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ORIGINAL PAPER

Quality testing of industrially produced essential oil of white pine (*Pinus sylvestris L.) from the Republic of Srpska*

Željka Marjanović-Balaban[1] | Vesna Gojković Cvjetković[2] | Marijana Kapović-Solomun[1] | Ljiljana Stanojević[3] | Jelena Stanojević[3] | Vesna Kalaba[4]

[1]University of Banja Luka, Faculty of Forestry, Vojvode Stepe Stepanovića 75a, 78000 Banja Luka, Republic of Srpska, Bosnia and Herzegovina

[2]University of East Sarajevo, Faculty of Technology Zvornik, Karakaj 34a, 75400 Zvornik, Republic of Srpska, Bosnia and Herzegovina

[3]University of Niš, Faculty of Technology, Bulevar Oslobođenja 124, 16000 Leskovac, Serbia

[4]Veterinary Institute of the Republic of Srpska "Dr Vaso Butozan" Banja Luka, Branka Radičevića 18, 78000 Banja Luka, Republic of Srpska, Bosnia and Herzegovina

Correspondence
V. Gojković Cvjetković
Email: vesna.gojkovic@tfzv.ues.rs.ba

Abstract

The quality of the essential oil obtained from white pine wood greenery from the territory of the Republic of Srpska was analyzed by examining the chemical composition and antimicrobial activity of samples produced by the hydro-distillation process in industrial production conditions. The chemical composition of the essential oil was analyzed by GC/MS and GC/FID analysis. The antimicrobial activity of the obtained white pine oil was investigated for its possible use as an antimicrobial agent. The obtained results prove the presence of 37 components in the analyzed sample, which represents 98.5% of the total components present. The most common are monoterpene hydrocarbons, followed by sesquiterpene hydrocarbons, much less represented are monoterpenes containing oxygen and aromatic compounds, while aliphatic esters are found only in traces. Antimicrobial activity was examined using the disk diffusion method. The results showed large zones of inhibition, both for the tested oil sample and for all eight analyzed bacterial strains (from 12.00 ± 0.00 to 17.33 ± 1.15mm), as well as for the fungal strain. The chemical composition and antimicrobial action of essential white pine oiled from plant material originating from the territory of the Republic of Srpska indicates its significant phytomedical potential.

Keywords: Essential oil, white pine, GC/MS analysis, antimicrobial activity.

1. INTRODUCTION

Pinus silvestris L. - white pine belongs to the family *Pinaceae*, order *Pinales*, class *Pinopsida*. The tree has a pyramidal canopy that can grow up to 35m. The bark is gray-brown and thick in the lower part of the mature tree, and scaly and recognizably orange in the higher parts. The bristles are 4 to 7 cm long and 2 mm wide, twisted around their axis. Male cones are very compact. Females ripen at the end of the next year. They have a clear stalk, and are gray-brown and dull, 2.5 to 7 cm long, 2 to 3.5 cm wide. The apophyses are variable, pyramidal, or flat. The seeds are small, 3 to 4 mm long. White pine inhabits its various habitats and is found from xerothermic forests with heather and black ash to peat forests. It climbs to the subalpine belt (Šumantić & Topalić-Trivunović 2012). It is an important forest species, it is often used in afforestation of difficult terrains (washed, shallow, degraded soils), sandy, hilly and mountainous areas. It is a fast growing species, common in parks and city greenery. It has a large application: construction and carpentry wood, water construction, mine wood, line poles, sleepers, propellers, cellulose wood, charring. Resin is obtained from its resin. Tar and turpentine oil are obtained from stumps and waste. Young twigs and conifers are used in medicine (Jovanović 1992). Essential oils are widely used in the pharmaceutical, medical and perfumery industries. They are aromatic, oily liquids obtained from various parts of plants, leaves, seeds, fruits, buds, flowers, woody greens, herbs, bark and roots (Burt 2004). Secondary are plant metabolites, which are synthesized by the plant, but are not necessary for the growth and development of the plant (Croteau, Kutchan, Lewis, et al. 2000). The content of essential oils can vary in quality, quantity and composition depending on geographical conditions, soil composition, plant parts, age, vegetation period (Angioni, Barra, Coroneo, Dessi, & Cabras 2006; Masotti, Juteau, Bessière, & Viano 2003). They can contain 20-60 compounds in different percentages, which eventually forms a very complex mixture. In general, usually two to three components are dominant with 20-70% representation (Bakkali, Averbeck, Averbeck, & Idaomar 2008). The scientific community has so far tested about 3000 essential oils, about 300 of which have commercial use (Silori, Kushwaha, & Kumar 2019). Owing to their antiseptic properties, essential oils have found wide bactericidal, fungicidal and medicinal applications, and are also used in the process of food preservation (Bakkali et al. 2008). Some key components of the essential oil are used in the production of fragrances and in the cosmetics industry, while agriculture and medicine are among the other main users of the essential oil. They have also found application in aromatherapy. Some studies claim that essential oils are also used in the treatment of dysfunction of some organs or systemic disorders (Hajhashemi, Ghannadi, & Sharif 2003; Perry, Bollen, Perry, & Ballard 2003; Silva et al. 2003). Despite the widespread use of essential oils, it is important to investigate their quality, their additional roles, all for the reason that they could be used to improve human health, in agriculture, food and other industries (Bakkali et al. 2008). Republic of Srpska has a great potential of wood greenery as a possible raw material for the industrial production of essential oils. It is indisputable that white pine wood greenery can be used as a good raw material for the production of essential oils in the Republic of Srpska, and, with a good organization of production, it could be successfully placed on the domestic and foreign markets (Miletić, Marjanović-Balaban, Oljača, & Grujić 2004). For this reason, the aim of this paper was to analyze the essential oil of white pine wood, originating from the Republic of Srpska, obtained in industrial production conditions, by determining its quality - chemical composition and antimicrobial properties, i.e. its phytomedical potential and possible application in various branches of industry.

2. MATERIALS AND METHODS

2.1. Plant material

The wood of the white pine tree (genus *Pinus* L., species *Pinus sylvestris* L.) was used as the raw material for the production of the essential oil. The feedstock for the hydro-distillation process contained all live fine twigs with cones up to 2 cm in diameter at the thicker end of the twigs, which were mechanically shredded for faster and better distillation of the essential oil. The plant material (up to 250 kg) was sampled in July 2019 at the Romanija Forest Holding, JPŠ "Republic of Srpska Forests", a.d. Sokolac (Lat. 43,938, Lng. 18,801) Republic of Srpska, Bosnia and Herzegovina. For the production of essential oil, the plant material was processed into essential oil immediately after felling.

2.2. Essential oil isolation

The essential oil of white pine was produced under industrial conditions by the process of hydro-distillation (hydro module water: plant material 3:1, respectively 750:250 kg) in a 2000 liter capacity distiller, in cooperation with JPŠ "Forests of Republic of Srpska", a.d. Sokolac. Hydro-distillation was done 3 times. For the purpose of faster and better distillation, the wood green of the white pine was passed through the chopper before the essential oil production process. The hydro-distillation process was carried out at a temperature of 102 °C, a pressure of 0.5 bar, for 3 hours. The obtained essential oil was briefly stored in glass vials with Teflon closure at 4 °C until laboratory analysis, in the absence of light.

2.3. GC/MS and GC/FID analysis

The GC/MS analysis of the isolated essential oils was performed on the Agilent Technologies 7890B gas chromatograph. One microliter of the sample dissolved in diethyl ether was injected in a split injector set at 250 °C in 20:1 split mode. Helium was used as the carrier gas, at a constant flow rate of 1 ml/min. Components were separated on weakly polar, silica capillary column, HP-5MS (5% diphenyl and 95% dimethyl-polysiloxane, 30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, USA). The oven temperature was programmed from 50 °C, held for 2.25 minutes, and then increased to 290 °C at the rate of 4 °C/min. The eluate was transferred by the MSD transfer line set at 300 °C to a selective 5977A quadrupole mass detector (Agilent Technologies, USA) with electron ionization. The temperatures of the ion source and quadrupole mass analyzer were set at 230 °C and 150 °C, respectively. The ionization voltage was 70 eV. The samples were analyzed using the Scan mode in the mass range from 45 to 650 m/z. The GC/FID analysis was carried out under identical experimental conditions as GC/MS. The temperature of the flame-ionization detector (FID) was set at 300 °C. Data processing was performed using MSD ChemStation Data Analysis (revision F.01.00.1903) in combination with AMDIS (version 2.70) and NIST MS Search (version 2.0g) Softwares (Agilent Technologies, USA). Retention indices of the components from the analyzed samples were experimentally determined using a homologous series of n-alkanes from $C_8 - C_{20}$ as standards. The identification of oil constituents was based on the comparison of their retention indices (RI^{exp}) with those available in the literature (Adams et al. 2007) (RI^{lit}); their mass spectra with those of the authentic standard as well as with those from Willey 6, NIST 2011 and RTLPEST3 libraries and wherever possible, by co-injection with an authentic standard. The percentage composition of a particular component in the essential oil was determined on the basis of calibration curves generated from the representative compounds'. Area normalization is one of the methods used for quantitative analysis. It is a calculation of area percentage which is assumed to be equal to weight percentage. In other words, the Area % calculation procedure reports the area of each peak in the run as a percentage of the total area of all peaks in the run. Area % does not require prior calibration and does not depend upon the amount of sample injected within the limits of the detector. No response factors ware used.

2.4. Antimicrobial activity

2.4.1. *Microorganisms and substrates*

Nine microorganisms were selected to determine the antimicrobial activity of essential oils: (eight bacterial strains) *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 8427), *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Listeria monocytogenes* (ATCC 19166) and (fungal strain) *Candida albicans* (ATCC 10259). Microorganisms are from the collection of the Microbiology Laboratory, Faculty of Technology, Leskovac.

2.4.2. *Disc-diffusion method*

The agar disc-diffusion method was used for testing the antimicrobial activity of the extract obtained (Kiehlbauch et al. 2000). The mediums were sterilized for 15 minutes in an autoclave at 121 °C under 110 kPa. Bacterial and fungal suspensions were prepared by direct colony method. The colonies were taken directly from the plate and suspended in 5 ml of sterile 0.85% saline. The turbidity of the initial suspension was adjusted by comparing it with 0.5 McFarland's turbidity (Andrews, 2005). After adjusting to the turbidity of the standard, the bacterium suspension contained about 108 colony forming units (CFU)/ml and the suspension of fungus contained 106 CFU/ml. Ten-fold dilutions of the initial suspension were additionally prepared in sterile 0.85% saline. Bacterial cell suspensions were inoculated to the nutrient agar plates (Torlak, Belgrade, Serbia) and the fungal suspension to the Sabouraud maltose agar plates (Torlak, Belgrade, Serbia). For screening, sterilised filter paper disks (9 mm dia., Schleicher Schuell) were placed on the surface of inoculated mediums and impregnated with 50 μl of the essential oil (1:10 V/V in DMSO). The plates were incubated for 24 hours at 37 °C for bacteria, and 48 hours at 25 °C for fungi. After incubation, the inhibition zone diameters were measured and expressed in mm. The presence of the inhibition zone indicates the activity of the tested samples against bacteria or fungi. Standardized discs of Ampicilin (10 μg/disc), Bactrim (25 μg/disc), Cefalexin (30 μg/disc) (Bio Rad) and Nystatin (100 U/disc) (Bioanalyse) were used as reference standards (Stanojević, Stanojević, Cvetković, & Danilović 2015; Stanojević, Cakić, Stanojević, Cvetković, & Danilović 2018). DMSO was used as negative control. All experiments were carried out in three replications and the results represent the mean value ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Qualitative and quantitative essential oil composition

The yield of industrially obtained white pine essential oil, light yellow in color, and with a pleasant odor, intended for further analysis, was 0.45%. Its chemical composition is shown in Table 1. The GC/MS analysis identified 37 components, representing 98.5% of the total components present in the sample. All identified compounds and their percentage in the essential oil are shown in Table 1, and the GC-FID chromatogram is given in Figure 1. The most common compounds are monoterpene hydrocarbons and sesquiterpene hydrocarbons, followed by monoterpenes containing oxygen and aromatic compounds, while aliphatic esters are found in traces.

Based on the obtained results, it can be concluded that the most common component of the tested white pine essential oil is α-pinene (44.6%), followed by limonene (14.6%), myrcene (14.0%), β-pinene (13.1%), camphene (2.5%) and (E) -caryophyllene (1.5%). α-pinene is one of the two isomers of pinene, an organic compound from the terpene family, which contains a reactive four-membered ring. It is found in oils of various types of conifers, especially pine. Limonene belongs to the class of colorless cyclic terpenes. It is commercially used for the synthesis of carvone, which is used in the food industry. The hydro-distillation process and centrifugal separation are the two primary techniques used to obtain limonene for commercial use (Sun 2007). Myrcene belongs to the monoterpene family and is an olefinic natural organic hydrocarbon.

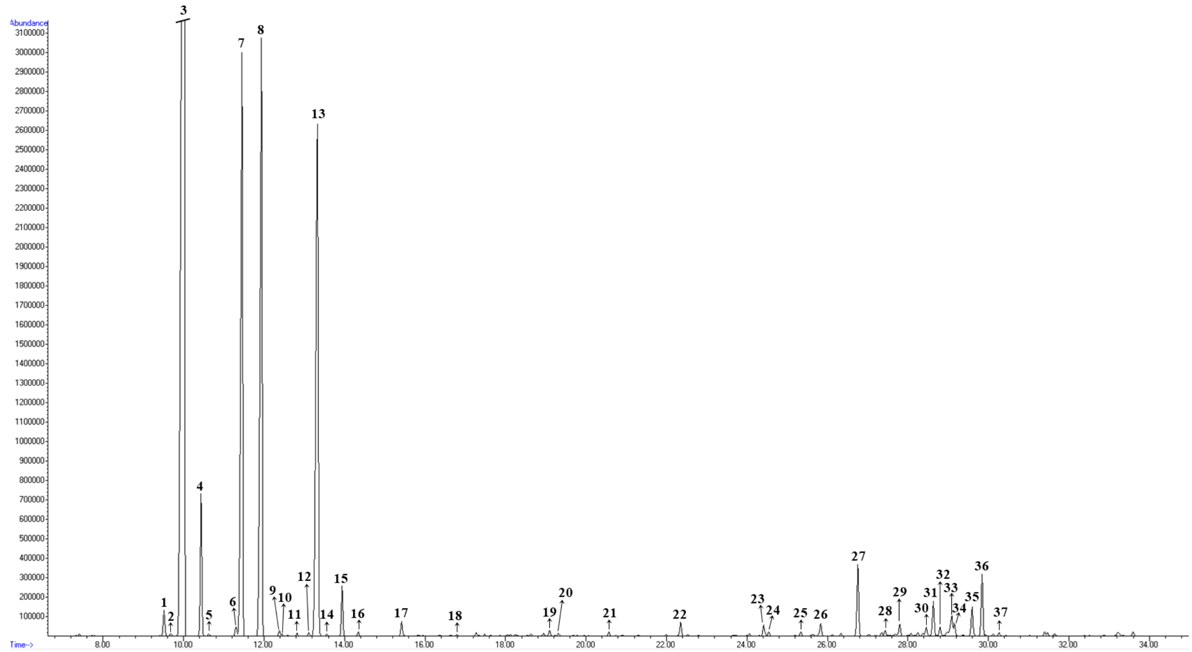

Figure 1. GC-FID chromatogram of *Pinus sylvestris* essential oil

It is obtained by pyrolysis of β-pinene at 400 °C, and very rarely directly from plants (Behr & Johnen 2009).

β-pinene is another isomer of pinene, a colorless organic liquid soluble in oil. It is a component of the essential oils of many plants and has the role of a plant metabolite. It is commercially obtained by distillation or conversion of α-pinene. Camphene is a volatile monoterpene, soluble in organic solvents and almost insoluble in water. It is produced for commercial purposes by catalytic isomerization of α-pinene. It is used as a food and fragrance supplement (Silori et al. 2019). Caryophyllene is a natural sesquiterpene found in medicinal plants and is used as an antirheumatic and analgesic (Mesquita et al. 2019).

The structures of the basic components of white pine essential oil are given in Figure 2. Ustun, Sezik, Kurkcuoglu, and Baser (2006) studied white pine essential oil originating from different parts of Turkey. They identified 43 components, with the highest content of α-pinene, camphene and β-pinene ranging between 19.44–56.88%, 2.87–17.09%, and 0.44–16.84%, respectively. Judzentiene and Kupcinskiene (2008) examined the chemical composition of white pine essential oil originating in northern Lithuania, using the GC/MS analysis, and found the highest prevalence of α-pinene and γ-3-carene. Using the GC/MS and GC/FID analyses, Stojiljković (2007) examined the composition of white pine essential oil from western Serbia and determined α-pinene as the dominant component that makes up 41.8% of the oil. Limonene content was 2.0%, myrcene 1.9%, β-pinene 3.2%, camphene 4.7% and (E) -caryophyllene 6.0%.

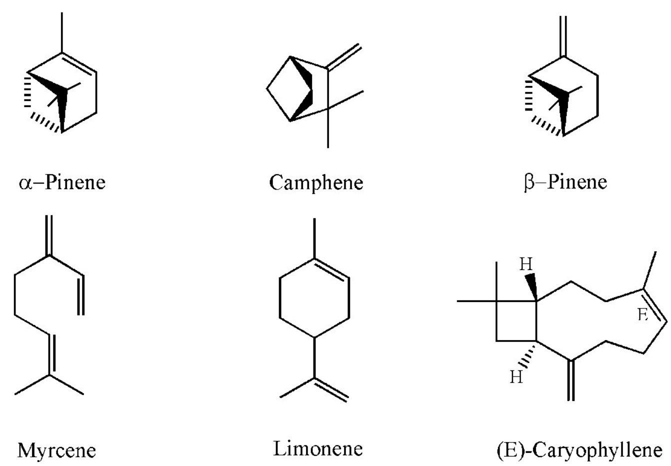

Figure 2. The most abundant components of *Pinus sylvestris* essential oil

The composition of the essential oil can be verified depending on several factors, and the most important one is the time of taking the plant material, geographical origin, genotype and chemotype of the plant species, environment and agronomic conditions, but also the method of technological processing of plant raw materials. Comparing the results of the chemical composition of white pine essential oil from the Republic of Srpska with the chromatographic profile prescribed by the European Pharmacopoeia (*European Pharmacopeia* 2009), it is noticed that the values of the content of α-pinene, β-pinene and E-caryophyllene are in accordance with the prescribed values, while the content of camphene, β–myrcene and limonene is above the prescribed values.

Table 1. Chemical composition of *Pinus sylvestris* essential oil.

No.	Compound	Molecular formula	Method of identification	RT min.	RI^{exp}	RI^{lit}	Content %
1	Tricyclene	$C_{10}H_{16}$	RI, MS	9.52	917	921	0.5
2	α-Thujene	$C_{10}H_{16}$	RI, MS	9.68	922	924	0.1
3	α-Pinene	$C_{10}H_{16}$	RI, MS, Co-I	10.02	931	932	44.6
4	Camphene	$C_{10}H_{16}$	RI, MS, Co-I	10.44	943	946	2.5
5	Thuja-2,4(10)-diene	$C_{10}H_{14}$	RI, MS	10.62	949	953	tr
6	Sabinene	$C_{10}H_{16}$	RI, MS	11.31	968	969	0.2
7	β-Pinene	$C_{10}H_{16}$	RI, MS, Co-I	11.46	972	974	13.1
8	Myrcene	$C_{10}H_{16}$	RI, MS	11.94	986	988	14.0
9	α-Phellandrene	$C_{10}H_{16}$	RI, MS	12.38	998	1002	0.1
10	(3Z)-Hexenyl acetate	$C_8H_{14}O_2$	RI, MS	12.44	1000	1004	tr
11	α-Terpinene	$C_{10}H_{16}$	RI, MS	12.82	1011	1014	0.1
12	p-Cymene	$C_{10}H_{14}$	RI, MS	13.11	1019	1020	0.1
13	Limonene	$C_{10}H_{16}$	RI, MS, Co-I	13.32	1025	1024	14.6
14	(Z)-β-Ocimene	$C_{10}H_{16}$	RI, MS	13.57	1032	1032	tr
15	(E)-β-Ocimene	$C_{10}H_{16}$	RI, MS	13.94	1042	1044	0.9
16	γ-Terpinene	$C_{10}H_{16}$	RI, MS, Co-I	14.34	1053	1054	0.1
17	Terpinolene	$C_{10}H_{16}$	RI, MS	15.42	1084	1086	0.3
18	α-Campholenal	$C_{10}H_{16}O$	RI, MS	16.79	1122	1122	tr
19	α-Terpineol	$C_{10}H_{18}O$	RI, MS, Co-I	19.10	1188	1186	0.1
20	Myrtenal	$C_{10}H_{16}O$	RI, MS	19.31	1194	1195	0.1
21	Thymol, methyl ether	$C_{11}H_{16}O$	RI, MS	20.58	1231	1232	0.1
22	Isobornyl acetate	$C_{12}H_{20}O_2$	RI, MS, Co-I	22.37	1283	1283	0.3
23	α-Terpinyl acetate	$C_{12}H_{20}O_2$	RI, MS	24.42	1346	1346	0.2
24	α-Longipinene	$C_{15}H_{24}$	RI, MS	24.55	1350	1350	0.1
25	α-Copaene	$C_{15}H_{24}$	RI, MS	25.34	1375	1374	0.1
26	β-Elemene	$C_{15}H_{24}$	RI, MS	25.84	1390	1389	0.3
27	(E)-Caryophyllene	$C_{15}H_{24}$	RI, MS	26.76	1420	1417	1.5
28	6,9-Guaiadiene	$C_{15}H_{24}$	RI, MS	27.43	1476	1475	0.1
29	α-Humulene	$C_{15}H_{24}$	RI, MS	27.81	1454	1452	0.3
30	γ-Muurolene	$C_{15}H_{24}$	RI, MS	28.46	1476	1478	0.3
31	Germacrene D	$C_{15}H_{24}$	RI, MS	28.63	1482	1484	0.7
32	β-Selinene	$C_{15}H_{24}$	RI, MS	28.80	1487	1489	0.2
33	Bicyclogermacrene	$C_{15}H_{24}$	RI, MS	29.10	1497	1500	0.9
34	α-Muurolene	$C_{15}H_{24}$	RI, MS	29.16	1499	1500	tr
35	γ-Cadinene	$C_{15}H_{24}$	RI, MS	29.60	1514	1513	0.6
36	δ-Cadinene	$C_{15}H_{24}$	RI, MS	29.85	1523	1522	1.3
37	α-Cadinene	$C_{15}H_{24}$	RI, MS	30.27	1537	1537	0.1
					Total identified (%)		98.5
Grouped components (%)							
Monoterpene hydrocarbons (1-9, 11-17)			91.2				
Oxygen-containing monoterpenes (18-20, 22, 23)			0.7				
Sesquiterpene hydrocarbons (24-37)			6.5				
Aliphatic esters (10)			Tr				
Aromatic compounds (21)			0.1				

RT-Retention time;
RI^{lit}-Retention indices from literature (Adams et al. 2007);
RI^{exp}-Experimentally determined retention indices using a homologous series of n-alkanes ($C_8 - C_{20}$) on the HP-5MS column.tr = trace amount (<0.05%).

3.2. Antimicrobial activity

The effect of white pine essential oil on the growth of eight different types of microorganisms and fungal strains was examined. The essential oil has an effect on all eight strains of bacteria, as well as on the fungal strain (Table 2). A good inhibitory effect was demonstrated with zones of inhibition from 12.00 ± 0.00mm (for *P. aeruginosa*) to 17.33 ± 1.15mm (for *E. coli*). The analyzed pine essential oil also showed good antifungal ability with an inhibition zone of 17.67 ± 0.58mm. Tables 2 and 3 show that white pine essential oil has a stronger antimicrobial effect on *E. coli, K. pneumonia, P. vulgaris* and *B. cereus* compared to certain antimicrobial agents, as well as on the fungal strain *C. albicans*. The results also showed that ampicillin, bactrim, cephalexin and penicillin are antimicrobial agents that have a significantly stronger inhibitory

Table 2. Antimicrobial activity of essential oils.

Microorganism	Inhibition zone (mm) *Pinus sylvestris* L. essential oil
E. coli	17.33 ± 1.15
K. pneumoniae	17.33 ± 0.58
P. vulgaris	14.33 ± 0.58
P. aeruginosa	12.00 ± 0.00
B. cereus	15.33 ± 1.53
S. aureus	14.33 ± 2.31
L. monocitogenes	12.67 ± 0.58
B. subtilis	17.00 ± 2.65
C. albicans	17.67 ± 0.58

Table 3. Antimicrobial activity of Ampicilin, Bactrim, Cefalexin, Nystatin and Penicilin.

Microor-ganism	Inhibition zone (mm), Antibiotics				
	A*	B*	C*	N*	P**
E. coli	n.i.	15.0	26.0	n.t.	n.t.
K. pneumoniae	n.t.	n.t.	13.0	n.t.	11.67
P. vulgaris	13.2	22.9	n.t.	n.t.	n.t.
B.cereus	n.t.	n.t.	n.t.	n.t.	13.0
L. monocitogenes	n.t.	n.t.	34.0	n.t.	n.t.
S. aureus	36.7	42.1	26.0	n.t.	32.33
B. subtilis	n.t.	n.t.	48.0	n.t.	n.t.
C. albicans	n.t.	n.t.	n.t.	17.0	n.t.
P. aeruginosa	n.t.	n.t.	n.t.	n.t.	15.67

n.i. – no influence; n.t. – not treated
A-Ampicilin; B-Bactrim; C-Cefalexin; N-Nystatin; P-Penicilin
*ref.-(Stanojević et al. 2015), **ref.-(Stanojević et al. 2018).

effect on *S. aureus* compared to essential oil. Penicillin has a stronger inhibitory effect on *P. aeruginosa*, and cefalexin on L. *monocytogenes* and *B. subtilis*compared to the tested essential oil. The obtained results of this study are in agreement with the results of other researchers (Leite et al. 2007; Stojiljković 2007). It is known that naturally active compounds of essential oils have antimicrobial activity when it comes to pathogenic microorganisms. A-pinene is a component of white pine essential oil that has a dominant effect on its antimicrobial activity (Kovač et al. 2015; Raman, Weir, & Bloomfield 1995; Wang, Chen, & Hou 2019).Di Pasqua et al. (2007) proved that limonene is also an active component of essential oil, especially in *E. coli, S. aureus, S. ententerica, P. fluorescens* and *B. thermosphacta*, Inoue et al. (2005) antimicrobial activity of myrcene in *S. aureus*, and Leite et al. (2007) antimicrobial activity of β-pinene. White pine essential oil produced from raw materials from the territory of the Republic of Srpska by the process of hydro-distillation in industrial production conditions is of good quality and can be a good alternative to synthetic antimicrobial and antifungal agents in the chemical, pharmaceutical, cosmetic and food industries.

4. CONCLUSIONS

The results of the work have proven that the essential oil of white pine, obtained from the raw material from the area of the Republic of Srpska, through the process of hydro-distillation in industrial production conditions, is of good quality. The most common component is α-pinene. The results of microbiological analyses indicate a good antimicrobial and antifungal effect of the tested oil, with *E. coli* and *K. pneumonia* being the most sensitive bacterial strains. Taking into account the chemical composition and antimicrobial activity of the analyzed white pine essential oil, it can be concluded that it has important phytomedical potential, which suggests the possibility and importance of its use as a natural antimicrobial agent in chemical, pharmaceutical, food and other industries. White pine from the territory of the Republic of Srpska has a higher content of dominant components that already have commercial usage in various industries than white pine from Europe and the USA.

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