

Antimicrobial activity of needle essential oil of *Pinus peuce* Griseb. (Pinaceae) from Macedonian flora

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Abstract

Chemical composition and antimicrobial activity of needle essential oil, obtained by hydrodistillation from wild *Pinus peuce* Griseb. (Pinaceae), growing on three different locations in R. Macedonia were investigated in period 2008/2009. Carried out GC/FID/MS analysis, one hundred and three constituents were identified belonging to the six different classes of components: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenes and other non-terpene components, representing 88.61/94.04% of the entire oil. The most abundant constituents were α -pinene (12.89/27.34%), β -pinene (6.16/13.13%), limonene + β -phellandrene (2.09/6.64%) and bornyl acetate (2.92/11.67%) as well as *trans*-(E)-caryophyllene (4.63/7.13%) and germacrene D (8.75/20.14%).

Antimicrobial screening of *Pinus peuce* needle essential oil was made by hole-plate diffusion and broth dilution method against 13 bacterial isolates of Gram positive and Gram negative bacteria and one strain of *Candida albicans*. The most sensitive bacteria against tested *Pinus peuce* essential oils were *Streptococcus pneumonia* encompassing *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Acinetobacter* spp. and *Streptococcus pyogenes*. Minimal inhibitory concentrations (MICs) of the oils ranged from 7.5 - 62.5 μ l/ml.

Key words: *Pinus peuce*, Macedonian pine, essential oil composition, GC/FID/MS, antimicrobial activity.

Introduction

Representatives of genus *Pinus* in flora of R. Macedonia include five different species: *Pinus nigra* Arnold, *Pinus sylvestris* L., *Pinus mugo* Turra, *Pinus heldreichii* Christ. subsp. *leucodermis* (Antoine) Blečić and *Pinus peuce* Griseb. (Micevski, 1985). Most of the *Pinus* species are trees or shrubs with specific morphological characteristics of leaves (needles) rich in strong and terpene-aromatic essential oil. Pine needle essential oils are mainly used in folk medicine for the treatment of respiratory in-

fections accompanied by cough, bronchitis, bronchial asthma, emphysema, tracheitis, sinusitis, laryngitis, pharyngitis, tonsillitis and influenza (Dervendzi, 1992). Up to now, there has been an increased interest in studying chemical composition as well as biological activity of the essential oils isolated from different pine species. Generally, monoterpenes and sesquiterpenes like α -pinene and β -pinene, camphene, Δ^3 -carene, β -myrcene, limonene, phellandrene, β -ocymene, β -caryophyllene, bornyl acetate, germacrene D, cadinene and muurolene are mentioned as dominant components of pine needle essential oils (Rousis, 1994; Ucar, 2004; Tognolini, 2006; Naydenov, 2006; Idzajtich, 2005; Grassmann, 2003; Grassmann, 2005; Stevanovic, 2005; Ustun, 2006; Maciag, 2007; Judzentiene, 2006; Kupcinskiene, 2008; Semiz, 2007; Kainulainen,

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2002, Holzke, 2006; Dob, 2007; Menkovic, 1993; Nikolic, 2007; Dob, 2006; Pagula, 2006; Dormont, 1998; Yong-Suk, 2005; Oluwadayo, 2008; Sacchetti, 2005; Barnola, 2000). Some of these oils like essential oil isolated from *Pinus caribaea* Morelet, *Pinus densiflora* S. and Z., and *Pinus radiata* D. Don have showed different antimicrobial activity against different microorganisms (Oluwadayo, 2008; Yong-Suk, 2005; Sacchetti, 2005). The antimicrobial activity of pine essential oils is of multipurpose thus they are used in manufacture of medicinal products and cosmetics as antimicrobial additives, etc. Nowadays, there are few 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 (Kamin, 2007).

Pinus peuce, known as "Molica" or "Macedonian pine" is an endemic species of Balkan Peninsula mostly spread in southern and western parts of R. Macedonia (Micevski, 1985), northern part of Greece and south-eastern parts of Bulgaria, as well as in parts of Albania, Serbia and Montenegro. Up to present, there are a lack of data that are related to the essential oil composition of *Pinus peuce* population from Macedonian flora and its antimicrobial activity, thus the aim to this study was to investigate the chemical composition of the needle essential oil of *Pinus peuce* from Macedonian flora and to assess its antimicrobial activity against certain types of microorganisms that affect respiratory, gastrointestinal and urogenital system and provoke pathological conditions on skin.

Material and methods

Plant material

Plant material was collected from four different localities in R. Macedonia: Baba Mtn. (Pelister), Nidze Mtn., Shara Mtn. and Karadzica Mtn. in July, 2008 and 2009, and was dried at room temperature and on draft for two weeks. Just before hydrodistillation, the needles were separated from the branches and were properly minced.

Determination of water content

Determination of water content was made by distillation according to Ph.Eur.7 regulations (2.2.13.) from 20 g dried plant material.

Essential oil isolation

Essential oil isolation was made by hydrodistillation in all-glass Clevenger apparatus (Ph.Eur 7, 2.8.12.). For that purpose, 20 g of minced needles were distilled for 4 hours. For purification purpose, anhydrous sodium sulfate was added to the isolated essential oil to remove residual water. For GC/FID/MS analysis, the essential oil was dissolved in xylene (Alkaloid, R. Macedonia) to obtain 1 µl/ml oil solution.

Gas chromatography

Essential oil samples were analyzed on 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, 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 that of the FID detector 270 °C. 1 µl of each sample was injected at 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 present in essential oils was made by comparing mass spectra of components in essential oils 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 mixture of homologous series of normal alkanes from C₉ to C₂₅ in hexane, under the same above mentioned conditions.

The percentage ratio of essential oils components was computed by the normalization method of the GC/FID peak areas and average values were taken into further consideration (n=3).

Antimicrobial screening

Antimicrobial activity of essential oils was studied against 14 different microorganisms, including 13 bacterial isolates representing both Gram-positive (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Enterococcus*) and Gram-negative bacteria (*Acinetobacter* spp, *Escherichia coli* ATCC 25927, *Salmonella enteritidis*, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* and *Proteus mirabilis*) and one strain of *Candida albicans* ATCC 10231.

Hole-plate diffusion method was used for screening the antimicrobial activity of all essential oils (determination of growth inhibition zones of studied microorganisms that occur around certain essential oil). This investigation was followed by broth dilution method (determination of minimal inhibitory concentration MIC of the particular oil that had revealed good antimicrobial activity by hole-plate diffusion method).

Hole-plate diffusion method

A nutrient agar (Mueller Hinton) was prepared by dissolving agar (28 g) in distilled water (1000 ml). The mixture was heated to dissolve and autoclaved at 121 °C for 15 minutes. The nutrient agar was poured into sterile Petri dishes at uniform depth of 3 mm and allowed to solidify. Blood agar (Oxoid) was used for testing the antimicrobial activity against *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Enterococcus*, while Sabouraud agar (bioMerieux) was used for testing the antimicrobial activity against *Candida albicans*. Microorganisms were suspended in sterile broth with turbidity corresponding to 0.5 and 1 Mc Farland (approximate by 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 a sterile cotton swabs to ensure uniform inoculation. After inoculation of microorganisms, holes of 6 mm in diameter were made at well-spaced intervals. They were filled with 85 µl of 50% solutions of essential oils in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany) and one hole was filled only with DMSO as a control. The plates were incubated at 37 °C, aerobically for 24 hours. 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. If the diameter of the growth inhibition zones was in the range between 0 and 14 mm, those species were accepted like resistant; if the diameter was between 14 and 19 mm, the species were accepted like moderate susceptible and in the cases where the diameter was above 19 mm, species were accepted like susceptible.

Broth dilution method

This method was used for some particular essential oil (50% solution in DMSO) that had revealed good antimicrobial activity by hole-plate diffusion method. For that purposes, 25 µl of those essential oils were diluted in equal quantities of 0.9% sodium chloride solution, to make them with the concentration of 25%. This concentration was decreased five times, subsequently, by adding 25 µl of each bacterial or fungal suspension, thus the final concentrations were: 12.5%, 6.25%, 3.125%, 1.562% and 0.75% or 125 µl/ml, 62.5 µl/ml, 31.25 µl/ml, 15.26 µl/ml and 7.5 µl/ml, respectively. 15 µl of each bacterial or fungal suspensions with these particular concentrations were inoculated on solid media (Miller-Hinton agar, blood agar, Sabouraud agar), depending on the microorganism. The growth of any microorganism was evaluated after its incubation under the optimal growth conditions. The lowest concentration of essential oil which was able to inhibit the growth of the particular microorganism was considered as its minimal inhibitory concentration (MIC).

Results and discussion

Essential oil yield, calculated on anhydrous needles of *Pinus peuce* is given in Table 1. The obtained essential oils were transparent, agile, light yellowish liquids with specific and very strong turpentine odor.

Table 1. The yield* of *Pinus peuce* needle essential oils calculated on anhydrous plant material.

Sample	Water content (ml/kg)	Yield* of essential oil (ml/kg) ± SD
Pelister Mtn.	78.21	5.04 ± 1.49%
Nidze Mtn.	68.33	9.93 ± 0.27%
Shara Mtn.	93.75	9.24 ± 2.86%
Karadzica Mtn.	80.00	2.86 ± 1.73%

*(n=3)

Table 2 shows components that were identified in the *Pinus peuce* needle essential oils with their percentage amount and Kovat's retention indices. Given percentage values are averages of percentages of the components that are present in the essential oils obtained from plant material collected from various altitudes on four different localities: Pelister, Nidze, Shara and Karadzica Mtn.

Total of one hundred and three components were identified in the investigated samples of *Pinus peuce* needle essential oils. Analysis of the essential oils composition shows seasonal variations in the content of components that are present, probably due to the influence of weather conditions throughout the year. The most abundant components in all samples of isolated essential oils were monoterpenes: α -pinene, β -pinene, limonene + β -phellandrene and bornyl acetate and sesquiterpenes: *trans*-(E)-caryophyllene and germacrene D. The content of each of these 6 components varies in relatively wide range but generally they are present in amounts from 2% to 28% and are likely to determine the physico-chemical characteristics as well as chemical profile of the oils. Large amount of monoterpenes camphene and α -terpenil acetate and sesquiterpenes δ -cadinene and α -cadinol was observed in some essential oils samples (Table 2).

Needle essential oil isolated from plant material collected in 2008/2009 on Baba Mtn. (Pelister) contains 12.89/19.72% α -pinene, 6.16/7.95% β -pinene, 3.08/3.97% limonene + β -phellandrene, 9.29/10.56% bornyl acetate, 4.88/7.13% *trans* (E)-caryophyllene and 8.75/19.90% germacrene D. These dominant components represent 54.56/59.72% of the whole needle essential oil. Essential oil obtained from *Pinus peuce* collected in 2008 from Nidze Mtn. contains larger amount of α -pinene (27.34%), β -pinene (10.73%) and limonene + β -phellandrene (6.64%), but smaller amount of bornyl acetate (7.71%). Among dom-

Table 2. Chemical composition of *Pinus peuce* needle essential oil (%).

No.	KIL	KIE	Component	P. Mtn.		N. Mtn.		Sh. Mtn.		K. Mtn.	
				2008	2009	2008	2009	2008	2009	2008	2009
1	926	959.9	Tricyclene	0.02	0.02	0.02	0.02	0.02	-	0.14	-
2	931	980.5	α -Thujene	-	-	0.02	0.02	-	-	-	-
3	939	983.7	α -Pinene	12.89	19.72	27.34	27.34	23.91	12.95	12.22	9.21
4	953	988.5	Camphene	2.69	5.03	6.41	6.41	1.56	4.95	1.5	0.85
5	-	990.1	Benzene acetaldehyde ^t	0.16	0.58	0.24	0.24	0.16	0.14	0.02	1.39
6	-	993.6	p-Ethyl methyl benzene ^t	0.04	2.64	0.12	0.12	0.05	-	-	7.81
7	-	998.1	1,2,5-Trimethyl benzene ^t	0.15	2.23	0.28	0.28	0.20	0.16	0.02	6.30
8	975	1000.3	Sabinene	0.05	0.08	0.07	0.07	0.08	-	0.09	0.30
9	980	1002.8	β -Pinene	6.16	7.95	10.73	10.73	13.13	8.40	6.03	4.88
10	991	1010.9	Myrcene	0.57	0.79	1.03	1.03	0.67	0.50	1.39	0.02
11	1000	1016.7	n-Decane	0.34	3.02	0.94	0.94	0.97	0.84	0.43	5.18
12	1003	1019.8	α -Phellandrene	0.14	0.34	0.45	0.45	0.05	0.15	0.75	0.67
13	1007	1024.0	Δ^3 -Carene	0.33	-	0.17	0.17	-	-	1.66	-
14	1018	1028.8	α -Terpinene	0.05	0.10	0.21	0.21	0.39	0.07	-	1.20
15	1023	1033.3	1,2,4-Trimethyl benzene ^t	0.02	0.42	0.02	0.02	-	0.02	-	1.26
16	1025	1035.2	p-Cymene	0.22	0.80	0.33	0.33	0.39	0.32	0.02	0.02
17	1031	1039.0	Limonene + β -Phellandrene	3.08	3.97	6.64	6.64	2.09	3.06	2.40	2.03
18	1033	1040.8	1,8-Cineole	0.13	0.20	0.13	0.13	0.15	-	-	0.02
19	1040	1064.3	β -cis-Ocymene	0.02	-	0.02	0.02	0.32	0.21	-	-
20	-	1057.2	2-Phenilpropanol ^t	0.12	0.02	0.07	0.07	0.23	0.20	-	0.40
21	1057	1058.2	trans-Decahydronaphthalene ^t	0.06	0.39	0.02	0.02	-	0.21	-	0.36
22	1062	1064.5	γ -Terpinene	0.13	0.37	0.10	0.10	0.28	-	-	0.02
23	1067	1084.0	m-Tolualdehyde	0.14	2.30	0.73	0.73	0.74	1.28	0.44	0.02
24	1079	1084.4	p-Tolualdehyde	0.50	1.37	0.02	0.02	0.39	0.62	0.02	0.02
25	1088	1092.2	Terpinolene	0.50	0.65	0.50	0.50	0.32	0.53	0.49	0.40
26	1099	1106.8	n-Undecane	1.10	1.75	1.37	1.37	1.44	1.26	0.63	1.94
27	-	1131.2	trans-p-Menth-2-en-1-ol	0.30	0.07	0.15	0.15	0.19	0.08	-	-
28	1120	1136.4	α -Campholenal	0.13	0.02	0.02	0.02	0.02	0.11	0.02	0.02
29	1139	1144.8	trans-Pinocarveol	0.27	0.02	-	-	-	-	-	-
30	1261	1145.0	2,6-Dimethylphenol ^t	0.76	1.50	0.51	0.51	0.41	0.68	0.20	-
31	-	1148.0	Viridene ^t	-	0.02	0.03	0.03	0.24	0.11	-	0.02
32	1143	1153.6	Camphor	0.06	-	-	-	-	0.20	-	-
33	1160	1169.1	trans-Pinocamphone	-	-	-	-	-	-	-	0.02
34	1162	1170.1	Pinocarvone	0.20	0.02	0.08	0.08	-	-	-	-
35	1165	1172.5	Borneol	0.64	0.76	0.48	0.48	0.17	2.80	-	-

No.	KIL	KIE	Component	P. Mtn.		N. Mtn.		Sh. Mtn.		K. Mtn.	
				2008	2009	2008	2009	2008	2009	2008	2009
36	1177	1181.9	Terpinene-4-ol	0.08	0.02	0.03	0.03	0.06	0.09	-	0.02
37	1183	1189.4	p-Cymene-8-ol	0.08	0.02	-	-	0.08	0.11	-	0.02
38	1189	1191.6	α -Terpineol	0.30	0.29	0.17	0.17	0.43	0.28	0.10	0.28
39	1193	1195.2	Myrtanal	0.29	0.16	0.20	0.20	0.11	0.18	0.13	0.35
40	1198	1199.2	Dodecane ¹	0.25	0.23	0.34	0.34	0.35	0.32	0.17	0.13
41	1205	1205.6	<i>trans</i> -Piperitol	0.19	0.20	0.04	0.04	-	0.08	-	-
42	-	1207.1	Verbenone	0.04	0.06	0.11	0.11	0.35	0.11	-	-
43	1212	1218.0	<i>trans</i> -Carveol	0.08	0.02	0.02	0.02	0.07	-	-	0.02
44	1220	1219.9	endo-Fenchyl acetate	0.03	0.02	0.03	0.03	0.02	0.11	-	-
45	-	1254.6	Piperitone	0.02	-	0.02	0.02	-	0.10	-	-
46	1256	1257.6	Linalool acetate	0.12	0.02	0.02	0.02	0.08	0.05	-	-
47	1261	1263.1	2-Decenal ¹	0.02	0.12	-	-	-	-	-	-
48	1285	1285.6	Bornyl acetate	10.56	9.29	7.71	7.71	2.92	11.67	0.20	0.17
49	1294	1296.2	Tridecane ¹	0.02	-	0.03	0.03	0.07	0.08	-	-
50	1314	1312.5	2E,4E-Decadienal ¹	0.02	0.02	0.02	0.02	-	-	-	-
51	1339	1336.1	δ -Elemene	0.21	0.11	0.19	0.19	0.18	0.17	0.91	0.22
52	1350	1347.5	α -Terpenyl acetate	2.97	1.20	1.10	1.10	1.63	1.80	0.17	-
53	1373	1371.1	α -Ylangene	0.02	-	0.02	0.02	0.02	-	-	0.02
54	1376	1374.0	α -Copaene	0.10	0.02	0.03	0.03	0.09	0.07	0.22	0.03
55	1383	1380.9	Geranyl acetate	0.04	-	-	-	-	-	-	-
56	1384	1383.3	β -Bourbonene	0.11	0.12	0.09	0.09	0.23	0.12	0.31	0.14
57	1391	1389.2	β -Elemene	0.47	0.36	0.51	0.51	0.35	0.11	1.30	0.50
58	1401	1400.7	Methyl eugenol	-	-	-	-	-	-	-	0.02
59	1402	1403.4	Longifolene	0.02	-	0.05	0.05	-	-	-	-
60	1418	1418.0	<i>trans</i> (E)-Caryophyllene	7.13	4.88	4.63	4.63	5.55	6.35	5.15	3.92
61	-	1430.1	β -Copaene	0.76	0.40	0.39	0.39	0.49	0.73	0.86	0.22
62	1439	1444.3	Aromadendrene	0.04	-	-	-	-	-	0.13	-
63	-	1446.8	γ -Amorphene	0.19	0.15	0.19	0.19	-	0.52	0.38	-
64	1454	1453.6	α -Hummulene	1.59	0.79	0.62	0.62	1.27	0.63	1.29	0.54
65	-	1469.1	Epi-bicyclosesquiphellandrene	0.06	0.52	0.54	0.54	0.14	0.84	0.24	0.30
66	1473	1474.5	Dodecanol	0.15	0.07	0.03	0.03	0.11	-	-	0.02
67	1477	1475.8	γ -Muurolene	0.15	0.22	0.30	0.30	0.50	0.30	1.18	0.79
68	1480	1481.7	Germacrene D	19.90	8.75	10.10	10.10	20.14	19.36	9.90	7.13
69	1485	1493.9	β -Selinene	0.03	-	-	-	-	-	0.35	0.02
70	1494	1496.3	Bicyclogermacrene	0.99	1.43	0.61	0.61	2.00	1.16	1.89	1.29
71	1495	1497.9	α -Muurolene	0.92	0.25	1.53	1.53	0.02	-	1.41	1.88
72	-	1507.5	<i>trans</i> -Cadina-1(6)4-diene	0.02	-	0.03	0.03	0.14	0.09	-	-

No.	KIL	KIE	Component	P. Mtn.		N. Mtn.		Sh. Mtn.		K. Mtn.	
				2008	2009	2008	2009	2008	2009	2008	2009
73	1513	1513.7	γ -Cadinene	0.45	0.39	0.49	0.48	0.50	0.50	2.68	0.96
74	1524	1525.3	δ -Cadinene	2.71	1.52	1.82	1.79	1.70	1.70	8.29	5.72
75	1532	1532.0	<i>trans</i> -Cadin-1,4-diene	0.06	0.39	0.08	0.05	0.23	0.23	0.23	0.30
76	1538	1540.6	α -Cadinene	0.13	0.31	0.33	0.20	0.25	0.25	0.31	0.32
77	1547	1546.4	α -Calacorene	0.02	0.14	0.02	0.02	0.02	0.02	0.02	0.02
78	1564	1561.4	Nerolidol-(E)	0.18	0.05	0.10	0.20	0.02	0.02	3.12	0.54
79	1568	1564.8	Dodecanoic acid ^t	0.50	0.06	0.03	0.08	0.09	0.09	0.02	-
80	-	1571.3	β -Calacorene	0.02	-	-	-	0.02	0.02	0.02	-
81	1570	1575.2	Hexenyl bezoate (3-Z-)	0.05	0.02	0.02	0.02	-	-	0.22	0.26
82	1574	1577.0	Germaecene-4-ol	0.10	-	0.02	0.29	0.23	0.23	3.71	0.42
83	1576	1583.0	Spathulenol	0.32	0.08	0.23	0.15	0.15	0.15	0.02	0.18
84	1581	1586.4	Caryophyllene oxide	0.57	0.07	0.19	0.41	-	-	0.68	0.33
85	1590	1598.0	Viridiflorol	0.03	-	-	-	-	-	-	-
86	1606	1613.0	Humulene epoxide II	0.12	-	0.10	0.12	0.25	0.25	0.43	-
87	1627	1637.8	1-epi-Cubenol	0.07	0.02	0.03	0.06	0.02	0.02	0.31	0.02
88	1640	1644.3	τ -Cadinol (epi- α -Cadinol) +	1.58	0.52	0.75	1.18	1.13	1.13	4.46	2.30
89	1641	1647.4	τ -Muurolool (epi- α -Muurolool)	0.24	0.23	0.02	0.13	0.70	0.70	0.76	0.16
90	1645	1650.1	α -Muurolool (Torreyol)	1.99	0.46	0.32	1.52	1.51	1.51	7.04	2.85
91	1653	1657.0	α -Cadinol	0.17	0.03	0.03	0.13	-	-	0.34	0.10
92	-	1693.1	Amorpha-4,9-dien-2-ol	-	-	0.02	-	0.06	0.06	-	-
93	1733	1746.3	Oplopanone	0.05	0.02	0.02	0.10	0.10	0.10	0.07	-
94	1762	1769.9	Benzyl benzoate	0.15	1.23	0.04	0.13	0.04	0.04	0.36	0.48
95	-	1884.5	Hexenyl cinnamate (3-Z-)	0.04	-	0.03	-	-	-	-	-
96	1929	1946.7	Cembrene	0.09	-	0.03	0.08	0.08	0.08	0.33	-
97	-	2009.4	Manool oxide	0.02	0.06	0.08	0.02	0.11	0.11	-	0.15
98	1967	2013.0	Sclarene	0.02	0.02	0.02	-	0.04	0.04	-	0.02
99	2054	2073.7	Abietatriene	0.02	0.02	0.04	-	0.04	0.04	0.11	0.13
100	2080	2101.4	Abietadiene	0.02	-	0.04	-	-	-	0.20	-
101	-	2166.4	Abienol	-	-	-	-	-	-	-	-
102	2220	2253.2	Sclareol	-	-	0.13	-	-	-	-	-
103	2263	2296.5	Dehydroabietal	0.02	-	0.02	-	0.05	0.05	0.41	0.02
103	2302	2332.1	Abietal	-	0.08	0.08	-	-	-	-	3.74
TOTAL				89.72	88.61	94.04	92.97	92.52	92.52	88.72	81.31

n=3; KIL - Kovat's index literature; KIE - Kovat's index estimated; P.Mtn., N.Mtn., Sh.Mtn., N.Mtn., N.Mtn. and K.Mtn. - Pelister, Nidze, Shara and Karadzica Mountain, respectively; (-) - not found, t - tentative identification, No - ordinal number of the component according to its retention time.

inant components in the essential oil obtained from plant material collected in 2008/2009 on Shara Mtn., α -pinene (12.95/23.91%), β -pinene (8.40/13.13%) and germacrene D (19.36/20.14%) were the most abundant while limonene + β -phellandrene (2.09/3.06%) and bornyl acetate (2.92/11.67%) were present in the smaller amount. These components represent 67.15% and 61.79/67.74% of whole needle essential oil isolated from *Pinus peuce* from Nidze and Shara Mtn., respectively. Essential oil composition of cultivated *Pinus peuce* from Karadzica Mtn. (samples collected in 2008/2009) is significantly different from the others essential oils mentioned above. This essential oil contains smaller amount of α -pinene (9.21/12.22%), β -pinene (4.88/6.03%), bornyl acetate (0.17/0.20%) and germacrene D (7.13/9.90%), but it contains larger amount for two more components, δ -cadinene (5.72/8.29%) and α -cadinol (2.85/7.04%). Large amount of diterpene abietal (3.74%) was measured in the essential oil isolated from plant material collected in 2009.

Data analysis of the chemical composition of *Pinus peuce* needle essential oils revealed six different classes of components: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenes and other non-terpene components. Among different classes of components that are present in the essential oil isolated from plant material from Baba (Pelister), Nidze, Shara and Karadzica Mtn, the major constituents were terpene hydrocarbons (62.44%, 76.59%, 69.86% and 54.46%, respectively) while oxygenated terpenes were present in smaller amount (Fig. 1.). The most abundant fraction among the total terpene amount were monoterpenes (47.2% in essential oil samples from

Pelister, 62.16% in essential oil samples from Nidze Mtn. and 49.35% in samples from Shara Mtn.) (Fig.2.). Dominant sesquiterpene fraction (45.23%) despite monoterpenes (23.96%) is present only in the needle essential oils isolated from cultivated *Pinus peuces* from Karadzica Mtn. This is probably due to the environmental conditions that affect terpene biosynthesis of *Pinus peuce* in this region.

Up to now, there are only few data related to the chemical composition of *Pinus peuce* needle essential oils (Henning, 1994; Papadopoulou, 1996; Koukos, 2000; Petrakis, 2001; Nikolic, 2008; Karapandzova, 2010a; Karapandzova, 2010b; Karapandzova, 2011). Generally, there are some differences in chemical composition of essential oils isolated from *Pinus peuce* growing in Greece, Serbia and Montenegro in terms of presence of the dominant components in the oil. Compared with essential oils isolated from *Pinus peuce* from Macedonia, the needle essential oil isolated from *Pinus peuce* from Greece contain larger amount of β -pinene (22.00%) and citronellol (13.42%), smaller amount of *trans* (E)-caryophyllene (3.05%), but it doesn't contain germacrene D (Koukos, 2000). On the other hand, the needle essential oil isolated from *Pinus peuce* from Montenegro and Serbia contain larger amount of α -pinene (36.5%) and camphene (8.5%) (Nikolic, 2008).

The most sensitive bacteria against tested needle essential oils were *Streptococcus pneumoniae*. *Streptococcus agalactiae* showed similar sensitivity as previous bacteria and was susceptible to the effects of needle essential oils isolated from *Pinus peuce* from Shara and Nidze Mtn. as well as moderate susceptible to the effects of the needle essential oil from Pelister. *Streptococcus pyogenes* was susceptible to the effects of essential oils isolated from

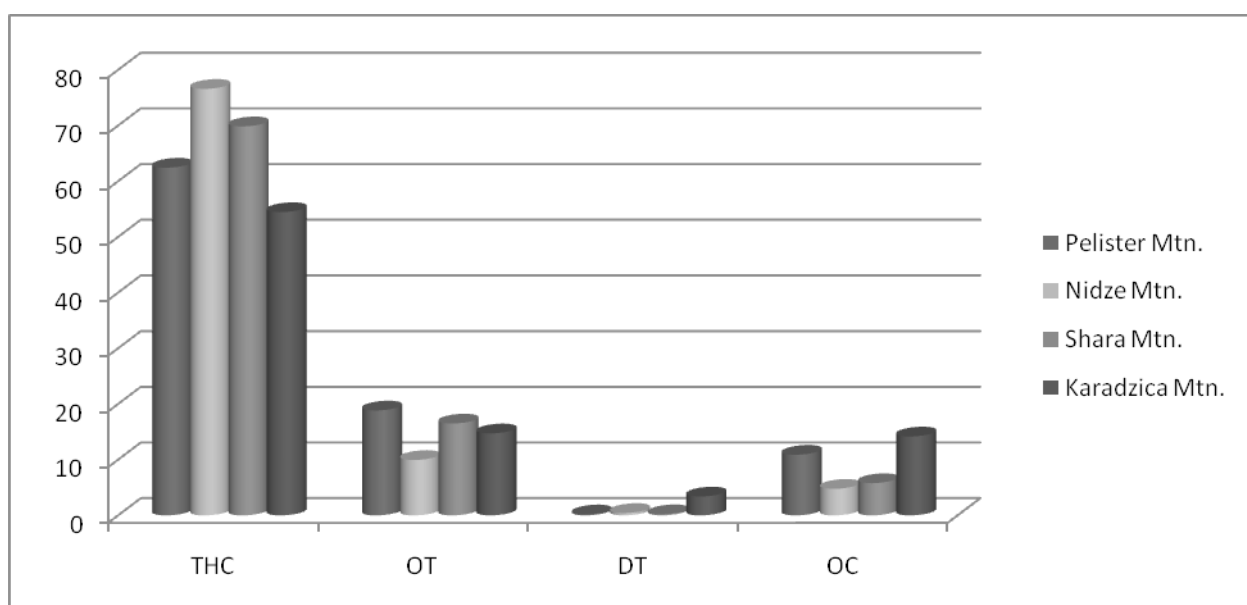


Fig. 1. Abundance of terpene hydrocarbons (THC) and oxygenated terpenes (OT) regarding diterpenes (DT) and other components (OC) in *Pinus peuce* needle essential oils.

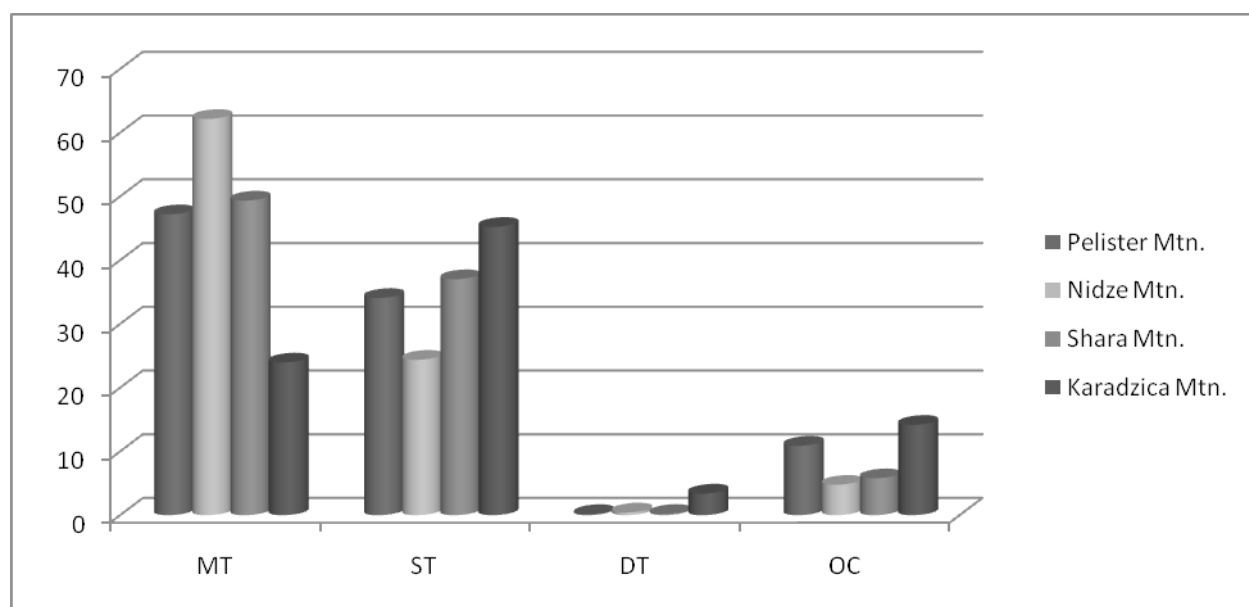


Fig. 2. Abundance of monoterpenes (MT), sesquiterpenes (ST), diterpenes (DT) and other components (OC) in *Pinus peuce* needle essential oils.

Pinus peuce from Shara Mtn. and moderate susceptible to the essential oils from Pelister and Nidze Mtn. Bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Acinetobacter* spp. showed sensitivity to the effects of essential oils isolated from plant material collected from Pelister and Nidze Mtn. Other investigated bacteria and *Candida albicans* were resistant to the antimicrobial effects of the *Pinus peuce* needle essential oils.

Summarizing the obtained results it is evident that the essential oil isolated from *Pinus peuce* from Pelister showed antimicrobial effect toward *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Acinetobacter* spp. Essential oil isolated from plant material from Shara Mtn. showed antimicrobial activity against *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Streptococcus pyogenes*, while the needle essential oil from *Pinus peuce* from Nidze Mtn. showed effects against *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae* and *Acinetobacter* spp. (Fig. 3.). This essential oil showed the best antimicrobial activity among all tested *Pinus peuce* needle essential oils.

Minimal inhibitory concentration (MIC) values of *Pinus peuce* needle essential oils for tested microorganisms determined by broth dilution method are given in Table 3. The lowest MIC (less than 7.5 $\mu\text{l/ml}$) is determined in essential oil isolated from *Pinus peuce* from Shara Mtn. against *Streptococcus pyogenes*, while the highest MIC value (62.5 $\mu\text{l/ml}$) is determined in essential oil obtained from plant material collected on Baba Mtn. (Pelister) toward *Staphylococcus aureus*.

Table 3. Minimal inhibitory concentrations (MIC) of *Pinus peuce* needle essential oils determined by broth dilution method ($\mu\text{l/ml}$).

Microorganism	MIC ($\mu\text{l/ml}$)		
	Pelister Mtn.	Shara Mtn.	Nidze Mtn.
<i>Streptococcus pneumoniae</i>	31.25	15.62	15.62
<i>Staphylococcus aureus</i>	62.5	n.a.	31.25
<i>Staphylococcus epidermidis</i>	31.25	n.a.	15.62
<i>Streptococcus agalactiae</i>	n.a.	31.25	15.62
<i>Streptococcus pyogenes</i>	n.a.	< 7.5	n.a.
<i>Enterococcus</i>	n.a.	n.a.	n.a.
<i>Haemophilus influenzae</i>	n.a.	n.a.	n.a.
<i>Acinetobacter</i> spp.	31.25	n.a.	31.25
<i>Escherichia coli</i>	n.a.	n.a.	n.a.
<i>Salmonella enteritidis</i>	n.a.	n.a.	n.a.
<i>Candida albicans</i>	n.a.	n.a.	n.a.

n.a. - no activity found

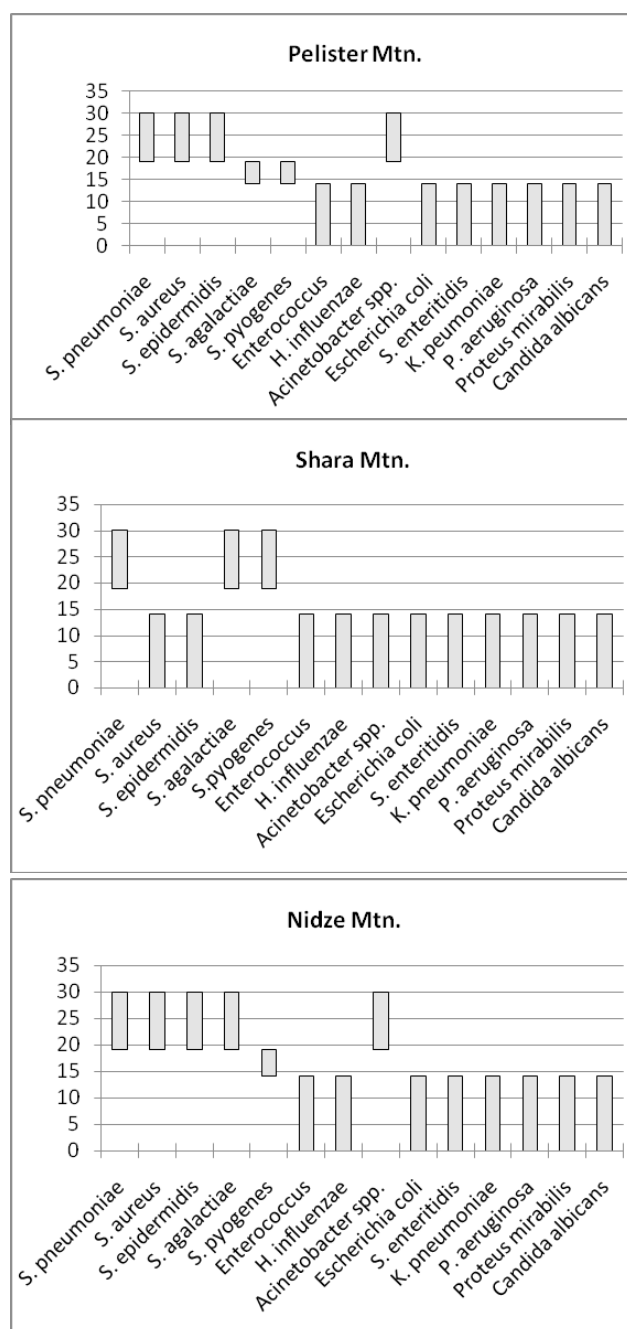


Fig. 3. Antimicrobial activity (growth inhibition zones in mm) of *Pinus peuce* needle essential oils determined by hole-plate diffusion method: 0-14 mm resistant, 14-19 mm moderate susceptible and 19-30 mm susceptible microorganisms.

Although the biological activity of essential oils is well known, the mode of their action is not completely understood. Generally, the activity of essential oils is the result of the combined effect both of their active and inactive components. Inactive components may affect resorption, speed of reaction and bioavailability of active components (Colgate, 1993; Svoboda, 1995), while the active

components can have a synergistic effect. Essential oils are familiar with antibacterial, antifungal and antiviral activity. Many studies have explained antibacterial activity of essential oils against Gram-positive and Gram-negative bacteria (Reichling, 1999a; Weseler, 2005; Reichling, 1999b; Youesf, 1980; Dorman, 2000; Takarada, 2004) and have confirmed their antifungal activity (Hammer, 1998). There are differences in the biological activity of essential oils, especially when it comes to different strains of Gram-positive and Gram-negative bacteria. The different susceptibility of bacteria to the action of essential oils are not only due to a different chemical composition of the oils, but the differences in the composition and structure of the cell wall, lipid and protein composition of cytoplasmic membrane and specific physiological processes in different bacterial species. The way and mechanism of antibacterial activity of essential oils are not sufficiently explained yet. Nowadays, it is accepted that some essential oils and their components mainly act on bacterial cytoplasmic membrane (Ulrich, 2008). According to the literature data, there are only three references that are related to the antimicrobial screening of pine needle essential oils. Oluwadayo et al. (2008) reported that the major constituents of essential oils isolated from the needles of *Pinus caribaea* Morelet are β -phellandrene (67.9%), β -caryophyllene (10.2%) and α -pinene (5.4%). This essential oil exhibited moderate activity against *Pseudomonas aeruginosa* at minimum inhibitory concentration of 1000 μ l/ml and no activity against other investigated microorganisms as *Candida albicans*, *Bacillus subtilis*, *Staphylococcus typhi*, *Bacillus aureus* and *Proteus mirabilis*. According to Sacchetti et al. (2005), the most abundant components of *Pinus radiata* D. Don are β -pinene (35.21%), α -pinene (21.9%), β -phellandrene (12.6%), iso-silvestrene (8.42%), α -terpineol (3.01%), terpinolene (2.21%), estragole (2.07%) and citronellol (1.87%). This essential oil showed a moderate inhibiting activity against tested yeasts as *Candida albicans*, *Rhodotorula glutinis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Yarrowia lypolitica* at minimum inhibitory concentrations of 0.14, 0.09, 0.02, 0.06 and 0.29 mg/ml, respectively. The antimicrobial effects of volatile components extracted from needles of *Pinus densiflora* S. and Z. was examined by Youg-Suk et al. (2005). α -Ocimene (29.3%), sabinene (10.9%), β -myrcene (9.6%), β -caryophyllene (8.0%), β -cadinene (7.3%), α -terpinolene (4.9%), 2-hexanal (4.5%) and β -pinene (4.3%) were considered as dominant components. Antimicrobial activity was screening against six different microorganisms: *Bacillus cereus*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. Among tested microorganisms, the growth of *Bacillus cereus*, *Salmonella typhimurium* and *Staphylococcus aureus* was completely inhibited.

Compared to our results, composition of *Pinus peuce* needle essential oil differs a lot from above tested pine oils. Antimicrobial screening was performed on different bacterial and fungi strains. All mentioned results indicate that the

antimicrobial activity of pine needle essential oils depend on the type of microorganism as well as chemical composition of the oil. For example, antimicrobial activity of *Pinus radiata* essential oil against *Candida albicans* is probably due to the higher amount of α -pinene (21.9%) and β -pinene (35.21%) in comparison with *Pinus peuce* needle essential oil which contains lower amounts of these two components and hasn't showed antimicrobial effect against tested fungi. On the other hand, the mechanism of antimicrobial activity of needle essential oil from different pine species has not been clarified yet thus the action of essential oils on bacterial cytoplasmic membrane may be considered as a superior mechanism of antimicrobial activity of *Pinus peuce* needle essential oil.

Conclusion

The yield of needle essential oils obtained by hydro-distillation of *Pinus peuce* from Pelister, Nidze, Shara and Karadzica Mtn. ranged from 5.04, 9.24, 9.93 and 2.86 ml/kg, respectively. The obtained essential oils were transparent, agile, light yellowish liquids with specific and very strong turpentine odor.

Using GC/FID/MS, one hundred and three components were identified in the *Pinus peuce* needle essential oils. The most abundant components in all samples of isolated oils were monoterpenes: α -pinene, β -pinene, limonene + β -phellandrene and bornyl acetate and sesquiterpenes: *trans*-(E)-caryophyllene and germacrene D.

The most sensitive bacteria against tested *Pinus peuce* needle essential oils were *Streptococcus pneumoniae*, while *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Acinetobacter* spp. showed different sensitivity to the antimicrobial effects of the essential oils deepening on the area of collection of plant material. Essential oil obtained from plant material collected from Nidze Mtn. showed the highest antimicrobial activity against tested microorganism.

As the most promising activity of *Pinus peuce* needle essential oil was found against *Streptococcus pneumoniae*, this oil could be recommend for inhalation for infections of upper respiratory tract or for treatment of different skin condition provoked by *Staphylococcus aureus*. Further examination of safety of *Pinus peuce* needle essential oil should be done before their final recommendation.

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Резиме

Антимикробна активност на етерично масло од иглички од молика (*Pinus peuce* Griseb., Pinaceae) од флората на Р. Македонија

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Клучни зборови: *Pinus peuce*, Македонски бор, хемиски состав на етерично масло, GC/FID/MS, антимикробна активност.

Хемиски состав и антимикробна активност се испитувани кај етерично масло добиено со дестилација со водена пара на иглички од молика (*Pinus peuce* Griseb., Pinaceae) која самоникнато расте на три различни локалитети во Р. Македонија. Со GC/FID/MS анализа, идентификувани се 103 компоненти што припаѓаат на шест различни класи соединенија: монотерпенски јаглеводороди, оксидирани монотерпени, сесквитерпенски јаглеводороди, оксидирани сесквитерпени, дитерпени и други нетерпенски компоненти и истите сочинуваат 86,61/94,04% од севкупното масло. Најзастапени компоненти во етеричното масло се α -пинен (12,89/27,34%), β -пинен (6,16/13,13%), лимонен + β -феландрен (2,09/6,64%) и борнил ацетат (2,92/11,67%), како и *trans* (E)-кариофилен (4,63/7,13%) и гермакрен D (8,75/20,14%).

Антимикробната активност на етерични масла од иглички од молика е испитувана со агар дифузиона и со агар дилуциона метода на 13 бактериски изолати на Грам позитивни и Грам негативни бакетии и еден изолат на габата *Candida albicans*. Најголема осетливост на дејството на маслата од молика покажа бактеријата *Streptococcus pneumoniae*, следена од *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Acinetobacter* spp. и *Streptococcus pyogenes*. Минималните инхибиторни концентрации на етеричните маслата се движат од 7,5 до 62,5 μ l/ml.