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New terpenoids in cultivated and wild chamomile (in vivo and in vitro)

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Abstract

The effect of *Chamomilla recutita* (L.) Rauschert is made up by several groups of active substances, among which terpenoids in the inflorescences are of greatest importance. Among cultivated species, the Hungarian *BK-2* contains more chamazulene in its essential oil than the German Degumil type, which is mainly cultivated for its (−)-α-bisabolol. Both components have important antiinflammatory activities. Among wild chamomile populations in Hungary, a population was found in the area of Szabadkigyós containing significant amounts—on average 48%—of (−)-α-bisabolol in its inflorescence oil. In vitro cultures were made from this population to obtain propagation material containing a high number of active substances. The intact roots contained no (−)-α-bisabolol but the sesquiterpene alcohol β-eudesmol as new compound was identified by our group. Sterile plantlets, cultured in vitro, were multiplied for phytochemical investigations. Pharmacologically important compounds of the essential oils were followed in great detail. The amount of in vitro cultured terpenoids and polyin compounds was compared with that of in vivo plants. These volatile compounds were identified by comparing their retention times with those of authentic standards, essential oils of known composition and peak enrichment. The confirmation of identity was done by comparison of their mass spectra with those reported in the literature and reference compounds. The percentage evaluation of each component was made by area normalisation. Gas chromatography (GC) and mass spectrometry (MS) showed that sterile chamomile cultures generated the most important terpenoid and polyin compounds characteristic of the parent plant. We identified germacrene-D, berkheyaradulene, 4-(2′, 4′, 4′-trimethyl-bicyclo[4.1.0]hept-2′-en-3′-yl)-3-buten-2-one, geranyl-isovalerate and cedrol as new components in these sterile cultures.

Keywords: *Chamomilla recutita*; Terpenoids

1. Introduction

In Hungary, chamomile, *Chamomilla recutita* (L.) Rauschert, Asteraceae, is one of the most common medicinal plants. The chamomile owes its therapeutical activity to different groups of effective substances, which make up the complex effect of the drug (Chamomillae anthodium). Essential oils are of greatest importance among all effective substances [1–3].

The essential oil content of plant parts under and above ground depends on different chemotypes [4]. According to the bisabolol-oxide content, commercial chamomile populations are classified as types of (−)-bisabolol, bisabolol-oxide A and B, and bisabolonoxide [5]. During the ontogenesis the essential oil content changes, reaching a maximum in the flower just before flowering (0.3–1.5%), and decreasing after the process of flowering. This is certainly the case for matricin, bisaboloid content and *E*-β-farnesene [6].

The root of chamomile contains only traces of essential oil (0.02–0.11%) [7]. Despite the fact that some therapeutically active compounds such as chamazulene and (−)-α-bisabolol are missing, other substances such as *trans*-β-farnesene, α-farnesene, chamomillaester, *cis* and *trans* en-in-dicycloethers, chamomillol, β-caryophyllene and caryophyllene-epoxide can be detected [8].

The main anti-inflammatory activity is due to chamazulene, which is formed during the steam distillation of the oil from matricin, and (−)-α-bisabolol, but also bisabolol-oxide A and B play a role [9–12]. Spasmolytical effects are attributed to apigenin and bisabolol-oxides [13] and wound healing properties to chamazulene, apigenin and (−)-α-bisabolol [14]. It should be noticed that chamomile flowers of the so called bisabolol-oxide B type also contain an allergenic compound: anthecotulide [15].

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Both cultivated and wild chamomile types are used in Hungary for therapeutically purposes. In earlier investigations Máthé [16] has found an increase of proazulenes in the flower of chamomiles, whereas Marczal and Petri [17] have found an increase in the bisaboloids content over the years. Based on examinations made 20 years ago chamomile populations from the best areas were studied again. Our aim was to study the features of the essential oil production of the chamomile types in these regions of Hungary in order to select chamomile types rich in therapeutically active substances, which can be then kept in a seed bank.

2. Experimental

2.1. Plant material

Wild chamomile populations were obtained from soily areas of *Szabadkígyós* and the National Park *Hortobágy* in Hungary. They have common morphological features and are rich in (−)-α-bisabolol. The Degumil and the polyploid *BK-2* types were cultivated in Kerepes. The plant material was collected in the end of May 1999 during three consecutive years [7].

2.2. Sterile chamomile cultures

Sterile chamomile plants were obtained by sterilization of seeds of intact plants with ethanol, then ethanol–mercury–chlorid and methyl–pyridine–chlorid solution [18]. The seeds were then rinsed three times with sterile distilled water. Young plantlets were then cultivated at 2500 lx (16 h light, 8 h dark photoperiod) at 26 °C, on solid 1/2 Murashige–Skoog (MS) [19] hormone-free media.

2.3. Isolation of the essential oil

The essential oil from roots, herbs (stem plus leaves), and flowers was isolated by steam distillation with apparatus according to the Pharmacopoea Hungarica (Ph.Hg.VII.) [20]. Fifteen to twenty grams of powdered drug, suspended in 500 ml of water, were distilled for 3 h. The essential oil content was measured gravimetrically [20].

2.4. Investigation of the essential oil

Gas chromatography (GC), standard addition and/or GC–MS methods were used to identify the oil components (Fig. 3 and Table 1). The compounds were identified by comparing their retention times with these of authentic standards, essential oils of known composition and peak enrichment. The confirmation of identity was done by comparison of their mass spectra with these reported in the literature and reference compounds. The standards originated from Fluka Chemie GmbH and Carl Roth firms, or were isolated and structure determined. The percentage evaluation of the oil components was made by area normalisation, on the basis of three parallel measurements. The deviation from average was (±) 6–8% at each compounds.

Table 1
Relative retentions to *trans*-β-farnesene (r_x) of oil components in chamomile on DB-1701 and β-DEXm stationary phases

Sign	Component	r_x	
		DB-1701	β-DEXm
a	4-(2′,4′,4′-Trimethyl-bicyclo[4.1.0] hept-2′-en-3′-yl)-3-buten-2-one (M^+ 204)**	0.91	0.92
b	Berkheyaradulene (M^+ 204)**	0.92	0.93
c	α-Selinene**	0.96	0.94
d	β-Caryophyllene	0.98	0.96
1	*trans*-β-Farnesene	1.00	1.00
2	Germacrene-D**	1.03	1.04
3	α-Murolene (M^+ 204)	1.06	1.05
3′	α-Farnesene	1.06	1.06
3″	α-Cadinene (M^+ 204)	1.08	1.08
e	Geranyl-isovalerate (M^+ 238)**	1.21	1.16
4	Spathulenol	1.24	1.25
x	Cedrol (M^+ 222)**	1.28	1.25
5	Bisabolol-oxide B	1.31	1.29
6	(−)-α-Bisabolol	1.34	1.32
6′	β-Eudesmol*	1.34	1.32
7	Bisabolon-oxide	1.36	1.34
8	Chamazulene	1.42	1.40
9	Bisabolol-oxide A	1.43	1.42
f	M^+ 220	1.61	1.60
10	*cis*-Spiroether	1.65	1.63
g	Chamomillaester	1.67	1.65
11	*trans*-Spiroether	1.71	1.69

Remark: Compounds identified at first in essential oil of chamomile ((*) intact plant and (**) in vitro cultures) by us.

2.4.1. Gas chromatographic (GC) parameters

Gas chromatograph: FISONS GC 8000; capillary column: 30 m × 0.32 mm i.d.; film thickness: 0.25 μm; stationary phases: DB-1701 and β-DEXm (latter is suitable to separate enantiomer monoterpene pairs [21]); oven temperature: 60–230 °C, 8 °C/min, 230 °C, isotherm 3 min; detector temperature: flame ionisation, 240 °C; carrier gas: nitrogen, pN_2=0.05 MPa, flow rate 6.8 cm^3/min; injector temperature: 200 °C; injection: splitless 10 s, split rate 1:10; injected volume: 0.4 μl of a 1:1000 dilution of oil in chloroform. For evaluation Chrom Card computer programme.

2.4.2. GC–MS parameters

The GC–MS analyses were performed on a Finnigan GCQ instrument (San José, CA, USA).

- *Gas chromatographic parameters*—Chromatograph type: Finnigan GC; capillary column: 30 m × 0.22 mm i.d.; film thickness: 0.25 μm; stationary phase: BPX5 (non-polar); oven temperature: 60–230 °C, 8 °C/min, 230 °C isotherm 3 min; detector: Finnigan MS; carrier gas: He, pHe = 0.20 MPa; injector temperature: 200 °C; the injector was

operated in the splitless mode 6 s, split rate 1:10; injected solution volume: 0.4 μl.

- *MS parameters*—Start: 3 min after injection; mode: electron–impact-ionisation (EI) positive ion with an electron energy of 70 eV; mass range: 40–650; scanning: 1 analyse/s; evaluation: Finnigan GCQ 2.0 computer programme and data of [22].

3. Results and discussion

3.1. Cultivated and wild chamomile populations

The total essential oil content and the percentage composition of the oils were evaluated in selected wild chamomile populations chosen according to the previous investigations [17]. In addition, comparison with cultivated *BK-2* type chamomile concerning the essential oil, was done. The highest amount of the total essential oil was obtained from the flowers of wild *Hortobágy* population (0.70%). A similar result was observed in the same chamomile type concerning the herbs (0.12%).

During 3 years according to the GC analysis (Figs. 1 and 2 and Table 2) it was clear that the oils from the *flowers* of the *Szabadkígyós* population were the richest in (−)-α-bisabolol (increasing 42 → 54%) and that from the *flowers* of *BK-2* in chamazulene (decreasing 24 → 18%). The ratio of (−)-α-bisabolol relatived to that chamazulene was always higher in wild samples. The highest bisabolol-oxide A content was found in the oil of *BK-2* (36%), whereas the *Hortobágy* population had the highest bisabolol-oxide-B concentration. Cycloethers occurred in about the same proportion in the studied samples; the content of *cis*-en-in-dicycloethers showed the higher amount of the *trans*-isomers, except for the *BK-2* type where the amount of *trans*-en-in-dicycloether exceeded that of *cis*-isomer.

It was interesting that the (−)-α-bisabolol content in the oil of *Szabadkígyós* population was 48% (average on three consecutive years, Fig. 2); therefore we planned to keep the genom of this type using biotechnological methods in order to produce chamomiles with high content of active substances.

Concerning the *herb*, its essential oil content was equally low both in cultivated and wild chamomile populations [7]. Both in the herbs of the cultivated *BK-2* and the wild populations from *Szabadkígyós*, *trans*-β-farnesene was the main component. *Cis*-spiroethers exceeded the content of *trans*-isomers in all cases. Further in the herbs four sesquiterpene compounds (M^+ 204): germacrene-D, α-selinene, berkheyaradulene and 4-(2′, 4′, 4′-trimethyl-bicyclo[4.1.0] hept-2′-en-3′-yl)-3-buten-2-one, were identified firstly by us. We could detected germacrene-D, a monocyclic sesquiterpene in the flower too (Fig. 3). A bicyclic sesquiterpene hydrocarbon: α-selinene and berkheyaradulene a tricyclic sesquiterpene hydrocarbon (Fig. 3) were earlier described in other species of Asteraceae family: such as in root of *Silphium* [23] and *Liabum* [24] species. The chamomile herbs contained one oxygenated sesquiterpene: 4-(2′,4′,4′-

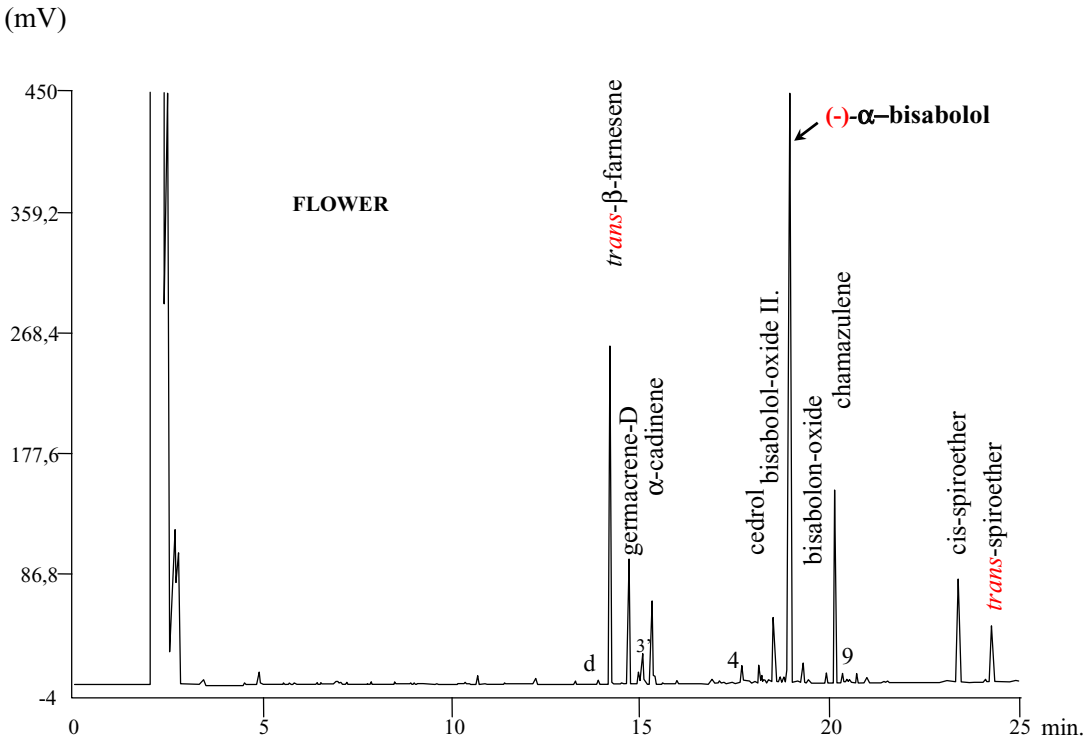

Fig. 1. GC of essential oil of the wild chamomile flower (occurring from "*Szabadkígyós*" location). For legends see Table 1.

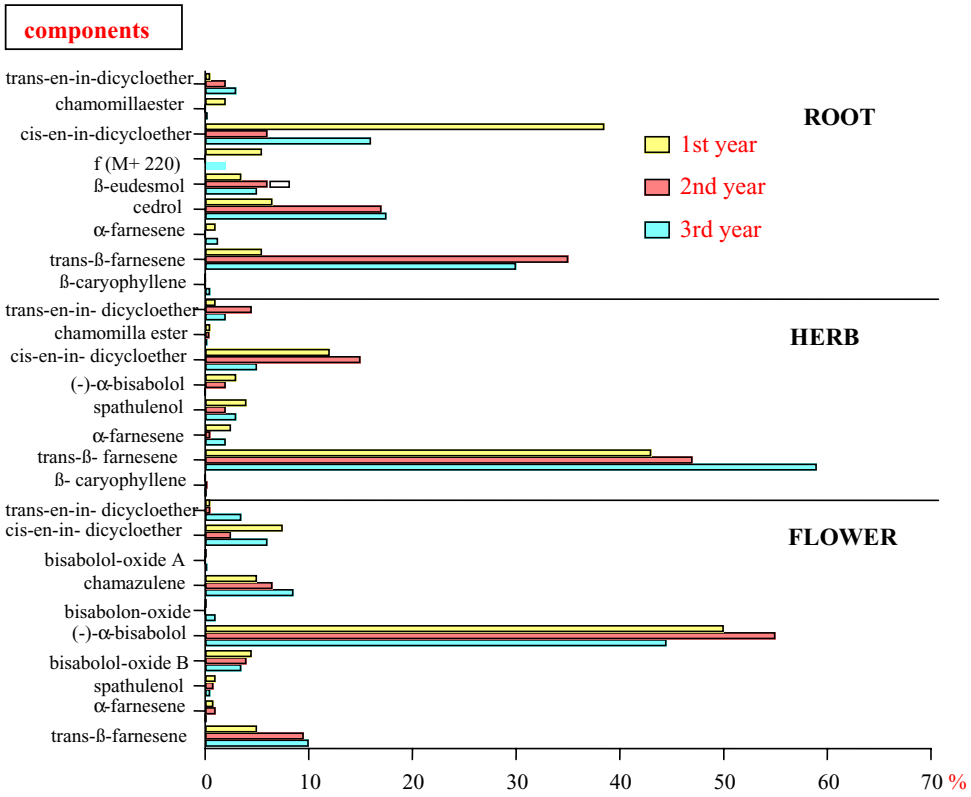

Fig. 2. Percentile distribution of some components in essential oil of flowers, herbs and roots of wild (area *Szabadkígyós*) chamomile (during 3 years).

trimethyl-bicyclo[4.1.0]hept-2′-en-3′-yl)-3-buten-2-one bicyclic sesquterpene as minor oil constituent (Fig. 3).

The characteristic main component of the *root* oil was again *trans*-β-farnesene, but its amount was lower than in herbs. In the essential oil of *BK-2* root it reached nearly 40%. Wild populations were also rich in percentile distribution of components similarly to the cultivated *BK-2* type. In the oil of the roots β-eudesmol was determined and identified by GC–MS, this component was characteristic for wild populations [25]. The highest value of β-eudesmol was found in the oil of roots of *Szeghalom*. The four sesquiterpenes (M^+ 204) above mentioned were present also in the root oil.

Summarizing our results we can conclude that, although a change was observed in the essential oil content and also

Table 2
Percentile distribution of oil components in the essential oil of *flowers* in cultivated (Degumil, *BK-2*) and wild ("*Hortobágy*", "*Szabadkígyós*") chamomile populations

	Degumil		*BK-2*		*Hortobágy*		*Szabadkígyós*	
	t_r (min)	Percentage	t_r (min)	Percentage	t_r (min)	Percentage	t_r (min)	Percentage
β-Caryophyllene	13.83	0.15	13.79	0.07	13.84	0.68	13.87	0.90
trans-β-Farnesene	14.17	12.80	14.12	8.22	14.17	11.07	14.20	15.28
Germacrene-D	14.64	0.55	14.59	0.16	14.66	0.62	14.68	5.78
α-Muurolene	15.01	2.86	14.95	1.00	14.92	0.33	15.03	1.43
α-Farnesene	15.07	0.30	15.03	0.27	15.02	2.43	15.11	0.15
α-Cadinene	15.25	0.90	15.21	0.85	15.28	2.99	15.29	3.75
Spathulenol	17.62	0.46	17.59	0.90	17.67	0.81	17.68	0.77
Bisabolol-oxide B	18.49	7.32	18.45	8.25	18.54	20.42	18.52	3.61
(−)-α-Bisabolol	18.93	30.00	18.83	1.59	18.94	24.00	18.96	41.54
Bisabolon-oxide	19.24	0.55	19.19	4.13	19.28	2.51	19.29	1.07
Chamazulene	20.12	24.50	20.08	23.41	20.12	9.31	20.13	8.71
Bisabolol-oxide A	20.29	6.00	20.32	36.27	20.34	11.24	20.32	0.42
cis-Spiroether	23.34	7.48	23.26	3.43	23.39	4.28	23.39	6.05
trans-Spiroether	24.17	0.90	24.17	6.01	24.25	1.87	24.27	3.70

t_r: reduced retention times on DB-1701 stationary phase; *Hortobágy* and *Szabadkígyós* are locations of Hungary. The percentage evaluation was carried out on the basis of three parallel measurements. The deviation from average was (±) 6–8% at each compounds.

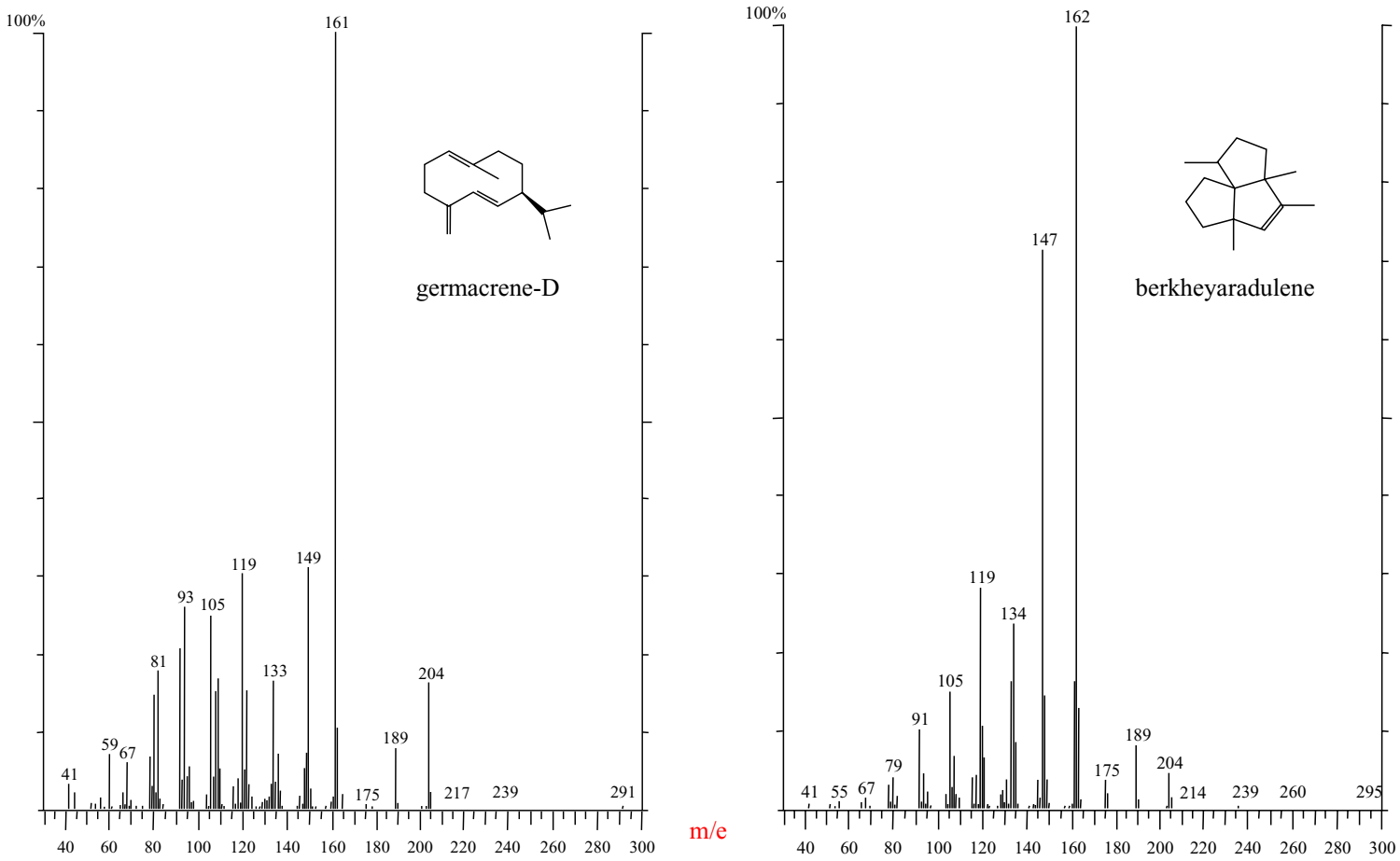

Fig. 3. Mass spectra of new compounds in the essential oil of chamomile (in vivo and in vitro).

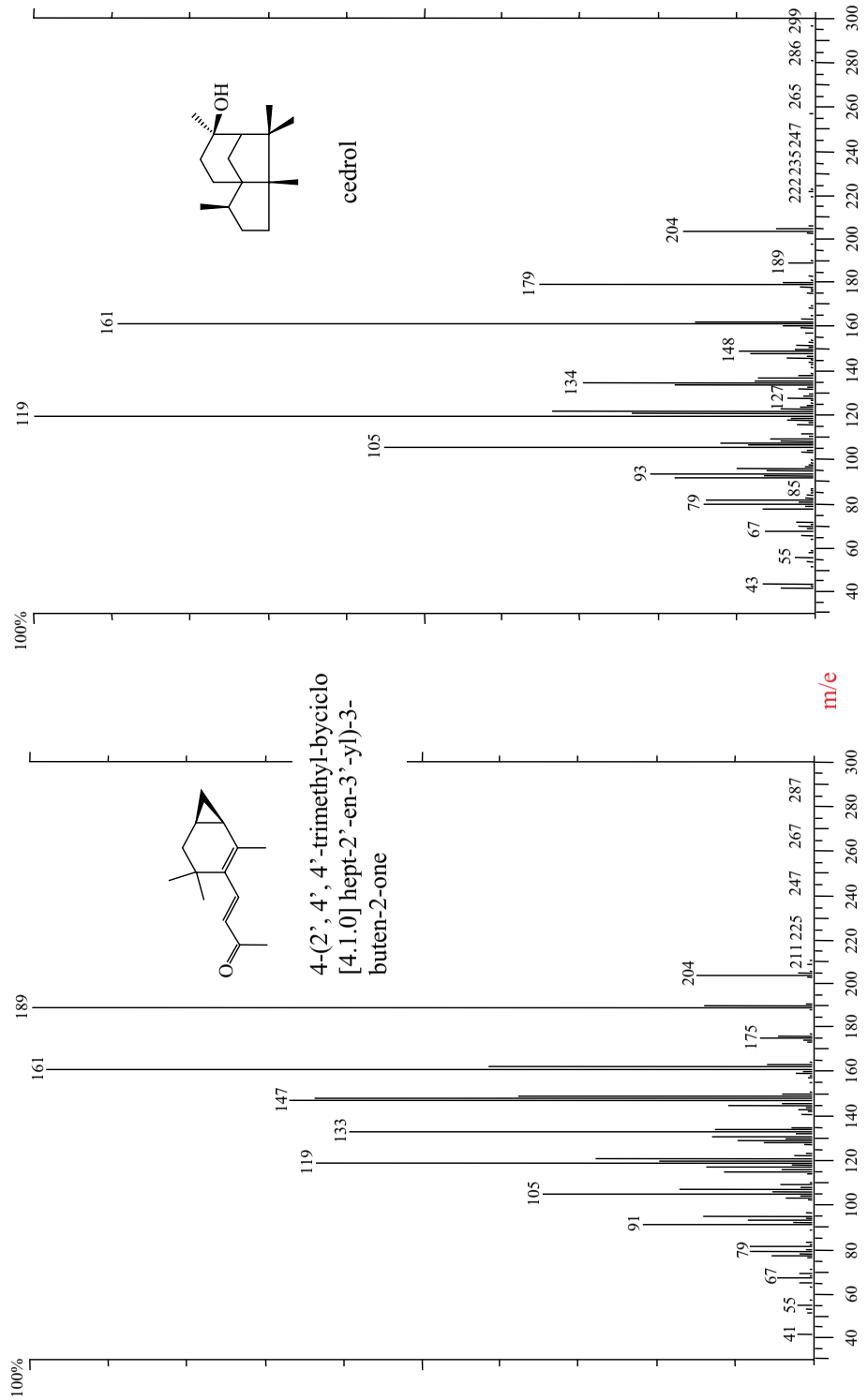

Fig. 3. (*Continued*).

in the proportion of pharmacologically active compounds in comparison with the results of the earlier survey, the characteristics of the oil of cultivated and wild chamomile populations remained the same.

3.2. Sterile in vitro chamomile cultures

Among wild chamomile populations in Hungary, a population was found in the area of *Szabadkígyós* containing significant amounts—on average 48%—of (−)-α-bisabolol in its inflorescences. We planned to keep the genom of this type using biotechnological methods [26] in order to produce chamomiles with high content of active substances.

Sterile organised chamomile cultures were cultivated on solid 1/2 Murashige–Skoog hormone-free media [19]. Table 3 shows the essential oil content (%) of herbs and roots in cultivated (Degumil) and wild chamomile (*Szabadkígyós*) populations in vivo and in vitro.

Gas chromatography and mass spectrometry showed that sterile chamomile cultures generated the most important terpenoid and polyin compounds characteristics of the parent plant. The four sesquiterpene compounds (M^+ 204): germacrene-D, α-selinene, berkheyaradulene and 4-(2′,4′,4′-trimethyl-bicyclo [4.1.0]hept-2′-en-3′-yl)-3-buten-2-one were found also in sterile cultures. The in vitro herb and root oils contained them in more significant quantity than the intact plant oils (Table 4 and Fig. 2). In sterile cultures we identified additional two oil compounds: the cedrol tricyclic sesquiterpene alcohol and the geranyl-isovalerate acyclic monoterpene ester (Fig. 3). The two compounds were earlier detected in the callus cultures: as M^+ 222 ($C_{15}H_{26}O$) and M^+ 238 ($C_{15}H_{26}O_2$) by us but the exact identification was carried out only now [27,28]. The organised root culture was especially rich in cedrol, contained 26% in the essential oil. The sterile root similarly to intact root oil contained also no (−)-α-bisabolol but a new sesquiterpene alcohol: β-eudesmol was firstly identified from the intact roots by us [25].

Furthermore, in vitro cultures were made from this population to obtain propagation material containing a high number of active substances.

Table 3
Total essential content (%) of herbs and roots in cultivated (Degumil) and wild (occurring from "*Szabadkígyós*" location of Hungary) chamomile populations in vivo and in vitro

Chamomile type	Total essential oil content (%)			
	Intact herb	Sterile herb	Intact root	Sterile root
Degumil	0.07	0.08	0.04	0.12
Szabadkígyós	0.07	0.08	0.12	0.14

Table 4
Comparing of percentile distribution of oil components in the total essential oil of sterile organised culture (1/2 MS medium) from cultivated (Degumil) and wild (area "*Szabadkígyós*" of Hungary) chamomile populations

Component	Sterile root oil		Sterile herb oil	
	Degumil	*Szabadkígyós*	Degumil	*Szabadkígyós*
M^+ 204	3.20	1.84	0.96	0.35
Berkheyaradulene	11.80	4.17	2.89	1.14
α-Selinene	2.41	1.25	0.53	0.25
β-Caryophyllene	1.80	1.20	0.76	0.68
trans-β-Farnesene	26.19	33.57	14.24	8.42
Germacrene-D	0.52	0.50	2.57	2.51
α-Muurolene	–	–	–	–
α-Farnesene	3.93	0.82	35.74	27.52
α-Cadinene	–	–	0.42	0.17
Geranyl-isovalerate (M^+ 238)	6.50	9.63	1.26	0.72
Spathulenol	–	–	0.68	0.55
Cedrol (M^+ 222)	8.71	1.40	3.62	0.51
Bisabolol-oxide B	+	0.24	–	+
(−)-α-Bisabolol	–	–	2.46	1.26
β-Eudesmol	2.81	1.23	–	
Bisabolon-oxide	+	1.00	+	0.4
cis-Spiroether	0.67	0.25	2.60	0.64
Chamomillaester	+	2.00	1.44	0.43
trans-Spiroether	+	+	1.34	1.55

(+) in traces: below 0.10%.

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