Chemical Composition and Antimicrobial Activity of Essential Oil from Shoots Spruce (*Picea abies* L)

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The aim of this paper was to evaluate the chemical composition, antimicrobial properties of volatile oil isolated from sprouts of Picea abies growing wild in Romania. The essential oil from young sprouts of Picea abies L. obtained by hidrodistillation was analysed by gas chromatography-mass spectrometry (GC-MS). Fifty four compounds accounting for 96.30 – 98.42% of the oil were identified. The main compounds found belong to monoterpenic hydrocarbons (α-pinene, camphene, limonene, myrcene), oxigenated monoterpenes (bornyl acetate), sesquiterpene hydrocarbons (δ-cadinene, muurolene), sesquiterpenic alcohols (cadinol, muurolol) and diterpenic alcohol (manool). The antimicrobial activity of the volatile oil was qualitatively tested against Gram-positive and Gram-negative bacteria and fungi. The susceptible bacterial strains were assessed by microdilution technique for minimal inhibitory concentration (MIC) values. The essential oil extracted from Picea abies possess antimicrobial activity of different intensity depending on the tested strains and in some cases on the solvent used for diluting the oil. The most evident inhibitory effect was noticed against the Gram-positive and fungi strains.

Keywords: Picea abies, essential oil, GC-MS analysis, antimicrobial activity

Picea abies (L.) H. Karst syn. *Picea excelsa* Link (Norway spruce or Siberian pine) belonging to *Pinaceae* family is the most widespread conifer tree in the Romanian forests. It can be used to avoid the erosion of the soil, and is of great economical importance, as construction wood, resonance material for musical instruments, paper manufacturing, and even in phytotherapy (some parts as shoots and needles) [1, 2].

The content and the chemical composition of volatile oil isolated from different species of the family *Pinaceae* depends on the geographic origin [2, 3-6] the type of the plant material (needles, twigs, cones), the isolation and determination techniques used for analysis [3, 7-13]. The volatile oil quality is also strongly affected by soil and air pollution [8, 10, 14-16].

There are a few investigations concerning qualitative chemical composition of volatile oil isolated from different species of conifers growing wild in Romania [17, 18].

It is well known that volatile oil produced by conifers possesses antibacterial, antifungal, antioxidant and cytotoxic effects [19-23]. The antibacterial activity of *Picea excelsa* essential oil was also tested by Canillac with the dilution method against Gram-positive and Gram-negative bacteria demonstrating the antibiofilm activity onto Gram-positive bacteria in stationary phase and the coliforms resistance, whatever the physiological age, since they grow with 8% of essential oil [24].

Norway spruce fir essential oil was used in Europe in the treatment of catarrhal diseases of children, by inhalation with hot water [2].

Bacterial resistance to existing antibiotics, the toxicity of antifungal drogs, combined with a decline in the development of new antibiotics, presents a significant threat to human health [25]. The identification of new antimicrobial agents is therefore of considerable importance.

The aim of this study was to investigate the composition of essential oil from shoots of *Picea abies* growing wild in Romania and to evaluate its antimicrobial activity. Data on the essential oil composition and on its biological activity can be allowed using this natural resource.

Experimental part

Chemicals and materials. Solvents and reagents were purchased from Merck, Darmstadt, Germany: dichloromethane was SupraSolv for gas-chromatography; anhydrous Na_2SO_4 granulated for organic trace analysis was used. The n-alkanes $C_6 – C_{24}$ used for the determination of the retention Kovats index were from Fluka, Buchs, Switzerland.

The standard antibiotic tobramycine (10 μg/ disk) from bioMérieux were used to control the sensitivity of the tested bacteria.

Plant material

The five shoots samples (5-6 cm long) of *Picea abies* L. were collected from 20-30 years old trees that were growing wild in different Buşteni locations (Silva, Calinderu, Azuga, Gura Diham) situated approximately 900-950 m altitude. Each sample was collected from each of six aged trees in May 2009. The antimicrobial activity tests of the essential oil used Silva sample.

Volatile Oil Extraction.

In a Clevenger-type apparatus 50 g of dried plant material were hydrodistilled without organic solvent for 4 h

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according to Romanian Pharmacopoeia (1993). The volatile oil was dried over anhydrous Na_2SO_4, stored in a dark glass bottle and kept at 4°C until analysis or biological test. The volatile oil was diluted in dichlorometane (1/200) for GC analysis and 1 µL were injected.

Volatile Oil Analysis by Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry analysis of *Picea abies* volatile oil was carried out using a Fisons Instruments GC 8000 with an electron impact quadrupole, MD 800 mass spectrometer detector. The electron ionisation energy was 70 eV. A fused silica column 5% phenylpoly(dimethylsiloxane) (SLB – 5 ms, 30 m x 0.32 mm i.d., film thickness = 0.25 µm) was employed. The operating conditions were the following: a split-splitless injector (split ratio, 1/30) at 280°C, ion-source temperature 200°C and the interface temperature 280°C; initial column temperature, 40°C for 3 min, raised at 4°/min to 280°C and finally held isothermally for 20 min; the carrier gas (helium) flow rate was 2 mL/min; sample volume injected, 1 µL. Data acquisition was performed with MassLab Software for the mass range 30-600 u with a scan speed of 1 scan/s.

The identity of volatile oil components was established from their GC Kovats retention indices and from mass spectra by computer matching with mass spectra library (NIST, WILEY and a personal library of 600 spectra). The linear retention indices were determined in relation to a homologous series of n-alkanes (C_6 – C_{24}). The experimental value of Kovats indices were compared with those reported in literature (NIST, Pherobase, Flavornet). Component relative concentrations were calculated from GC peak without using correction factors.

Microbial strains

The antimicrobial activity of the essential oil was tested against bacterial and fungal strains recently isolated from clinical specimens as well as reference strains belonging to the following species: Echerichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC 25923, Proteus vulgaris, Bacillus cereus, Candida albicans, Aspergillus niger.

Antimicrobial assay

Qualitative screening of the antimicrobial properties of the essential oil . For testing in vitro antimicrobial activity of essential oil we used an adapted disk diffusion method, standardised for testing antimicrobial drugs. The solid media used where Mueller Hinton recommended for bacterial strains, Sabouraud for yeasts and starch glucose medium for molds. The stock solution of essential oil was diluted (1:1, 1:10, 1:20) using two solvents, dimethyl sulfoxide (DMSO) and triethylene glycol (TEG), 5 µL of each dilution sample being distributed on 5 mm paper disks previously placed onto the seeded culture media. The used solvents were also tested for their antimicrobial activity. The antimicrobial activity of the tested oil was referred to standard antibiotic disks (tobramycin) used as positive controls. The positive results were read as the occurrence of an inhibition zone of microbial growth around the disk [26].

Quantitative assay of the antimicrobial activity

MIC value for essential oil was determined by twofold microdilution technique in liquid medium (Muller Hinton broth) using 96 multi-well plates, starting from 0.75µL/mL to 0.024 µL/mL. 40 µL of bacterial suspension at the standard density of 0.5 McFarland was added in each well. Simultaneously, there were achieved serial dilutions for tobramycin starting from 40µg/ mL to 1.25µg/ mL, in order to obtain the antibiotic sensitivity of tested strains. Negative (MH broth) and positive controls (MH broth and microbial inoculum) were used. The plates were incubated for 24 h at 37°C. While the TEG stock solution oil gave a turbidity reaction at the contact with the MH broth, the MIC value could not be read by the classical way (MIC corresponding to the last well where the microbial growth was absent and the culture medium remained clear), all wells content being opalescent. In order to establish the MIC viable cell counts method was used [27].

Results and discussions

The content in volatile oil of shoots spruce (*Picea abies*) samples varies between 0.95 to 1.15% (relative to dried material) with a medium value 1.01%.

The gas-chromatogram of volatile oil (sample A) is presented in figure 1.

The volatile oil composition of the four samples A-D is reported in table 1. In all analysed samples fifty four compounds were identified adding up between 96.30 and 98.42% of the total area.

The chemical composition of the four analyzed oil samples is characterized by equilibrium between monoterpenic and sesquiterpenic compounds. The total monoterpenic compounds are predominant in B and C samples accounting for 56.78, respectively 62.96% from

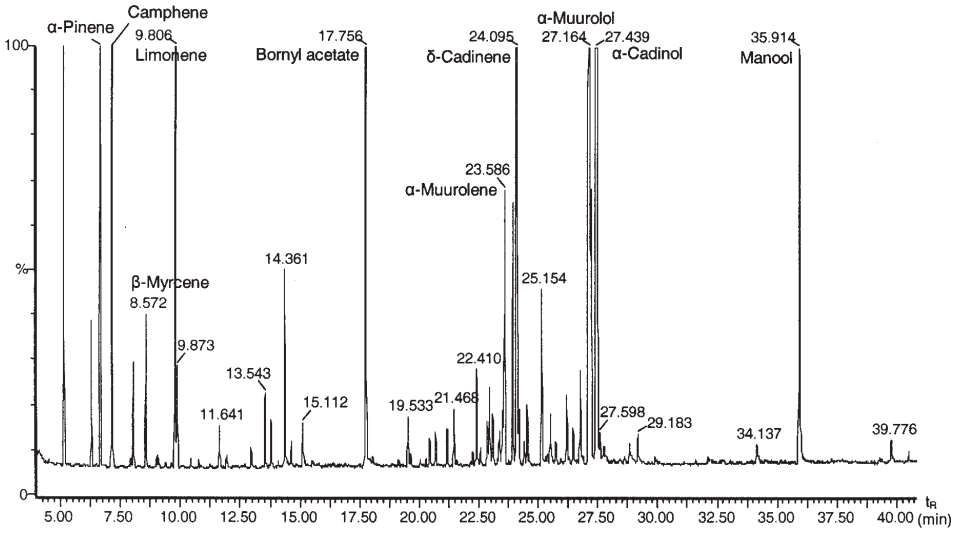

Fig. 1. The chromatogram of Picea abies volatile oil

Table 1
CHEMICAL COMPOSITION OF VOLATILE OIL FROM *PICEA ABIES*

No.	Compound	KI exp	KI lit	Relative area (%)			
				Silva (A)	Azuga (B)	Calinderu (C)	Gura Diham (D)
1	Santene	887	888	2.27	2.91	3.09	0.77
2	Tricyclene+α-Thujene	921	926	0.74	1.05	1.30	0.35
3	α-Pinene	934	939	5.40	7.31	10.42	2.44
4	Camphene	950	952	7.55	11.07	14.07	3.89
5	Sabinene	973	973	0.05	0.08	0.05	0.05
6	β-Pinene	976	980	0.52	0.70	2.61	0.32
7	β-Myrcene	991	991	0.75	0.98	10.12	0.44
8	α-Phellandrene	1003	1005	0.06	0.10	0.05	0.05
9	δ-3-Carene	1006	1009	0.05	0.06	0.05	0.05
10	α-Terpinene	1016	1017	0.05	0.05	0.05	0.05
11	p-Cymene	1024	1026	0.05	0.05	0.05	0.05
12	Limonene	1029	1031	9.29	12.98	7.32	6.27
13	1,8-Cineole	1032	1033	0.45	0.80	0.11	0.42
14	(Z)-β-Ocimene	1040	1040	0.05	0.05	0.05	0.05
15	(E)-β-Ocimene	1050	1050	0.06	0.07	0.05	0.05
16	γ-Terpinene	1060	1062	0.05	0.08	0.05	0.05
17	α-Terpinolene	1085	1084	0.24	0.28	0.33	0.14
18	Linalool	1100	1100	0.05	0.05	0.05	0.05
19	α-Campholenal	1126	1027	0.14	0.13	0.05	0.08
20	Camphor	1145	1145	0.40	0.63	0.16	0.36
21	β-Terpineol	1154	1159	0.28	0.36	0.14	0.33
22	Borneol	1171	1171	1.11	1.35	0.43	1.17
23	α-Terpineol	1193	1197	0.24	0.45	0.25	0.30
24	β-Citronellol	1231	1230	0.05	0.05	0.05	0.05
25	Bornyl acetate	1283	1285	11.78	15.04	12.05	12.44
26	*Cis*-3-hexenyl tiglate	1321	1322	0.05	0.05	0.05	0.05
27	Terpenyl acetate	1347	1351	0.32	0.44	0.05	0.36
28	Citronellyl acetate	1351	1354	0.08	0.13	0.05	0.10
29	α-Copaene	1375	1376	0.05	0.05	0.05	0.05
30	Geranyl acetate	1380	1382	0.17	0.24	0.07	0.05
31	β-Elemene	1387	1391	0.20	0.14	0.13	0.19

32	*Trans*-caryophyllene	1416	1415	0.33	0.25	1.09	0.25
33	α-Humulene	1453	1452	0.60	0.46	1.05	0.52
34	β-Cadinene	1470	1472	0.29	0.21	0.19	0.24
35	γ-Muurolene	1473	1477	0.46	0.35	0.29	0.47
36	Germacrene D	1478	1480	0.38	0.28	0.40	0.33
37	β-Selinene	1493	1490	0.42	0.23	0.23	0.48
38	α-Muurolene	1496	1499	1.61	1.28	1.14	1.66
39	γ-Cadinene	1510	1514	1.54	1.08	1.07	1.70
40	δ-Cadinene	1517	1523	9.49	6.55	6.06	9.80
41	Cadina-1,4-diene	1531	1532	0.14	0.09	0.08	0.13
42	α-Cadinene	1536	1538	0.37	0.25	0.19	0.41
43	α-Calacorene	1539	1546	0.07	0.05	0.05	0.09
44	Nerolidol	1560	1565	1.01	0.70	0.48	1.22
45	3-Hexenyl benzoate	1569	1574	0.05	0.08	0.16	0.05
46	1,10-di-epi-Cubenol	1614	1619	0.23	0.19	0.13	0.36
47	1-epi-Cubenol	1626	1628	0.52	0.45	0.41	0.75
48	Epi-α-cadinol	1640	1641	0.05	0.05	0.05	5.10
49	α-Muurolol	1643	1645	11.01	8.06	6.42	8.06
50	δ-Cadinol	1646	1646	1.48	1.15	0.89	1.82
51	α-Cadinol	1655	1656	21.39	14.69	11.19	25.28
52	Pentadecanal	1718	1714	0.08	0.09	0.05	0.25
53	Manool	2053	2056	3.58	2.45	3.40	6.18
54	Trieicosane	2300	2300	0.05	0.09	0.05	0.13
TOTAL				97.70	96.81	98.42	96.30

the total essential oil, while the total content of sesquiterpenic compounds was 51.69 and 58.90% in A, respectively D samples.

The total percentage of monoterpenic hydrocarbons ranged from 15.02 (sample D) to 49.66% (sample C). The main compounds of monoterpenic hydrocarbons in A-D samples are: limonene, camphene, α-pinene. The sample C is reach also in β-myrcene (10.12%). The content in sesquiterpenic hydrocarbons has a small variation range between 11.27% (sample B) and 16.32% (sample D), the most abundant compound being δ-cadinene. The monoterpenic ester bornyl acetate was present in appreciable amounts in all samples (from 11.78 to 15.04%).

Sesquiterpenic alcohols were rather abundant; the total value varies from 19.57% for sample C to 42.59% for sample D. Other important constituent of the volatile oil was manool, a diterpenic alcohol (from 2.45 to 6.18%).

The amounts of carbonyl compounds such as campholenal and camphor in volatile oil samples were found to be low.

Our results are in accordance with other authors [28]. Most of the components identified in *Picea abies* sprouts essential oil were previously reported as being contained by other conifers too [29, 30].

The antimicrobial activity of the essential oil extracted from members of *Pinaceae* family was investigated by many authors [30 - 32].

Some volatile compounds from composition of *Picea abies* essential oil were related with antimicrobial activity: α- and β-pinene, δ-3-carene [33], *p*-cymene [34], ocimene [35], limonene [35, 36], γ-terpinene [34], camphene [28], 1.8-cineol [28], linalool [37], bornyl acetate [34], and nerolidol [38].

The analysis of antimicrobial components (fig. 2) of essential oils (specimens A-D) revealed that the average content of the antimicrobial activity related compounds closely superposed to the biocide content evidenced in the specimen A (36.36%), which, based on this consideration, was further selected for biological tests.

The qualitative screening of the antimicrobial properties of the essential oil extracted from *Picea abies* revealed inhibition zones of microbial growth. The antimicrobial activity was noticed against Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*), Gram-negative (*Proteus vulgaris*) and fungal strains (*Candida albicans*, *Aspergillus niger*). The two solvents used for solubilising essential oil (DMSO and TEG) exhibited no antimicrobial activity against the

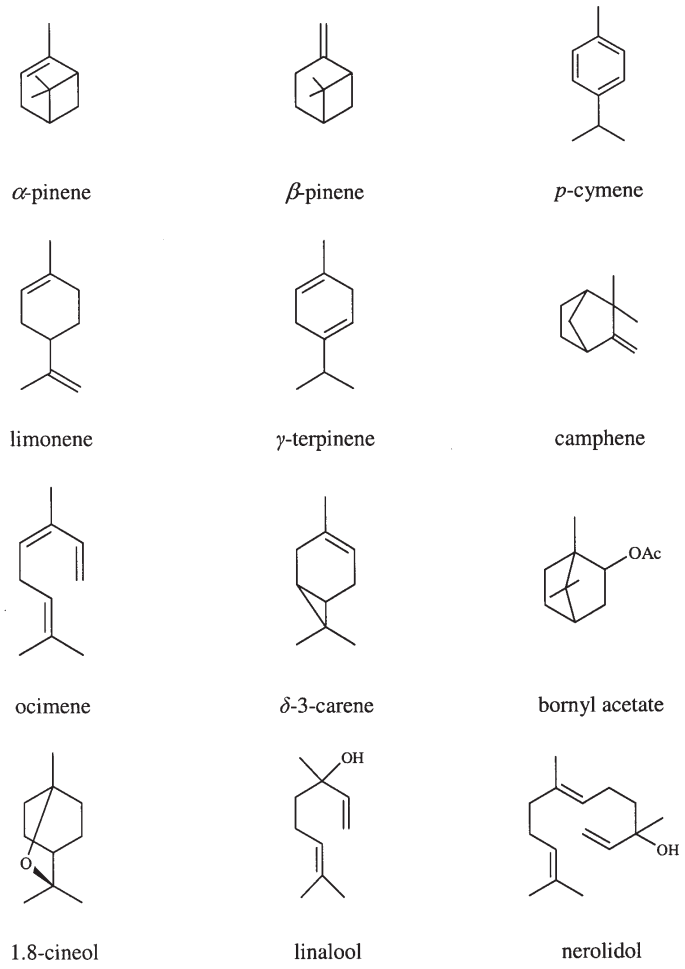

Fig. 2. Compounds with antimicrobial activity

Bacterial strain	MIC value (μL/ mL) for tested essential oil	MIC value (μg/ mL) for tobramycin (control of antibiotic susceptibility)
Staphylococcus aureus	0.375	4
Bacillus cereus	0.187	3
Proteus vulgaris	0.750	4

Table 2
MIC VALUES OF TEG DILUTED ESSENTIAL OIL AGAINST SOME BACTERIAL STRAINS AS COMPARED WITH THE STANDARD ANTIBIOTIC

tested strains. In exchange the intensity of the essential oil antimicrobial effect depended on the used solvent in case of *Proteus vulgaris*, *Staphylococcus aureus*, *Candida albicans*. The antimicrobial effect was maximum against *Bacillus cereus* strain, in both working variants for all dilutions. The yeast strain (*Candida albicans*) proved to be more susceptible than the tested mold strain (*Aspergillus niger*), for the first one growth inhibition zones being obtained for all tested dilutions in both working variants (all samples), while for the second only for 1:1 dilution, and the inhibition zones diameters being different for the two solvents.

The quantitative assay of the antimicrobial activity was performed only for the tested bacterial strains which proved susceptible to the tested oil in the screening assay, in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations. MIC values could be thus estimated for *Proteus vulgaris*, *Staphylococcus aureus* and *Bacillus cereus* (table 2).

The quantitative assay confirmed that the intensity of the antimicrobial activity of *Picea abies* essential oil is different and dependent on the tested microbial strain. The most susceptible strain proved to be the Gram positive ones (*Bacillus cereus* and *S. aureus*), leading us to the hypothesis that the outer membrane of Gram-negative bacteria could be an efficient barrier for the internalisation of the active compounds contained by the essential oils. The higher sensitivity of Gram-positive bacteria could be attributed to their outer layer chemical structure (peptidoglycan) which is not an effective permeability barrier. The outer membrane of Gram-negative bacteria being normally negatively charged and hydrophilic, due to its structural lipopolysaccharide components, could be less permeable to lipophilic substances, while porins represent a selective barrier to high molecular weight hydrophilic compounds [39].

Conclusions

The chemical composition of the four analyzed essential oil samples is characterized by equilibrium between monoterpenic and sesquiterpenic compounds. The total monoterpenic compounds were predominant in two samples: Azuga and Calinderu (56.78% and respectively 62.96% from the total essential oil) and the sesquiterpenic compounds were present in larger quantity in Silva and Gura Diham essential oils (51.69% and respectively 58.90% from the total essential oil).

The average content of the antimicrobial activity related compounds was 36.36% from total amount of the identified components.

The essential oil extracted from *Picea abies* possesses antimicrobial activity of different intensity depending on the tested strains. The most evident inhibitory effect was noticed against the Gram-positive and fungi strains.

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