NPC Natural Product Communications

2018
Vol. 13
No. 8
1035 - 1037

Terpenes and *n*-Alkanes in Needles of *Pinus cembra*

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Received: May 21st, 2018; Accepted: June 8th, 2018

Simultaneous hydrodistillation and extraction of *Pinus cembra* needles from Slovakia was done (via Likens Nickerson apparatus) for the first time. In essential oil extracts 55 compounds were identified, comprising 99.6% of the extract. The most abundant were monoterpene hydrocarbons (71.0%). In the terpene profile α-pinene, limonene/β-phellandrene, germacrene D, β-pinene, and δ-cadinene dominated (53.2%, 11.4%, 9.4%, 4.6%, and 4.3%, respectively). Seven new compounds for *P. cembra*, such as methyl daniellate (0.5%), 1,8 cineole (0.2%) and *trans*-cadina-1(6),4-diene (0.2%), etc. were found. In needle cuticular wax of *P. cembra* the amount of nonacosan-10-ol was 75.8%. *n*-Alkanes ranged from C_{20} to C_{35} with the most dominant C_{31}, C_{29} and C_{33} (33.4%, 16.9%, and 9.6%, resp.). Differences in terpene profiles between Slovakian and Greece from one side and Romanian and Polish cembran pines on the other side could be the consequence of its disjuncted areal in Carpathian Mountains caused by glaciation and survival of species in different ecological niches. Obtained differences in *n*-alkane profiles among our and literature results could be the consequence of different age of trees.

Keywords: *Pinus cembra*, Terpenes, *n*-Alkanes, Nonacosan-10-ol.

Pinus cembra L., known as cembran pine, Swiss stone pine or Arolla pine, is five-needle soft pine which belongs to family Pinaceae, genus *Pinus*, subgenus *Strobus*, section *Quinquefoliae*, subsection *Strobus* (classification of Gernandt et al. [1]). It is glacial relict naturally widespread at high altitudes in the Europaean Alps as well as in the Carpathian Mountains [2]. It grows very slow in its natural area, but could live more than 500 years [3]. There are many references about essential oil composition of pines [4–8], even on population level [9–11], etc. However, there are only few reports of Swiss stone pine dealing with composition of terpenes [12, 13], their enantiomers [14] and/or their antioxidant and antimicrobial activities [2].The most detailed is the report of Lis et al. [15] who examined essential oils from different parts of *P. cembra*.

Cuticular waxes and *n*-alkanes of various pines had already been published [16–19], even on population level [20]. In the case of *P. cembra* cuticle thickness [21], wax composition [22], and *n*-alkanes of young trees [23], have been published.

Table 1: Terpene compounds (in %) in the needles of *Pinus cembra*

Entry	RT[a)]	RI[b)]	Compds	Area(in %)	Class
1	3.274	801	Hexanal	0.2	AC
2	5.561	923	Tricyclene	0.1	MH
3	5.654	926	α-Thujene	tr[c)]	MH
4	5.843	934	α-Pinene	53.2	MH
5	6.249	949	Camphene	0.6	MH
6	6.390	953	Thuja-2,4(10)-diene	tr	MH
7	6.931	973	Sabinene	0.1	MH
8	7.041	978	β-Pinene	4.6	MH
9	7.460	984	Myrcene	0.8	MH
10	7.900	1007	α-Phellandrene	tr	MH
11	8.094	1010	δ-3-Carene	tr	MH
12	8.324	1016	α-Terpinene	tr	MH
13	8.580	1023	*p*-Cymene	tr	MH
14	8.711	1030	Limonene/β-Phellandrene	11.4	MH
15	**8.857**	**1032**	**1,8-Cineole**	**0.2**	**OM**
16	9.838	1057	γ-Terpinene	tr	MH
17	10.973	1090	Terpinolene	0.2	MH
18	14.669	1175	Terpinen-4-ol	tr	OM
19	15.263	1192	α-Terpineol	0.2	OM
20	17.191	1235	Thymol methyl ether	0.1	BC
21	19.443	1285	Bornyl acetate	0.3	OM
22	21.734	1139	δ-Elemene	tr	SH
23	22.292	1351	α-Longipinene	0.5	SH
24	23.442	1377	α-Copaene	0.2	SH
25	24.079	1391	β-Cubebene	0.2	SH
26	24.171	1392	β-Elemene	0.1	SH
27	**24.468**	**1401**	**β-Longipinene**	**0.1**	**SH**
28	**24.720**	**1407**	**Longifolene**	**tr**	**SH**
29	25.338	1421	*(E)*-β-Caryophyllene	0.4	SH
30	25.757	1431	β-Copaene	0.2	SH
31	26.169	1441	Aromadendrene	0.3	SH
32	26.495	1449	*trans*-Muurola-3,5-diene	tr	SH
33	26.662	1453	*cis*-Muurola-3,5-diene	tr	SH
34	26.788	1456	α-Humulene	tr	SH
35	27.203	1465	*cis*-Muurola-4(14),5-diene	0.1	SH
36	27.415	1471	n.i.[d)] 1	0.2	-
37	**27.655**	**1477**	***trans*-Cadina-1(6),4-diene**	**0.2**	**SH**
38	27.779	1480	γ-Muurolene	1.0	SH
39	27.973	1485	Germacrene D	9.4	SH
40	28.191	1489	β-Selinene	tr	SH
41	**28.437**	**1495**	***trans*-Muurola-4(14),5-diene**	**0.1**	**SH**
42	28.578	1499	n.i. 2	0.4	-
43	28.632	1500	Bicyclogermacrene	2.1	SH
44	28.787	1504	α-Muurolene	0.6	SH
45	**29.074**	**1510**	**δ-Amorphene**	**tr**	**SH**
46	29.351	1517	γ-Cadinene	2.3	SH
47	29.744	1526	δ-Cadinene	4.3	SH
48	30.098	1535	*trans*-Cadina-1,4-diene	0.1	SH
49	30.348	1540	α-Cadinene	0.1	SH
50	30.559	1546	n.i. 3	tr	-
51	31.712	1574	n.i. 4	tr	-
52	31.863	1578	Germacrene D-4-ol	1.4	OS
53	31.939	1580	Spathulenol	0.1	OS
54	33.439	1617	1,10-di-epi-Cubenol	tr	OS
55	33.955	1630	1-epi-Cubenol	0.1	OS
56	34.504	1644	epi-α-Murrolol	1.3	OS
57	34.614	1647	α-Muurolol	0.4	OS
58	34.983	1657	α-Cadinol	1.3	OS
59	**57.223**	**2337**	**Methyl daniellate**	**0.5**	**OT**

[a)]RT – retention time; [b)]RT – retention index; [c)]traces (< 0.1%); [d)]n.i.–nonidentifed.

P. cembra from Carpathian Mountains has disjuncted areal. Up to know *n*-alkanes were studied from territory of Romania [2] and Poland [15]. The aim of this study is to examine for the first time essential oil composition, amount of nonacosan-10-ol content and *n*-alkane profile of cembran pine from Slovakia.

In essential oil extracts among 59 compounds detected, 55 were identified, comprising together 99.6% of the extract (Tables 1,2). The most abundant were monoterpene hydrocarbons (71.0%). Seven new compounds for *P. cembra*, such as methyl daniellate (0.5%), 1,8 cineole (0.2%) and *trans*-cadina-1(6),4-diene (0.2%), etc. were found. Terpene profile was as following: α-pinene>>> limonene/β-phellandrene >germacrene D >β-pinene = δ-cadinene. (53.2%, 11.4%, 9.4%, 4.6%, 4.3%, respectively. Symbols were explained by Petrakis et al. [24].

Table 2: Terpene classes (in %) in the needles of *P. cembra*.

Class	Area (in %)
Total monoterpenes	**71.7**
Monoterpene hydrocarbons (MH)	71.0
Oxygenated Monoterpenes (OM)	0.7
Total sesquiterpenes	**27.1**
Sesquiterpene hydrocarbons (SH)	22.5
Oxygenated sesquiterpenes (OS)	4.6
Others	**0.8**
Aliphatic compounds (AC) [a)]	0.2
Benzenoid compounds (BC) [b)]	0.1
Others (OT)	0.5
Unknown	**0.4**
Total (%)	**100.0**

[a)] Aliphatic aldehydes and hydrocarbons; [b)] Cyclic hydrocarbons.

It is obvious that chemotype of *P. cembra* from Slovakia (present results) has more abundant germacrene D and β-pinene (at third and fourth place in profile) comparing to same species from Poland (where δ-cadinene is more abundant than germacrene D) [15]. Furthermore, *P. cembra* from Romania [2] differed from both of them with abundant α-cadinene, γ-cadinene and camphene (at third, fourth and fifth place, resp.).

In needle cuticular wax of *P. cembra* the amount of nonacosan-10-ol was 75.8%, which is significantly higher than in the same species from Swiss Alps [20] where is nearly 60% for one-year and nearly 50% for 2–4 year old needles. In presented work *n*-alkanes ranged from C_{20} to C_{35} with the most dominant C_{31}, C_{29}, C_{30} and C_{33} (33.4%, 16.9%, 10.6% and 9.6%, resp.). In nursery conditions [21] *n*-alkanes of *P. cembra* ranged from C_{18} to C_{33}, where the most dominant were C_{31}, C_{27}, C_{26}, C_{29} and C_{25} (20.0%, 6.64%, 5.26%, 5.17% and 5.14%, resp.). Interestingly, in its var. *glauca* range of *n*-alkanes is narrower (C_{21} to C_{31}), and the most dominant was also C_{31}, but with significantly lower abundance (4.51%), followed by C_{25}, C_{24} and C_{23} (4.23%, 4.00% and 3.38%, resp.).

Differences in terpene profiles and *n*-alkane composition among Slovakian and Greek [25] from one side and Romanian and Polish cembran pines on the other side could be the consequence of disjuncted area of *P. cembra* in Carpathian Mountains caused by glaciation as well as survival and development of species in ecological niches. Obtained differences in *n*-alkane profiles among our and literature results could be the consequence of different age of trees.

Experimental

Plant material: Twigs with needles from the lowest third of the full tree crown (up to 60 years old) were collected in autumn 2015 from Slovakia, Mt. Vysoké Tatry, locality Štrbské Pleso (elevation about 1500 m). The collected twigs were stored at –20°C prior to further analyses.

Extraction of essential oil: Essential oil was obtained by simultaneous distillation and extraction with dichloromethane via Likens-Nickerson apparatus.

GC-FID and GC/MS analyses of essential oil: GC-FID and GC/MS analyses were carried out with an Agilent 7890A apparatus equipped with an 5975C MSD, FID, and a HP-5MSI fused-silica cap. col. (30 m × 0.25 mm × 0.25 μm).The oven temperature was programmed linearly rising from 60 to 315°C for 15 min; injector: 250°C; FID detect.: 300°C; carrier gas, He (1.0 mL/min at 210°C), injection vol. 1 μL split ratio,10:1. EI-MS (70 eV), *m/z* range 40–550.

Identification of essential oil components: Identification of all compounds in essential oil was match by comparison of their linear retention indices (relative to C8–C36 *n*-alkanes on the HP-5MSI column) and MS spectra with those of authentic standards from NIST11 and homemade MS library data bases.

Extraction of needle wax: The total wax of each sample was extracted by immersing 3 g of leaves in 5 mL of hexane for 45 sec. After extraction the solvent was removed under vacuum at 60°C and the remaining wax dissolved in 1.0 mL hexane. These wax samples were stored at –20°C until further analysis.

GC and GC-MS analyses of needle wax: Gas chromatography (GC) and gas chromatography–mass spectrometric (GC-MS) analyses were performed using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass selective detector (MSD) and flame ionization detector (FID) connected by capillary flow technology 2-way splitter with make-up. A HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The GC oven temperature was programmed from 60 to 300 °C at a rate of 3 °C min^{-1} and held for 15 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1 μL of sample. The sample was analyzed in the splitless mode. The injector temperature was 300 °C and the detector temperature 300 °C. MS data was acquired in the EI mode with scan range 30–550 *m/z*, source temperature 230 °C, and quadrupole temperature150 °C; the solvent delay was 3 min.

Identification of needle wax components: The components were identified based on their retention indices and comparison with reference spectra (Wiley and NIST databases) as well as by the retention time locking (RTL) method and the RTL Adams database. The retention indices were experimentally determined using the standard method of Van Den Dool and Kratz [26] involving retention times of *n*-alkanes, injected after the sample under the same chromatographic conditions. The relative abundance of the *n*-alkanes was calculated from the signal intensities of the homologues in the GC-FID traces.

Acknowledgements – This research was supported by Grants No. 173029, 173021 and 172053 by the Ministry of Education, Science and Technological Development of the Republic of Serbia. Authors are grateful to Ing. Ján Kučera, NLC-ÚHÚL Zvolen, Slovakia, who collected plant material.

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