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Chemical Composition and Antimicrobial Activity of the Essential Oils of *Pinus peuce* (Pinaceae) Growing Wild in R. Macedonia

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The chemical composition and antimicrobial activity of the essential oils isolated from twigs with needles (T+N) and from twigs without needles (T-N) from wild *Pinus peuce* Griseb. (Pinaceae), from three different locations in R. Macedonia, were investigated. Essential oil yields of T+N ranged from 7.5 mL/kg to 12.5 mL/kg and for T-N from 13.8 mL/kg to 17.3 mL/kg. GC/FID/MS analysis of the essential oils revealed eighty-four components, representing 93.7-95.7% and 91.2-92.0% of the T+N and T-N oils, respectively. The major components in T+N and T-N oils were monoterpenes: α-pinene (23.8-39.9%, 21.2-23.3%), camphene (2.2-5.5%, 0.7-2.0%), β-pinene (10.1-17.1%, 8.2-16.4%), myrcene (1.2-1.41%, 1.6-2.5%), limonene+β-phellandrene (6.8-14.0%, 8.8-23.6%) and bornyl acetate (2.3-6.9%, 1.1-3.4%), followed by the sesquiterpenes: *trans*-(*E*)-caryophyllene (3.6-4.3%, 3.2-7.3%), germacrene D (7.1-9.5%, 5.0-10.3%) and δ-cadinene (2.1-3.1%, 3.3-4.2%, respectively). Antimicrobial screening of the essential oils was made by disk diffusion and broth dilution methods against 13 bacterial isolates of Gram-positive and Gram-negative bacteria and one strain of *Candida albicans*. T-N essential oils showed antimicrobial activity toward *Streptococcus pneumoniae*, *Staphylococcus aureus*, *S. epidermidis* and *Candida albicans* as well as *Streptococcus agalactiae, Acinetobacter* spp. and *Haemophilus influenzae*. The antimicrobial activity of T+N essential oils was greater, especially against *Streptococcus agalactiae*, *S. pyogenes*, *Enterococcus* and *Candida albicans*, followed by *Haemophilus influenzae*, *Acinetobacter* spp., *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus* and *S. epidermidis*. Minimal inhibitory concentrations (MICs) of all tested essential oils ranged from 15-125 μL/mL. Summarizing the obtained results, the antimicrobial activity of *Pinus peuce* T+N and T-N essential oils collected from different localities in R. Macedonia varied considerably. These alterations in the antimicrobial activity can be attributed to the differences in the quantitative composition and percentage amounts of the components present in the respective essential oils, although it was evident that there were no differences in the qualitative composition of the essential oils, regardless of the locality of collection, or the type of plant material (T+N or T-N).

Keywords: *Pinus peuce*, Macedonian pine, Essential oil composition, Twigs, Needles, GC/FID/MS, Antimicrobial activity.

The genus *Pinus* is comprised of more than 100 species divided into three subgenera, based on cone, seed and leaf characteristics: *Strobus* (white or soft pines), *Ducampopinus* (pinyon, lacebark and bristlecone pines) and *Pinus* (typical, yellow or hard pines). Among all pine species that belong to the white or soft pine group (subgenus *Strobus*, section *Strobus*, subsection *Strobi*), *Pinus peuce* or Macedonian pine is the only one that is native to the Balkan Peninsula. The natural habitat range of this endemic conifer consists of two parts, separated by the valley of the Vardar River. The eastern part is in south-western Bulgaria and the western part in Macedonia, south-western Serbia, south-eastern Montenegro, eastern Albania and north-western Greece. This species was discovered in 1839 on Baba Mtn. (Pelister), R. Macedonia and described as *Pinus peuce* in 1844 by A. Grisebach [1-3].

Essential oils from different *Pinus* species have various therapeutic effects and are widely used either for health promoting purposes as a folk medicine or as a food [4,5]. Mainly, they are used for the treatment of pulmonary infections accompanied by cough, catarrh, bronchitis, bronchial asthma, emphysema, tracheitis, sinusitis, laryngitis, pharyngitis, tonsillitis and influenza, and for curing skin and muscle disorders of infectious, rheumatic or neuralgic origin [6,7].

The chemistry and the biological effects of the essential oils isolated from different pine species have been intensively studied, in particular, the pine needles. Generally, components like α-pinene and β-pinene, camphene, Δ^3-carene, β-myrcene, limonene, β-phellandrene, β-ocymene, β-caryophyllene, bornyl acetate, germacrene D, cadinene and muurolene are declared as dominant [1,2,4,8a-r]. On the other hand, there are a few studies that refer to the chemical composition of the essential oils isolated from pine twigs [8c,8n,8l,9a-c]. Also, it has been reported that pine essential oils isolated from needles and from twigs possess antimicrobial activity against different types of microorganisms [4,8i-k,8m,8n]. In addition, there are some registered pharmaceuticals, such as Pinimenthol®, an ointment which contains pine needle essential oil and is particularly suitable for the treatment for upper respiratory tract infections both in adolescents and adults [10].

However, despite the abundant literature on this topic, very little is known about the chemistry [11a-e,12a-b] and biological activity of *P. peuce* [12a-b]. Thus the main objective of the present study was to investigate the chemical composition of the essential oils isolated from young twigs with needles (T+N) and from twigs without needles (T-N) of *P. peuce* from Macedonia and to assess their antimicrobial activity against certain types of microorganisms that affect respiratory, gastrointestinal and urogenital systems, as well as provoking pathological conditions of the skin. The information gained will help to define possible applications and therapeutic uses of these essential oils as antimicrobial agents.

Table 1: The yield of *Pinus peuce* essential oils isolated from T+N and T-N, calculated in mL/kg of dry plant material.

Sample	Yield* of essential oil (mL/kg) T+N	T-N
Pelister Mtn.	7.5 ± 0.2	17.3 ± 0.2
Nidze Mtn.	9.2 ± 0.2	13.8 ± 0.2
Shara Mtn.	12.5 ± 0.2	16.7 ± 0.1

*Data represent mean ± SD of three independent experiments

Essential oil yield, calculated from the dried plant material is given in Table 1. The obtained essential oils were transparent, agile, light yellowish liquids with specific and very strong turpentine odors. Table 2 shows the percentage amounts and Kovat′s retention indices of components identified in the *P. peuce* essential oils isolated from T+N and from T-N collected from three different localities in R. Macedonia: Baba (Pelister) Mtn., Nidze Mtn. and Shara Mtn.

Eighty-four components were identified in the investigated samples, representing 93.7-95.7% and 91.2-92.1% of the T+N and T-N oils, respectively. Data analysis of the chemical composition revealed six different classes of components: monoterpene hydrocarbons (MH), oxygen containing monoterpenes (OM), sesquiterpene hydrocarbons (SH), oxygen containing sesquiterpenes (OS), diterpenes (D) and non-terpene components (NT). Among the different classes of components that were present in the tested samples of essential oils isolated from plant material from Baba Mtn. (Pelister), Nidze Mtn. and Shara Mtn., the most abundant fraction was MH (for T+N essential oils 56.0%, 59.30% and 68.6% and for T-N essential oils, 49.6%, 58.2% and 57.2%, respectively), (Table 2).

Table 2: Chemical composition of *Pinus peuce* essential oils (%).

No.	KIL	KIE	Component	T+N Pelister Mtn.	T+N Nidze Mtn.	T+N Shara Mtn.	T-N Pelister Mtn.	T-N Nidze Mtn.	T-N Shara Mtn.
1	926	959.9	Tricyclene	0.2	0.3	0.1	tr	tr	tr
2	931	980.5	α-Thujene	tr	tr	tr	tr	-	tr
3	939	983.7	α-Pinene	23.8	29.9	39.9	23.3	21.2	22.1
4	953	988.5	Camphene	3.7	5.5	2.2	0.7	2.0	1.8
5	975	1000.3	Sabinene	tr	tr	0.1	tr	tr	0.1
6	980	1002.8	β-Pinene	11.9	10.1	17.1	13.0	8.2	16.4
7	991	1010.9	Myrcene	1.2	1.2	1.4	2.5	1.6	2.3
8	1003	1019.8	α-Phellandrene	0.2	1.1	0.2	0.1	0.1	0.3
9	1007	1024.0	Δ^3-Carene	0.6	1.1	0.3	0.8	1.1	0.2
10	1018	1028.8	α-Terpinene	0.1	0.4	0.1	tr	-	0.1
11	1025	1035.2	*p*-Cymene	0.1	0.1	0.1	0.1	0.1	0.1
12	1031	1039.0	Limonene+β-Phellandrene	14.0	9.5	6.8	8.8	23.6	13.3
13	1062	1064.5	γ-Terpinene	tr	tr	0.1	0.1	tr	0.1
14	1088	1092.2	Terpinolene	0.4	0.4	0.3	0.2	0.3	0.4
15	1120	1136.4	α- Campholenal	0.1	0.1	0.1	0.1	0.1	0.1
16	1139	1144.8	*trans*-Pinocarveol	0.3	0.3	0.3	0.4	0.3	0.5
17	1143	1153.6	Camphor	tr	tr	tr	-	tr	-
18	1144	1155.5	*trans*-Verbenol	tr	0.1	tr	0.1	0.1	0.1
19	1160	1169.1	*trans*-Pinocamphone	tr	tr	tr	0.1	0.1	tr
20	1162	1170.1	Pinocarvone	0.3	0.1	0.1	0.1	0.1	0.1
21	1165	1172.5	Borneol	0.6	0.8	0.4	0.1	0.3	0.3
22	1177	1181.9	Terpinene-4-ol	0.3	0.1	0.1	0.1	0.1	0.2
23	1183	1189.4	*p*-Cymene-8-ol	0.1	0.1	0.2	0.1	0.1	-
24	1189	1191.6	α-Terpineol	0.5	0.3	0.7	0.5	0.5	0.6
25	1193	1195.2	Myrtenal	0.3	0.4	0.3	0.4	0.3	tr
26	1204	1207.1	Verbenone	0.1	0.1	0.1	0.2	0.2	0.3
27	1212	1218.0	*trans*-Carveol	0.1	0.2	0.1	0.1	0.3	0.1
28	1220	1219.9	endo-Fenchyl acetate	tr	tr	-	-	-	-
29	1235	1237.1	Thymol methyl ether	-	-	-	tr	-	-
30	1243	1246.2	Carvone	0.1	0.1	tr	tr	0.1	tr
31	1249	1254.6	Piperitone	tr	tr	-	-	-	tr
32	1256	1257.6	Linalool acetate	tr	0.1	0.1	0.1	0.1	0.1
33	1261	1263.1	2-Decenal	tr	-	-	tr	tr	tr
34	1285	1285.6	Bornyl acetate	5.6	6.9	2.3	1.1	3.4	2.6
35	1314	1312.5	2E,4E-Decadienal	tr	-	-	-	tr	-
36	1339	1336.1	δ-Elemene	0.1	0.1	0.1	0.2	0.2	0.2
37	1350	1347.5	α-Terpenyl acetate	1.0	0.9	0.8	0.4	0.6	0.5
38	1373	1371.1	α-Ylangene	0.1	0.1	0.1	0.1	0.2	0.1
39	1376	1374.0	α-Copaene	0.2	0.2	0.1	0.3	0.2	-
40	1383	1380.9	Geranyl acetate	-	-	tr	-	tr	-
41	1384	1383.3	β-Bourbonene	0.1	0.1	0.1	0.1	tr	0.1
42	1391	1389.2	β-Elemene	0.4	0.3	0.4	0.4	0.1	0.2
43	1401	1400.7	Methyl eugenol	-	-	-	-	tr	0.1
44	1402	1403.4	Longifolene	0.2	0.6	tr	0.2	2.6	0.1
45	1418	1418.0	*trans*-(*E*)-Caryophyllene	4.1	3.6	4.3	7.3	3.2	4.9
46	1430	1430.1	β-Copaene	0.5	0.3	0.3	0.6	0.3	0.4
47	1439	1444.3	Aromadendrene	tr	0.1	0.1	tr	tr	0.3
48	1454	1453.6	α-Humulene	1.0	0.9	0.9	1.7	1.0	1.3
49	1473	1474.5	Dodecanol	0.2	0.2	0.1	-	-	-
50	1477	1475.8	γ-Muurolene	1.3	0.8	0.8	2.2	1.2	1.5
51	1480	1481.7	Germacrene D	9.5	7.8	7.1	10.3	5.0	7.1
52	1485	1493.9	β-Selinene	0.3	0.2	0.2	0.3	0.2	0.2
53	1494	1496.3	Bicyclogermacrene	0.1	0.5	-	tr	0.1	0.4
54	1495	1497.9	α-Muurolene	0.9	0.5	0.5	1.6	0.7	0.9
55	1513	1513.7	γ-Cadinene	1.0	1.0	0.8	1.9	1.5	1.6
56	1524	1525.3	δ- Cadinene	3.1	2.4	2.1	4.2	3.3	4.1
57	1532	1532.0	*trans*-Cadina-1,4-diene	0.1	0.1	0.1	0.2	0.1	0.2
58	1538	1540.6	α- Cadinene	0.2	0.2	0.2	0.3	0.2	0.2
59	1547	1546.4	α-Calacorene	0.1	tr	tr	0.1	0.1	0.1
60	1561	1561.4	E-Nerolidol	0.2	0.3	0.1	0.1	-	tr
61	1564	1571.3	β-Calacorene	0.1	tr	tr	0.1	0.1	0.1
62	1570	1575.2	3Z-Hexenyl bezoate	tr	0.1	0.1	-	-	-
63	1574	1577.0	Germacrene-4-ol	-	0.1	tr	-	-	-
64	1576	1583.0	Spathulenol	0.5	0.5	0.3	0.4	0.4	0.3

65	1581	1586.4	Caryophyllene oxide	0.7	0.9	0.4	1.2	1.3	1.0
66	1590	1598.0	Viridiflorol	-	-	-	-	0.1	-
67	1592	1609.0	Longiborneol	tr	-	-	0.1	0.2	0.1
68	1606	1613.0	Humulene epoxide II	0.1	0.5	-	0.4	1.1	0.7
69	1627	1637.8	1-*epi*-Cubenol	0.2	0.1	0.1	0.3	0.2	0.2
70	1640	1644.3	τ-Cadinol (epi-α-Cadinol)+τ-Muurolol (*epi*-α-Muurolol)	0.6	0.7	0.5	0.7	0.7	0.9
71	1645	1650.1	α-Muurolol (Torreyol)	0.1	0.1	0.2	0.3	0.2	tr
72	1653	1657.0	α-Cadinol	1.2	1.1	0.8	1.4	1.1	1.1
73	1700	1693.1	Amorpha-4,9-dien-2-ol	0.4	0.3	0.2	0.4	0.2	0.2
74	1733	1746.3	Oplopanone	tr	tr	tr	-	-	-
75	1762	1769.9	Benzyl benzoate	tr	tr	tr	tr	-	-
76	1880	1884.5	3Z-Hexenyl cinnamate	tr	0.1	tr	tr	-	-
77	1929	1946.7	Cembrene	0.1	0.1	0.1	0.1	0.2	0.2
78	1987	2009.4	Manool oxide	0.2	0.1	0.1	0.2	0.1	0.1
79	2054	2073.7	Abietatriene	tr	tr	tr	0.1	tr	tr
80	2080	2101.4	Abietadiene	tr	tr	tr	0.1	tr	tr
81	2149	2166.4	Abienol	0.1	tr	0.1	0.3	0.1	0.2
82	2220	2253.2	Sclareol	tr	tr	-	0.1	-	-
83	2263	2296.5	Dehydroabietal	0.1	0.1	0.1	0.2	0.1	0.1
84	2302	2332.1	Abietal	tr	-	-	0.1	-	0.1
			Monoterpene hydrocarbons (MH)	56.0	59.3	68.6	49.6	58.2	57.2
			Oxygen containing monoterpenes (OM)	9.4	10.6	5.6	3.9	6.7	5.6
			Sesquiterpene hydrocarbons (SH)	23.4	19.8	18.2	32.1	20.3	24.0
			Oxygen containing sesquiterpenes (OS)	4.0	4.6	2.6	5.3	5.5	4.5
			Diterpenes (D)	0.5	0.3	0.4	1.2	0.5	0.7
			Non-terpene components (NT)	0.4	0.7	0.3	-	-	-
			TOTAL	**93.7**	**95.3**	**95.7**	**92.1**	**91.2**	**92.0**

KIL - Kovat's (retention) index - literature data [13]; KIE – Kovat's (retention) index experimentally determined (AMDIS); (-) - not found; tr – trace < 0.05; No. - ordinal number of the component according to its retention time; T+N – twigs with needles; T-N – twigs without needles

Based on the presence of the total monoterpene (M) and sesquiterpene (S) fractions (both hydrocarbons and oxygen containing derivatives) in terms of the presence of other classes of components, it can be concluded that *P. peuce* essential oils from Baba Mtn. (Pelister), Nidze Mtn. and Shara Mtn are mainly composed of mono (M) (for T+N essential oils 65.4%, 69.9% and 74.2% and for T-N essential oils, 53.5%, 64.9% and 62.8%, respectively) and sesquiterpene (S) fractions (for T+N essential oils 27.4%, 24.4% and 20.8% and for T-N essential oils, 37.4%, 25.8% and 28.5%, respectively). Consequently, the trend of presence of the indicated fractions is the same in all tested samples (M > S > D > NT).

The most abundant components in all tested T+N and T-N essential oil samples were the monoterpenes α-pinene, β-pinene and limonene+β-phellandrene, followed by bornyl acetate, camphene and myrcene, and the sesquiterpenes germacrene D, *trans*-(*E*)-caryophyllene and δ-cadinene (Table 2). Generally, T+N essential oils contained larger amount of α-pinene, camphene and bornyl acetate than myrcene, limonene+β-phellandrene, *trans*-(*E*)-caryophyllene and δ-cadinene. Regarding T-N oils, the percentage representation of other dominant components is approximately the same. Additionally, amounts up to 1.0% of the sesquiterpenes α-humulene, γ-muurolene, γ-cadinene, caryophyllene oxide and α-cadinol were observed in T-N essential oil samples (Table 2). According to Koukos *et al*., the main components in the T-N oil isolated from *P. peuce* from Greece were α-pinene (7.3%), β-pinene (12.4%), β-phellanderene (26.9%) and β-caryophyllene (4.4%). Compared with these results, our tested T-N essential oils contain larger amounts of α-pinene and smaller amounts of β-phellanderene. Additionally, Koukos found that citronellol was present in large amount (12.4%), and declared it as dominant [11a]. This component was not identified in our tested samples. Furthermore, Papadopoulou *et al*. reported α-pinene (27.5-40.6), β-pinene (12.0-15.3%) and limonene (10.5-15.7%) as major constituents of *P. peuce* T-N oils [11b]. Then again, our samples contained smaller amount of α-pinene. Different environmental conditions and the time of collection of plant material could be one of the probable explanations for these differences in the chemical composition of the T-N essential oils of *P. peuce*.

Based on the available date, the chemical composition of *P. peuce* T+N essential oil differs a lot compared with that obtained from needles (N). According to our findings [12b], N essential oils isolated from plant material collected from the same localities in R. Macedonia contained smaller amounts of α-pinene, β-pinene and limonene+β-phellandrene, but larger amounts of bornyl acetate, *trans*-(*E*)-caryophyllene and germacrene D, which indicates that the presence of twigs (T) in the plant material during distillation affects the composition of the essential oil obtained from the needles (N).

The antimicrobial activity of the T+N and T-N essential oils was evaluated against Gram-positive and Gram-negative bacteria, as well as fungi. Their activity potentials were assessed qualitatively and quantitatively by the presence of inhibition zones, zone diameters and minimum inhibitory concentration (MIC) values. The results are presented in Table 3.

T-N essential oils showed antimicrobial activity towards *Streptococcus pneumoniae*, *Staphylococcus aureus*, *S. epidermidis* and *Candida albicans* as well as *Streptococcus agalactiae, Acinetobacter* spp. and *Haemophilus influenzae*. The susceptibility of the test microorganisms to the effects of *P. peuce* T+N essential oils differs in terms of the above mentioned oils. Generally, T+N essential oils showed greater antimicrobial activity, especially against *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Enterococcus* and *Candida albicans*, followed by *Streptococcus pneumoniae*, *Staphylococcus aureus*, *S. epidermidis*, *Haemophilus influenzae* and *Acinetobacter* spp. Furthermore, T+N essential oils revealed antimicrobial activity towards *Escherichia coli* and *Salmonella enteritidis*. T+N oils contain large amounts of α-pinene (23.8-39.9%), a component which has been found to have relatively strong antimicrobial properties and which may contribute to the antimicrobial activity, but probably this was not the only component responsible for the antimicrobial potency. Additionally, synergistic, additive or antagonistic interactions between volatile constituents in the essential oils may also affect the antimicrobial activity of the oil [14]. Other tested bacteria, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* showed resistance to the antimicrobial activity of both T+N and T-N essential oils. Minimal inhibitory concentration (MICs) values for all tested essential oil samples ranged from 15-125 μL/Ml, depending on the type of microorganism (Table 3).

Table 3: Antimicrobial activity of *Pinus peuce* essential oils[a].

Microorganism		T-N			T+N		
		Pelister Mtn.	**Shara Mtn.**	**Nidze Mtn.**	**Pelister Mtn.**	**Shara Mtn.**	**Nidze Mtn.**
Streptococcus pneumoniae	DD	27.5±0.1	27.6±0.2	28.3±0.2	n.a.	28.4±0.1	28.5±0.1
	MIC	15	31	15	n.a.	31	31
Staphylococcus aureus	DD	26.8±0.4	27.3±0.2	28.4±0.3	n.a.	n.a.	27.7±0.2
	MIC	31	62	62	n.a.	n.a.	15
Staphylococcus epidermidis	DD	27.1±0.2	27.5±0.4	28.5±0.2	n.a.	n.a.	28.2±0.2
	MIC	31	31	31	n.a.	n.a.	15
Streptococcus agalactiae	DD	n.a.	26.3±0.2	n.a.	28.8±0.1	28.0±0.1	28.5±0.1
	MIC	n.a.	31	n.a.	15	31	31
Streptococcus pyogenes	DD	n.a.	n.a.	n.a.	28.6±0.3	28.4±0.2	28.5±0.1
	MIC	n.a..	n.a.	n.a.	15	15	7
Enterococuus	DD	n.a.	n.a.	n.a.	28.6±0.2	28.1±0.1	28.3±0.2
	MIC	n.a..	n.a.	n.a.	125	125	125
Haemophilus influenzae	DD	n.a.	n.a.	26.3±0.3	n.a.	27.9±0.3	28.1±0.3
	MIC	n.a.	n.a.	31	n.a.	7	7
Acinetobacter spp.	DD	n.a.	26.2±0.3	n.a.	n.a.	n.a.	26.9±0.2
	MIC	n.a.	31	n.a.	n.a.	n.a.	31
Escherichia coli	DD	n.a.	n.a.	n.a.	n.a.	26.9±0.2	n.a.
	MIC	n.a.	n.a.	n.a.	n.a.	125	n.a.
Salmonella enteritidis	DD	n.a.	n.a.	n.a.	n.a.	27.7±0.2	n.a.
	MIC	n.a.	n.a.	n.a.	n.a.	125	n.a.
Klebsiella pneumonie	DD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	MIC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Pseudomonas aeruginosa	DD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	MIC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Proteus mirabilis	DD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	MIC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Candida albicans	DD	27.2±0.2	27.8±0.2	27.3±0.2	28.9±0.2	28.6±0.3	28.4±0.1
	MIC	62	62	62	31	31	31

[a]Data represent mean ± SD of three independent experiments; n.a. - no activity found; DD – Disc diffusion (zone of inhibition including the diameter of disc 6 mm); MIC – Minimum inhibitory concentration (μL/mL).

Up to now, there is a lack of data related to the antimicrobial activity of *P. peuce* essential oils, and this is the first report of antimicrobial screening of its T+N and T-N essential oils. Additionally, only a few studies on the antimicrobial activity of the T-N or T+N collected from some other pine species could be found. Antimicrobial screening of T-N essential oil isolated from *P. cembra* L. against six different microorganisms (*Staphylococcus aureus*, *Sarcina lutea*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*) was made by Apetrei *et al*. [8n]. They reported high antimicrobial activity against *Staphylococcus aureus* and *Sarcina lutea*, with minimum inhibitory concentrations (MIC) of 1.9 and 0.1 mg mL-1, respectively. Additionally, the essential oil exhibited moderate activity against *Candida albicans*, with a MIC value 7.8 mg mL-1, but no activity toward the other investigated microorganisms, such as *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. These differences between the antimicrobial effects of *P. peuce* and *P. cembra* T-N essential oils might be attributed to the variations in the chemical compositions of the oils. In this regard, *P. peuce* N essential oil showed antimicrobial activity toward *Streptococcus pneumoniae*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus agalactiae, S. pyogenes* and *Acinetobacter* spp., with MIC values from 7.5 to 62.5 μL/mL [12b]. This lower antimicrobial activity of *P. peuce* (N) essential oils in terms of both T+N and T-N essential oils is probably due to the smaller amount of α-pinene, as well s possible interactions between constituents of the oils.

Summarizing the results, it is evident that there were no differences in the qualitative composition of the essential oils, regardless of the locality of collection, or the type of plant material (T+N or T-N). On the other hand, the antimicrobial activity of *P. peuce* T+N and T-N essential oils collected from different localities in R. Macedonia differed a lot. These alterations in the antimicrobial activity of the tested essential oils can be attributed to the differences in the quantitative composition and percentage amounts of the components present in the respective essential oils.

Experimental

Plant material: The terminal twigs with needles (T+N) were collected from 3 different localities in R. Macedonia: Baba Mtn. (Pelister), Nidze Mtn. and Shara Mtn. in July, 2008. Plant identity was verified as *Pinus peuce* Griseb. and a herbarium voucher specimen N°2008/Pp was deposited at the Department of Pharmaceutical Botany, Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, R. Macedonia.

Before isolation of the essential oil, plant material was dried at room temperature. One part was minced to obtain T+N, while with the other part, the needles were separated to obtain T-N (twigs without needles); this was also minced before essential oil isolation.

Chemicals: Dimethylsulfoxide was purchased from Sigma-Aldrich (Steinheim, Germany), sodium chloride from Merck (Darmstadt, Germany), anhydrous sodium sulfate from Kemica (Zagreb, Croatia), and xylene from Alkaloid (Skopje, R. Macedonia).

***Essential oil isolation*:** Essential oil isolation from plant material was made by steam distillation in a special all-glass Clevenger type apparatus. For that purpose, 20 g of minced plant material (T+N and T-N) was distilled for 4 h. After isolation, anhydrous sodium sulfate was added to remove residual water from the oil.

The essential oil yield was calculated based on the dried plant material and was expressed in mL/kg. For GC/FID/MS analysis, the essential oil was dissolved in xylene to obtain a 1 μL/mL oil solution.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS): Essential oil samples were analyzed on an Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector, as well as capillary flow technology, which enables simultaneous analysis of the samples on both detectors. For that purpose, a HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm) was used. Operating conditions were as follows: oven temperature at 60°C (5

min), 1°C/min to 80°C (2 min) and 5°C/min to 280°C (5 min); helium as carrier gas at a flow rate of 1mL/min; injector temperature 260°C and FID 270°C. One μL of each sample was injected at a split ratio 1:1. The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 230°C, transfer line temperature 280°C and mass range from 50 - 500 Da. The MS was operated in scan mode.

Identification of the components: Identification of the components present in the essential oils was made by comparing mass spectra of components with those from NIST, Wiley and Adams mass spectra libraries, by AMDIS (Automated Mass Spectral Deconvolution and Identification System) and by comparing literature and estimated Kovat′s (retention) indices that were determined using a mixture of a homologous series of normal alkanes from C_9 to C_{25} in *n*-hexane, under the above mentioned conditions. The percentage ratio of essential oils components was computed by the normalization method of the GC/FID peak areas without any correction factors.

Microbial strains and cultures: Thirteen bacterial isolates representing both Gram-positive and Gram-negative bacteria and one strain of *Candida albicans* were used for antimicrobial screening. Five isolates were standard strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* 25927, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231). The remaining 9 bacterial strains (*Staphylococcus epidermidis, Enterococcus, S. pyogenes, S. agalactiae, S. pneumoniae, Haemophilus influenzae, Proteus mirabilis, Salmonella enteritidis* and *Acinetobacter* spp.) were clinical isolates provided from the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, R. Macedonia.

A nutrient (Mueller Hinton) agar (Merck, Darmstadt, Germany) was used for growing the bacteria, with the exception of *Streptococcus pyogenes, S. agalactiae, S. pneumoniae* and *Enterococcus*, which were grown on blood agar (Oxoid, Basingstoke, UK), and *Candida albicans*, which was grown on Sabouraud agar (bioMerieux, Durham, NC).

Disc diffusion method: The disc diffusion method was used for screening the antimicrobial activity of all the essential oils. In this regard, microorganisms were suspended in sterile broth with turbidity corresponding to 0.5 and 1 Mc Farland (approximately 10^7-10^8 CFU/mL) for all bacteria and for *Candida albicans*, respectively. The microbial suspensions were streaked over the surface of the agar media using sterile cotton swabs to ensure uniform inoculation. After inoculation, discs of 6 mm in diameter were placed at well-spaced intervals. They were filled with 85 μL of 50% solutions of essential oils in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany) and one disc was filled only with DMSO as a control. The plates were incubated at 37^{O}C, aerobically for 24 h. The growth inhibition zones were measured after incubation of the isolates under their optimal growth conditions and were ranged between 6 mm and 30 mm in diameter. The antimicrobial activity was determined according to the diameters of the inhibition zones (0-14 mm resistant, 14-19 mm moderately susceptible, and 19-30 susceptible microorganisms).

Broth dilution method: This method was used to determine the minimal inhibitory concentration (MIC) of essential oils (50% solution in DMSO) that had revealed good antimicrobial activity in the disc diffusion method (diameter of the inhibition zone 19-30 mm). For this purpose, 25 μL of each essential oil was diluted in equal quantities of 0.9% sodium chloride solution, to make a concentration of 25%. This was decreased 5 times, subsequently, by adding 25 μL of each bacterial or fungal suspension, and thus the final concentrations were: 12.5%, 6.2%, 3.1%, 1.5% and 0.7% or 125 μL/mL, 62 μL/mL, 31 μL/mL, 15 μL/mL and 7 μL/mL, respectively. Fifteen μL of each bacterial or fungal suspension with these particular concentrations was inoculated on solid media (Miller-Hinton agar, blood agar, Sabouraud agar), depending on the type of microorganism. The growth of any microorganism was evaluated after its incubation under the optimal growth conditions. The lowest concentration of essential oil that was able to inhibit the growth of the particular microorganism was considered as its minimal inhibitory concentration (MIC).

Statistical analysis*:* Data obtained from determination of essential oil yield and zones of inhibition are expressed as mean values. Statistical analysis were carried out by employing one way ANOVA (p<0.05). Statistical package STATGRAPH version 21.0 was used for data analysis.

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