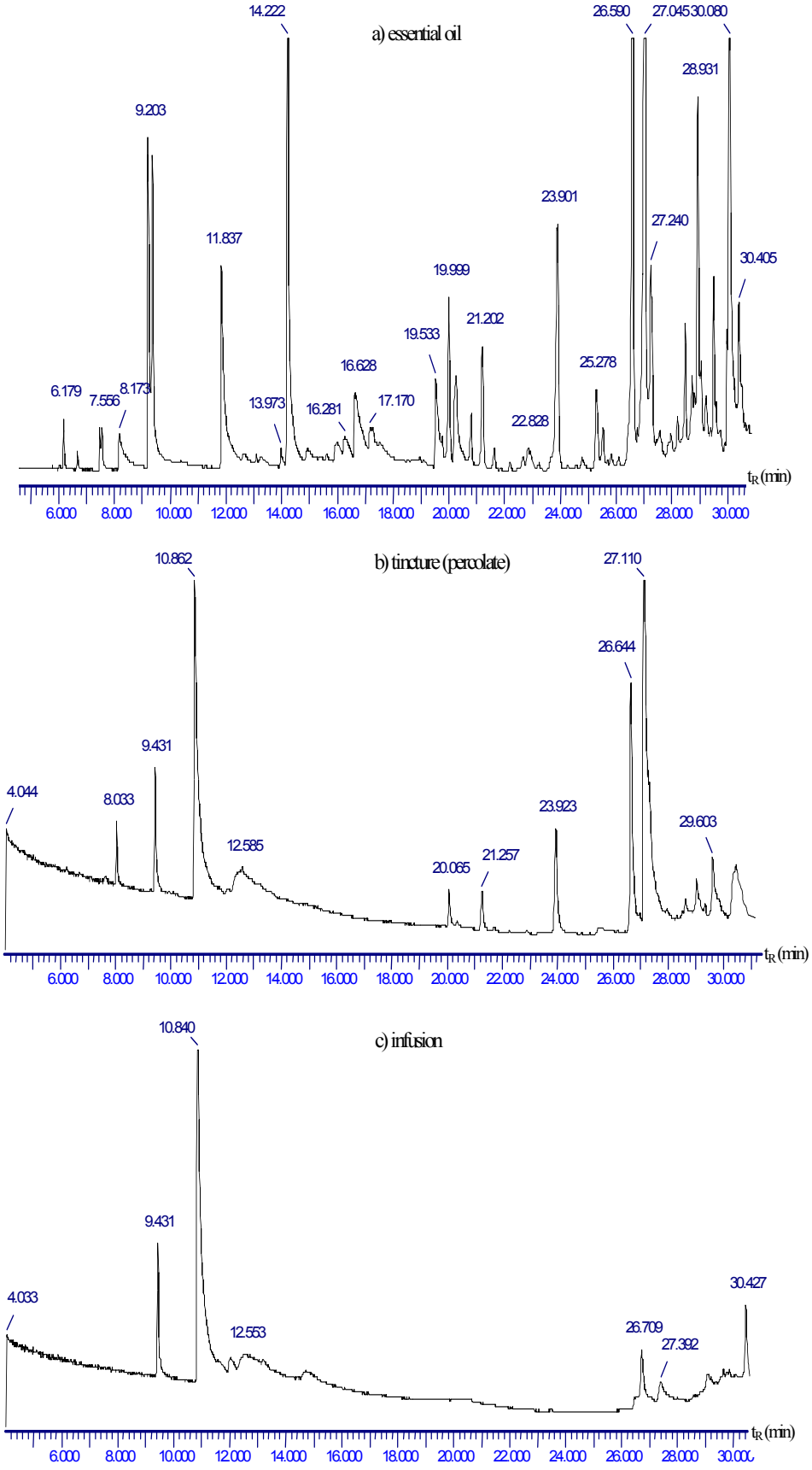

Fig. 1 – Chromatograms of *Thymus glabrescens*: essential oil (a), tincture (b) and infusion (c).

Table 1

Chemical composition of *Thymus glabrescens* essential oil

No.	Compounds	t_R (min)	Kovats Retention Indexes	Area (%)
1	*α*-pinene	6.179	916	0.35
2	camphene	6.667	931	0.12
3	sabinene	7.480	955	0.28
4	*β*-pinene	7.556	958	0.42
5	myrcene	8.173	976	1.30
6	*p*-cymene	9.203	1007	3.21
7	1.8-cineol	9.366	1013	3.82
8	linalool	11.837	1087	4.29
9	p-menth-2-en-1-ol	12.628	1112	0.60
10	camphor	13.095	1127	0.18
11	borneol	13.973	1132	0.21
12	terpin-4-ol	14.222	1164	7.49
13	*α*-terpineol	14.927	1188	0.34
14	nerol	15.989	1217	0.60
15	*Z*-citral	16.281	1225	0.93
16	geraniol	16.628	1234	2.80
17	*E*-citral	17.257	1256	1.79
18	neryl acetate	19.533	1347	1.45
19	*α*-copaene	19.761	1355	0.25
20	*β*-bourbonene	19.999	1362	1.67
21	*β*-elemene	20.249	1371	1.87
22	longifolene	20.791	1389	0.53
23	*β*-caryophyllene	21.202	1402	1.49
24	*β*-copaene	21.636	1412	0.26
25	*α*-humulene	22.666	1436	0.20
26	aromadendrene	22.828	1440	0.32
27	germacrene D	23.901	1465	4.02
28	*β*-bisabolene	25.278	1497	0.99
29	*δ*-cadinene	25.528	1505	0.35
30	elemol	26.590	1545	7.40
31	nerolidol	27.045	1562	13.83
32	spathulenol	27.240	1570	2.83
33	*β*-eudesmol	28.476	1623	1.20
34	*T*-muurolol	28.725	1637	0.65
35	*γ*-eudesmol	28.931	1648	3.68
36	intermedeol	29.061	1655	0.62
37	1.4-*cis*-1.7-*cis*-acorenone	29.484	1678	1.25
38	caryophyllene oxide	29.581	1684	0.38
39	(*Z,E*)-farnesal	29.972	1709	0.89
40	farnesol	30.080	1718	14.59
45	(*E,E*)-farnesal	30.405	1747	1.83
	Identified from total area			91.28

In the essential oil from *Thymus glabrescens*, 45 compounds – with the minimum content of 0.1% – were identified, adding up to 91.28% of the total area. The percentage of monoterpenic hydrocarbons varies between 0.12% for camphene and 3.21% for *p*-cymene, with a total of 5.68% of

the area. Sesquiterpenic hydrocarbons have values between 0.2% (α-humulene) and 4.02% (germacrene D), accounting for 11.95% of the total area.

The content of monoterpenic alcohols is high, varying from 0.21% for borneol to 7.49% for terpin-4-ol; the total percentage of monoterpenic alcohols is 16.33%. Sesquiterpenic alcohols are the main compounds of the volatile oil, their added values reaching as high as 44.8%. Thus, the following alcohols account for more than 1%: spathulenol, β- and γ-eudesmol, elemol, nerolidol, farnesol. Among the monoterpenic ethers, 1,8-cineol is present with 3.82%, and from the sesquiterpenic ones, caryophyllene oxide with only 0.38%.

Carbonyl compounds with a monoterpenic and sesquiterpenic structure are also present in small quantities.

A single ester, neryl acetate, was found in the volatile oil of *Thymus glabrescens*, with a content of 1.45% from the total area.

Comparing this with literature data, the content in volatile oil determined for our samples is smaller, with an average value of 0.4%, whereas 1.13% was determined in samples of *Thymus glabrescens* Willd. from the Sofia floral region, Bulgaria. [20] The composition of the volatile oil analysed by us is also essentially different from the results obtained by Bulgarian researchers, which showed that the essential oil consisted mainly of monoterpenoids, totalling 86.9%, from which: linalool 39.86%, thymol 14.68%, α-terpenyl acetate 11.69%, geranyl acetate 6.15%.

In table 2 is shown the content in volatile and semivolatile compounds of the tincture and infusion samples. It can be noticed that the number of identified volatile compounds is significantly smaller in tincture (18 compounds) and infusion (11 compounds), compared to the essential oil (45 compounds).

Table 2

Chemical composition of the tincture and infusion from *Thymus glabrescens*

No.	Compounds	t_R (min)*	% from total area		
			Tincture (macerate)	Tincture (percolate)	Infusion
1	1.8-cineol	9.431	5.28	3.80	7.87
2	*cis*-sabinene hydrate	10.862	29.45	23.63	62.23
3	linalool	11.937	0.53	1.98	2.78
4	*p*-menth-2-en-1-ol	12.733	0.22	0.33	2.09
5	camphor	13.214	0.23	0.72	1.47
6	camphene hydrate	14.709	-	-	0.46
7	β-bourbonene	20.064	2.49	1.51	-
8	β-elemene	20.357	0.42	0.29	-
9	β-caryophyllene	21.268	2.22	1.62	-
10	β-copaene	21.712	0.30	0.24	-
11	aromadendrene	22.893	0.34	0.17	-
12	germacrene D	23.956	2.69	5.86	-
13	elemol	26.677	11.22	11.03	4.63
14	nerolidol	27.197	10.73	20.84	-
15	spathulenol	27.327	9.63	13.03	1.80
16	caryophylla-2(12),6-dien-5-one	28.660	3.62	0.46	-
17	intermedeol	29.148	1.86	1.73	1.36
18	caryophyllene oxide	29.625	8.30	4.43	0.76
19	farnesol	30.118	1.78	1.30	-
20	(E,E)-farnesal	30.427	-	-	4.67
	Identified from total area		91.31	92.97	90.12

* retention times correspond to the chromatogram for tincture (percolate).

It can be observed from experimental data that neither the tincture not the infusion retain the monoterpenic hydrocarbons. However, both in tincture and infusion the compound *cis*-sabinene

hydrate is present in high quantities, reaching 23.63% in the tincture obtained through percolation and 29.45% in the one obtained through maceration, whereas in infusion it is the majority compound with 62.23%.

Hydrocarbons with sesquiterpenic structure are present in the tinctures, with a total content of 8.46% in the macerated product and 9.69% in the one obtained through percolation.

Monoterpenic alcohols are found in smaller quantities in tinctures, having only 0.22% p-menth-2-en-1-ol and 0.53% linalool. However, the relative content in sesquiterpenic alcohols elemol, nerolidol and spathulenol is quite large in tinctures, with values of 11.22%, 10.73% and 9.63%, respectively, in the macerated tincture, and 11.03%, 20.84% and 13.03% in the tincture obtained through percolation. It can be noticed a drop in the relative content of farnesol (1.78% and 1.30% respectively), compared to 14.59% in essential oil.

The monoterpenic ether 1,8-cineol is present in tinctures with a relative content comparable with the one from essential oil (3.80% and 5.28% for tinctures and 3.82% in oil).

The infusion lacks both the hydrocarbons with monoterpenic structures and the ones with a sesquiterpenic one. This fact was pointed out in previous research made on *Salvia officinalis*. [29]

In the infusion, the compound *cis*-sabinene hydrate (4-thuianol) has the majority; its presence in the volatile oil hasn't been detected, but it appears in the tinctures also with a relatively high content. Experimental data could lead to the conclusion that in the macerating and percolating processes, as well as during the preparation of infusion, some hydrocarbons with monoterpenic structure may undergo hydratation reactions, leading to the formation of 4-thuianol (*cis*-sabinene hydrate).

Since the experimental data from table 2 represents the content of volatile compounds relative to the total area, we tried to make a quantitative estimation (as mg/g plant) for the main compounds present in tinctures and infusion. To this end, the compound linalyl acetate was used as internal standard, as presented in paragraph 2.6. The quantitative estimation was made by using only the area of the compound and the area of the internal standard, without a correction factor, in other words considering this factor equal to 1 for all analysed compounds.

From the chromatograms of spiked tincture and infusion samples with internal standard, the quantity of the main compound expressed in mg / g plant was calculated with the following formula:

$$quantity\ (mg/g) = \frac{A_C \cdot V_f \cdot c_{IS}}{A_{IS} \cdot m} \cdot a \cdot 10^{-3}$$

where A_C is the area of the compound, A_{IS} the area of the internal standard, c_{IS} the concentration of the internal standard solution, V_f the final volume of sample (mL), m the amount of plant sample (g) and a the dilution factor (a = 1 for infusion and 40 for tincture).

Table 3 shows the content of compounds in mg/100g dry plant, for nine of the most important compounds present in macerated tincture, percolated tincture and infusion. The results represent the average of two determinations. This data expresses more realistically the efficiency of using volatile compounds from plants, depending on experimental conditions used at tincture and infusion preparation. It is interesting to notice the fact that although the two tinctures used the same solvent, 70% ethanol, the different methods of preparation was responsible for a differential extraction of some compounds. Thus, an essential difference is the quantity of camphor (a cyclic monoterpenic ketone) in the two tinctures: 0.53mg/100g in macerate and 1.39mg/100g in percolate.

Table 3

The content of main compounds determined in the tincture and infusion of *Thymus glabrescens*

No.	Compounds	mg compound / 100g plant		
		Tincture (macerate)	Tincture (percolate)	Infusion
1	1.8-cineol	5.74	4.59	0.52
2	*cis*-sabinene hydrate	34.36	31.37	4.33
3	linalool	3.17	2.71	0.44
4	camphor	0.53	1.39	0.37

Table 3 (continued)

5	germacrene D	1.19	3.62	-
6	elemol	5.87	8.01	0.19
7	nerolidol	7.73	18.95	-
8	spathulenol	4.06	5.78	0.13
9	caryophyllene oxide	7.41	4.06	0.02

In the case of nerolidol, the method of percolation also has a higher efficiency than maceration, the quantity of nerolidol in the percolated tincture being almost three times larger than in the macerate.

Regarding the infusion, one can notice a low presence of the volatile compounds, way under 10% in comparison with tincture. These quantitative estimations confirm the results of previous research performed on other plants. [30]

CONCLUSIONS

In the essential oil were identified 45 compounds with monoterpenic (30.22%) and sesquiterpenic (59.85%) structures. Thymol and carvacrol are not the main compounds of the essential oil from *Thymus glabrescens* Willd. growing in Romania, as opposed to data obtained for other species of thyme.

The content of volatile compounds determined for the tincture samples, obtained through two different procedures (b and c), shows that the chemical composition depends on the experimental conditions used for preparation of these tinctures.

During the processes of maceration, percolation and infusion, chemical reactions may take place, which determine the appearance of compounds not found in the essential oil. Thus, the alcohol 4-thuianol (or *cis*-sabinene hydrate), while absent in the essential oil, represents the majority compound in infusion (61.30%) and has between 23.6% and 28.22% in tinctures.

In the tinctures the number of volatile compounds is smaller and no monoterpenic hydrocarbons were found. Through infusion, the highly volatile compounds were lost and therefore neither monoterpenic hydrocarbons nor sesquiterpenic hydrocarbons were identified here.

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