

Phytochemical Composition and Biological Activities of Essential Oils from *Pinus nigra* J. F. Arnold and *Pinus sylvestris* L.

Fitohemijski sastav i biološka aktivnost eteričnih ulja iz *Pinus nigra* J. F. Arnold i *Pinus sylvestris* L.

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ABSTRACT

This research aimed to examine the chemical composition, as well as the antioxidant, antibacterial, and antibiofilm properties of essential oils (EOs) derived from black pine (*Pinus nigra* J. F. Arnold) and white pine (*Pinus sylvestris* L.). The essential oils were extracted using the hydrodistillation method and analyzed using gas chromatography-mass spectrometry (GC-MS). The dominant components identified in the EOs were monoterpenes and sesquiterpenes. The antioxidant capacity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, which revealed that both EOs exhibited moderate antioxidant activity. The antibacterial activity was evaluated against *Staphylococcus aureus* ATCC 6538 and Extended-Spectrum Beta-lactamase (ESBL) - producing *Escherichia coli* ATCC 35218, using the agar diffusion method and the microdilution technique. Antibiofilm activity was assessed using the Tissue Culture Plate (TCP) method. The results of the agar diffusion method suggested that *P. sylvestris* exhibited stronger antibacterial activity compared to *P. nigra*. This finding aligns with the results of the broth microdilution method, which showed lower minimum inhibitory concentration (MIC) values for the essential oil of *P. sylvestris*. *Staphylococcus aureus* investigated in this study formed a moderately adherent biofilm, while ESBL *E. coli* was identified as strong biofilm former. Tested EOs performed the capacity to change the biofilm-forming category in a species-specific and concentration-dependent manner, with *P. nigra* EO showing more pronounced antibiofilm effects.

Keywords: Essential oil, black pine, Scots pine, chemical composition, antibacterial activity

INTRODUCTION - Uvod

Essential oils (EOs) are complex mixtures of volatile, naturally occurring compounds with strong aroma, classified as secondary plant metabolites. They exhibit diverse biological activities, including antimicrobial, anti-in-

flammatory, sedative, analgesic, and spasmolytic effects. Based on their biosynthetic origin, the main groups of EO compounds are terpenes (oxidized and non-oxidized) and low-molecular-weight aromatic or aliphatic compounds (Bakkali et al., 2008). Terpenoids, in particular, have demonstrated antibacterial effects, often exhibi-

ting stronger activity against Gram-positive bacteria than Gram-negative (Chouhan et al., 2017). Some monoterpenes, such as limonene, can act synergistically with antibiotics, inhibiting the growth of multidrug-resistant strains, including *Staphylococcus aureus* and *Escherichia coli* (Costa et al., 2019; Guimarães et al., 2019). *Pinus nigra* J. F. Arnold (black pine) and *Pinus sylvestris* L. (Scots pine) are widely distributed in Europe and the Balkans, including Bosnia and Herzegovina. Traditionally, preparations from these species have been used to treat respiratory conditions, skin disorders, and infections (Mirković et al., 2024). Their essential oils are rich in monoterpenes and sesquiterpenes, including α -pinene, β -pinene, germacrene D, and β -caryophyllene (Agić et al., 2011; Mirković et al., 2024). These compounds contribute to both antimicrobial and antioxidant effects. Due to the rising resistance of microorganisms to conventional antibiotics, natural products such as pine EOs are being investigated as alternative therapeutic agents. This study aims to evaluate the chemical composition, antioxidant, antibacterial, and antibiofilm activities of *P. nigra* and *P. sylvestris* essential oils, with a focus on their potential application against multidrug-resistant bacteria.

MATERIAL AND METHODS -

Materijal i metode

Essential Oils

The essential oil samples were obtained directly from distillers located in the southern Herzegovina region (Eterična ulja d.o.o. Ljubuški; Biohalilović, Ilijaš, Eko Aromatik d.o.o. Ljubuški). The oils were produced for pharmaceutical and related commercial applications. All samples were prepared and bottled by the distillers and delivered to the laboratory in tightly sealed amber glass vials. Upon receipt, the vials were refrigerated until analysis to ensure preservation of chemical integrity. EOs were tested in pure form (100%) and 25% dilution (250 μ L/mL) in 1% dimethyl sulfoxide (DMSO) $\geq 99.9\%$ analytical reagent grade (Fisher Scientific, UK). Stock solutions of the test substances were kept at room temperature in the dark.

GC/MS Analysis of White and Black Pine Essential Oils

The GC/MS analysis of black pine (*P. nigra*) and Scots pine (*P. sylvestris*) essential oils was performed using an Agilent 7820A gas chromatograph equipped with a HP5-MS ultra-inert capillary column (30 m $\times$ 250 μ m i.d., film thickness 0.25 μ m, temperature range -60 $^{\circ}$ C to 325 $^{\circ}$ C) and interfaced with an Agilent 5977E mass selective detector (MSD). High-purity helium (5.0 grade) was used

as the carrier gas at a constant flow rate of 1.02 mL/min, with an inlet pressure of 8.4599 psi and an average linear velocity of 36.987 cm/s. A 1 μ L aliquot of each EO sample (diluted in n-hexane) was injected in splitless mode at 220 $^{\circ}$ C. The GC oven temperature was programmed as follows: initial temperature of 60 $^{\circ}$ C (1 min hold), ramped to 246 $^{\circ}$ C at 3 $^{\circ}$ C/min (no hold), and further increased to 280 $^{\circ}$ C at 10 $^{\circ}$ C/min. The total runtime was 86.4 minutes, with an equilibration time of 3 minutes. A solvent cut-off time of 4 minutes was applied to exclude the solvent peak. The autosampler needle was washed three times with n-hexane before and after each injection. The MSD operated in electron ionization mode (EI, 70 eV), scanning in the m/z range of 40-400. The transfer line, ion source, and quadrupole temperatures were maintained at 250 $^{\circ}$ C, 230 $^{\circ}$ C, and 150 $^{\circ}$ C, respectively. Data acquisition and processing were conducted using ChemStation software.

Identification and Semi-Quantitative Analysis

Following the acquisition, chromatograms were processed using RTE-mode integration with predefined parameters (minimum peak area threshold: 0.1% of the largest peak; integration via baseline-drop or tangent method), ensuring consistent peak detection and integration. Component identification was based on mass-spectral matching against NIST (NIST14, NIST05) and Wiley7 libraries, where only matches with a similarity score above 80% were accepted. Retention indices (RI) were assigned using corresponding library data obtained through similarity-search identification and further compared with literature values reported for essential oils of the same *Pinus* species. All identified compounds are expected constituents of essential oils based on published compositional profiles.

Antioxidant Activity (DPPH)

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was used to evaluate antioxidant activity (Meda et al., 2005). EOs were tested at concentrations of 25% and 100%. After 30 minutes of incubation with DPPH solution, absorbance was measured at 517 nm. Radical inhibition was calculated as:

$$\text{Inhibition (\%)} = \frac{A_0 - A_{30}}{A_0} \times 100$$

Where A_0 is the initial absorbance, and A_{30} is the absorbance after 30 minutes.

Bacterial Species

Investigation of antibacterial and antibiofilm properties of selected EOs included Gram-positive bacterium: *Staphylococcus aureus* ATCC 6538 and Gram-negative

bacterium: Extended-Spectrum Beta-lactamase (ESBL) - producing *Escherichia coli* ATCC 35218, a multidrug-resistant (MDR) strain. Investigated bacterial strains were purchased from the American Type Culture Collection (ATCC). Inocula were set in accordance with EUCAST (2017). After incubating overnight, bacterial cultures were diluted in sterile saline solution to achieve a final turbidity of 0.5 McFarland standard (1.5×10^8 CFU/mL).

Agar Diffusion Method

The initial assessment of bacterial sensitivity to EOs was conducted using the agar diffusion method (Balouiri et al. 2016). Müller-Hinton agar (MHA; Sigma-Aldrich, St. Louis, MO) plates were inoculated with bacterial suspension using sterile swabs, and such plates were left at room temperature for 15 minutes to achieve better absorption of the inoculum into the medium. Plates were then drilled to make 8 mm-diameter wells. Wells were filled with 100 μ L of EO (250 μ L/mL) and incubated for 16 to 18 hours at 37 °C. Both EOs were triple tested. At the same time, an antibiogram was performed using the following antibiotics as positive controls: Ampicillin (2 μ g), Erythromycin (15 μ g), Ceftazidime (30 μ g), Clindamycin (2 μ g), Rifampicin (30 μ g), Tetracycline (3 μ g), Gentamicin (10 μ g), Ciprofloxacin (5 μ g), Chloramphenicol (30 μ g), and Amikacin (30 μ g) (Oxoid™, Great Britain). DMSO at a concentration of 1% was used as a negative control. The antibacterial activity was evaluated based on the diameter of inhibition zones (in mm), and the following categories were defined: <10 mm = *insensitive*, 10-15 mm = *weak sensitivity*, 16-20 mm = *moderate sensitivity*, >20 mm = *strong sensitivity* (Pirvu et al., 2014).

Broth Microdilution Method

Values of the minimum inhibitory concentration of *P. nigra* and *P. sylvestris* EOs were determined through the Broth microdilution method, following CLSI guidelines (2018). The 96-well microplate was filled with Tryptic Soy Broth (TSB) (Sigma Aldrich St. Louis, MO), followed by the addition of 200 μ L of two-fold dilutions of EO, forming the final range of 250-0.00006 μ L/mL. The amount of 20 μ L of bacterial suspension was used to inoculate each well. Positive controls were bacteria in TSB only, while the uninoculated media were taken as the negative controls. Plates were incubated overnight and analysed using a microplate reader (BioTek Epoch Microplate Spectrophotometer) at 595 nm. Experiments were performed in quadruplets.

Impact on Bacterial Biofilms

Evaluation of the impact of *P. nigra* and *P. sylvestris* EOs on bacterial biofilms was performed through the Tissue

Culture Plate (TCP) method in 96-well plates with the Tryptic Soy Broth, TSB as the diluting medium (Merritt et al., 2005). The initial concentration of EOs (250 μ L/mL) was twofold diluted in TSB to an end concentration of 0.00006 μ L/mL, and 100 μ L of such dilution was added to each well. Inoculation is performed with 20 μ L of bacterial culture, prepared as previously described. The inoculated TSB medium was taken as the positive control, while the sterile medium was the negative control. The biofilm formation was determined by the bacterial adherence in the presence of TSB only. After incubating overnight, the plates were decanted, washed in Phosphate Buffered Saline, PBS (Sigma Aldrich St. Louis, MO), and stained with 0.1% crystal violet solution for 10 minutes. This step was followed by washing the plates and filling them with 96% ethanol. Results were read on a microplate reader (BioTek Epoch Microplate Spectrophotometer) at the wavelength of 595 nm. The experiment was performed in quadruplets, and the results are presented as mean values $\pm$ standard deviation (STDEV). The biofilm-forming category was determined according to Stepanović et al. (2007). The optical density cut-off value (OD_c) was calculated as three standard deviations above the mean OD of the negative control, while the biofilm-forming categories were determined as follows: OD $\leq$ OD_c: *non-adherent*, OD_c < OD $\leq$ 2 $\times$ OD_c: *weakly adherent*, 2 $\times$ OD_c < OD $\leq$ 4 $\times$ OD_c: *moderately adherent*, and 4 $\times$ OD_c < OD: *strongly adherent*. The percentage of biofilm inhibition achieved by the activity of the investigated EOs was calculated in accordance with Jadhav et al. (2013).

Statistical analysis

All descriptive statistical computations were conducted by use of Microsoft Excel 2019 (Microsoft Corporation, Redmond, USA). One-way ANOVA with the post hoc multiple comparison tests (LSD) in terms of determining significant differences between the test compounds and the controls was performed using Statistica 10 (StatSoft, Inc., Tulsa, OK, USA). Differences were considered statistically significant at the $p < 0.05$ level. For the determination of MIC values, as well as in antibiofilm assays, data were presented descriptively, and interpretation was done according to predefined threshold values/percent of inhibition.

RESULTS AND DISCUSSION -

Rezultati i diskusija

GC-MS analysis confirmed that α -pinene was the dominant constituent in both oils (Table 1), accompanied by β -pinene, D-limonene and *o*-cymene in *P. nigra*, and α -terpineol, verbenone, isothujol, and isoborneol in *P. syl-*

vestris. The high α -pinene content correlates with much of the observed antimicrobial and antioxidant activity. Minor compositional differences may explain functional variations, particularly the stronger antibiofilm activity of *P. nigra* EO. Previous studies have shown that the content of pine EO compounds can vary. Thus, Salem et al. (2015) noticed that α -phellandrene and α -pinene were the only common compounds in oil obtained from the bark and wood of *P. sylvestris*. The chemical composition of EOs has been attributed to antimicrobial activity. Specifically, the elevated content of sesquiterpenes and oxygenated monoterpenes in *P. nigra* has been associated with interference in bacterial adhesion processes and the destabilization of biofilm structures (Roy et al., 2018). Overall, *P. sylvestris* EO demonstrated superior antibacterial activity, while *P. nigra* EO exhibited more pronounced antibiofilm effects. Both oils displayed mo-

derate antioxidant activity, highlighting their potential as natural agents for managing multidrug-resistant bacteria and biofilm-associated infections.

The DPPH radical scavenging assay revealed weak to moderate antioxidant activity (Table 2) for both EOs, which increased with concentration. *Pinus nigra* EO displayed slightly stronger activity compared to *P. sylvestris*. These findings align with previous studies showing that the antioxidant activity in pine species varies based on species, plant part, and analytical method (Kurti et al., 2019; Koutsaviti et al., 2021).

Table 1. Chemical compounds of the *P. nigra* and *P. sylvestris* EOs

Tabela 1. Hemijski spojevi eteričnih ulja *P. nigra* i *P. sylvestris*

	RI	Compound	<i>P. nigra</i>		<i>P. sylvestris</i>	
			Conc. (%)	RT	Conc. (%)	RT
1	929	α -pinene	88.908	5.872	63.420	5.855
2	979	β -pinene	1.578	7.077	2.090	7.077
3	1022	<i>o</i> -cymene	2.166	8.641	1.108	8.641
4	1030	D-limonene	3.560	8.794	2.631	8.789
5	1090	<i>p</i> -cymenene	/	/	0.942	11.073
6	1113	fenchol	/	/	0.485	12.020
7	1139	trans-pinocarveole	0.527	13.056	1.467	13.056
8	1142	cis-verbenol	/	/	1.116	13.297
9	1143	sabinol	1.153	13.297	/	/
10	1153	β -terpineole	/	/	1.335	14.105
11	1157	isoborneol	/	/	3.159	14.181
12	1168	isothujol	/	/	3.874	14.689
13	1183	<i>p</i> -cymene-8-ol	0.453	15.010	/	/
14	1189	α -terpineol	/	/	6.064	15.259
15	1193	myrtenal	/	/	0.847	15.505
16	1195	myrtenol	/	/	1.744	15.627
17	1205	verbenone	1.657	16.037	4.219	16.042
18	1285	bornyl acetate	/	/	2.010	19.370
19	1376	α -copaene	/	/	0.479	23.180
20	1384	β -bourbonene	/	/	1.466	23.548
21	1419	trans-caryophyllene	/	/	0.696	24.960
22	1477	γ -muurolene	/	/	0.849	27.315
Sum concentration			99.022		95.703	

Table 2. DPPH antioxidant activity of *P. nigra* and *P. sylvestris* EOs

Tabela 2. DPPH antioksidativna svojstva esencijalnih ulja
P. nigra i *P. sylvestris*

EO/ concentration	Diluted EO (25%)	Pure EO (100%)
<i>P. nigra</i>	9.27	23.13
<i>P. sylvestris</i>	7.21	15.73

Both *P. nigra* and *P. sylvestris* EOs exhibited weak to moderate inhibitory effects against *S. aureus* and the ESBL strain of *E. coli*. Agar diffusion assays showed inhibition zones of 13.67 ± 1.15 mm (*P. nigra*) and 17.0 ± 1.0 mm (*P. sylvestris*) against *S. aureus*. On the contrary, both EOs had a weaker effect on the ESBL strain of *E. coli* (Table 3), where inhibition zones were measured as 10.33 ± 0.58 (*P. nigra*) and 12.00 ± 0.00 (*P. sylvestris*). Results of the antimicrobial screening generated in the agar diffusion method indicated a stronger antibacterial potential of Scots pine when compared to black pine.

Table 3. Obtained inhibition zones due to the activity of *P. nigra* and *P. sylvestris* EOs

Tabela 3. Postignute zone inhibicije usljed djelovanja eteričnih ulja *P. nigra* i *P. sylvestris*

Essential oil	Inhibition zone, IZ (mm)*	
	<i>S. aureus</i> ATCC 6538	ESBL <i>E. coli</i> ATCC 35218
<i>P. nigra</i>	13.67 ± 1.15	10.33 ± 0.58
<i>P. sylvestris</i>	17.00 ± 1.00	12.00 ± 0.00
Controls		
Ampicillin	30.00 ± 0.00	10.00 ± 0.00
Erythromycin	22.00 ± 0.00	9.00 ± 0.00
Ceftazidime	18.00 ± 0.00	25.00 ± 0.00
Clindamycin	23.00 ± 0.00	NI
Rifampicin	31.00 ± 0.00	16.00 ± 0.00
Tetracycline	24.00 ± 0.00	19.00 ± 0.00
Gentamicin	20.00 ± 0.00	19.00 ± 0.00
Ciprofloxacin	24.00 ± 0.00	27.00 ± 0.00
Chloramphenicol	20.00 ± 0.00	19.00 ± 0.00
Amikacin	19.00 ± 0.00	18.00 ± 0.00
Results are presented as mean $\pm$ STDEV. *All generated IZ values differ significantly after performing a <i>post-hoc</i> LSD test. NI=No inhibition. DMSO=NI		

The MIC values of the investigated EOs are presented in Table 4. *Pinus nigra* against *S. aureus* had an MIC at $3.90 \mu\text{L/mL}$, while the ESBL strain of *E. coli* was less susceptible to this EO, with the MIC at $15.62 \mu\text{L/mL}$. Results of the Broth microdilution method were in accordance with the initial antibacterial screening, where *P. sylvestris* performed stronger antibacterial activity. MIC values of the Scots pine were $1.95 \mu\text{L/mL}$ against *S. aureus* and $3.90 \mu\text{L/mL}$ against ESBL *E. coli*. Average OD values of quadruplets ($\pm$ STDEV) used in the determination of MIC values of EOs against tested bacteria are presented in Figure 1.

Table 4. Determined MIC values of investigated EOs

Tabela 4. Određene vrijednosti MIK istraživanih eteričnih ulja

Essential oil	MIC value ($\mu\text{L/mL}$)	
	<i>S. aureus</i> ATCC 6538	ESBL <i>E. coli</i> ATCC 35218
<i>P. nigra</i>	3.90	15.62
<i>P. sylvestris</i>	1.95	3.90

Research on the antimicrobial properties of *Pinus* species native to Bosnia and Herzegovina is still limited, particularly regarding the essential oils (EOs) and their potential synergistic effects with antibiotics. Several *Pinus* species were examined, revealing that the essential oil extracted from *P. sylvestris* cones displayed the strongest antimicrobial activity (Mirković et al., 2024). In contrast, EOs from *P. sylvestris* needles, *P. mugo*, and *P. nigra* needles and cones showed weaker antimicrobial potential against the tested microorganisms. Interestingly, *P. nigra* was identified as the most effective of all the tested EOs against *E. coli*. Gojković Cvjetković et al. (2020) reported inhibition zones of 14.33 mm for *S. aureus*, compared to inhibition zones of 17.33 mm for both *E. coli* and *Klebsiella pneumoniae*, and 17.00 mm for *Bacillus subtilis*. Similar findings were observed in our research, where *P. sylvestris* demonstrated better antibacterial activity against both types of bacteria. Bacteria that produce Extended-Spectrum Beta-Lactamases (ESBLs) exhibit resistance to β -lactam antibiotics as well as other classes of antibiotics (Chong et al., 2018). This resistance leads to the development of a multiple-drug resistance phenotype (Kyo et al. 2021), making these bacteria a significant cause of morbidity and mortality worldwide (Jouini et al. 2013; Klibi et al. 2019).

All replicates of the positive control demonstrated that *S. aureus* ATCC 6538 investigated in this study formed a moderately adherent biofilm. After the application of the *P. nigra* EO, the first two subinhibitory concentrati-

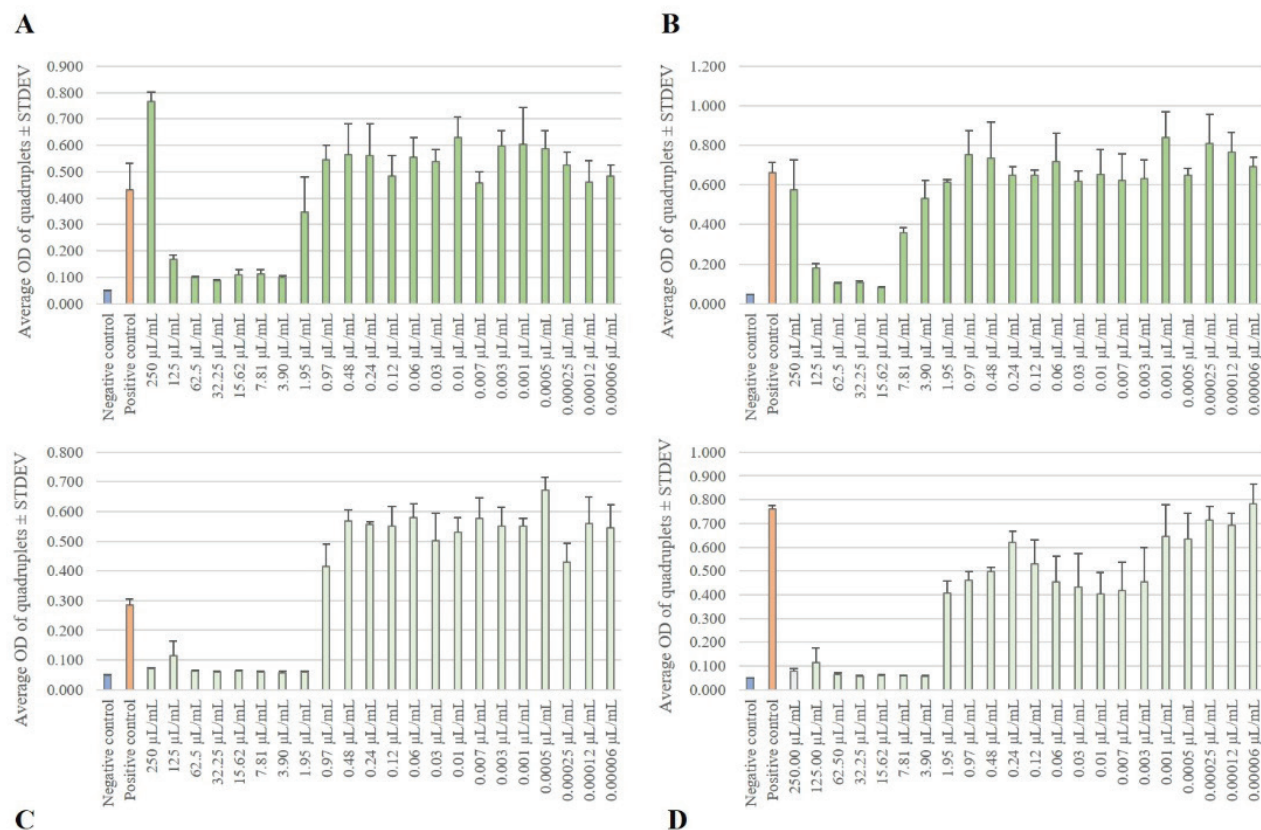


Figure 1. Average OD of quadruplets $\pm$ STDEV in the determination of MIC values of investigated EOs against tested bacteria. A. *P. nigra* against *S. aureus*; B. *P. nigra* against *E. coli*; C. *P. sylvestris* against *S. aureus*; D. *P. sylvestris* against *E. coli*.

Slika 1. Prosječne OD vrijednosti kvadrupleta $\pm$ STDEV u determinaciji MİK vrijednosti istraživanih eteričnih ulja protiv testnih bakterija. A. *P. nigra* protiv *S. aureus*; B. *P. nigra* protiv *E. coli*; C. *P. sylvestris* protiv *S. aureus*; D. *P. sylvestris* protiv *E. coli*.

ons (1.95 $\mu\text{L/mL}$ and 0.97 $\mu\text{L/mL}$) did not cause a change in the biofilm-forming capacity, while the rest of the dilution (up to 0.00006 $\mu\text{L/mL}$) induced the occurrence of strong biofilm. A similar pattern was observed in this bacterium after the application of *P. sylvestris* EO, where at first, subinhibitory concentrations (0.97-0.24 $\mu\text{L/mL}$) biofilm stayed moderately-adherent. Other serial dilutions led to the formation of strong biofilm. An extended-spectrum β -lactamase-producing strain of *E. coli* ATCC 35218 in this study was determined as a strong biofilm-former in all replications of the positive control. All subinhibitory concentrations of *P. nigra* EO, starting from 7.81 $\mu\text{L/mL}$, led to the elimination of this strong biofilm formation. The percentage of inhibition ranged from 77.74% to 96.56%. *P. sylvestris* EO eliminated this biofilm at 1.95 $\mu\text{L/mL}$ and 0.97 $\mu\text{L/mL}$, with the inhibition rate of 81.31-87.62%. Following serial dilutions (from 0.48 $\mu\text{L/mL}$ to 0.003 $\mu\text{L/mL}$) decreased the strong biofilm for two categories (to weakly-adherent), and the inhibition percent ranged from 59.94% to 72.29%. Lowest concentrations of this EO decreased biofilm for one category, to moderately-adherent, with

inhibition of 20.37% to 43.40%. Mean absorbance values ($\pm$ STDEV) observed in the evaluation of the impact of *P. nigra* and *P. sylvestris* EOs on the bacterial biofilms are presented in Tables 5 and 6.

These findings suggest that *P. nigra* essential oil may serve as a potential natural agent to inhibit biofilm formation in certain bacteria, particularly those responsible for infections caused by MDR strains like ESBL (Mirković et al., 2025). These actions contribute to the antimicrobial activity of EOs by impairing the ability of microorganisms to form and maintain biofilms, making them more susceptible to conventional antimicrobial agents. Furthermore, numerous studies have shown that EOs can degrade key components of the extracellular matrix, including polysaccharides and proteins, thereby weakening the structural integrity of biofilms (Touati et al., 2025). This study showed that *S. aureus* exhibits increased biofilm-forming capacity after the application of EOs in subinhibitory dilutions. Similar results have been observed in other Gram-positive bacteria, such as *Bacillus subtilis* and *Bacillus subtilis* (Beauregard et

Table 5. Absorbance values (mean $\pm$ STDEV) in evaluating the impact of *P. nigra* EO on the bacterial biofilmsTabela 5. Vrijednosti absorbanci (mean $\pm$ STDEV) u evaluaciji uticaja eteričnog ulja *P. nigra* na bakterijske biofilmove

Bacteria	Negative control	Positive control	Subinhibitory concentrations of EOs (μ L/mL)						
<i>Staphylococcus aureus</i> ATCC 6538	0.063 $\pm$ 0.009	0.303 $\pm$ 0.127	1.95	0.97	0.48	0.24	0.12	0.06	0.03
			0.341 $\pm$ 0.520	0.331 $\pm$ 0.184	0.576 $\pm$ 0.079	0.632 $\pm$ 0.091	0.640 $\pm$ 0.082	0.664 $\pm$ 0.272	0.589 $\pm$ 0.167
			0.01	0.007	0.003	0.001	0.0005	0.00025	0.00012
			0.661 $\pm$ 0.104	0.539 $\pm$ 0.152	0.601 $\pm$ 0.131	0.674 $\pm$ 0.072	0.555 $\pm$ 0.167	0.600 $\pm$ 0.043	0.643 $\pm$ 0.103
			0.00006	-	-	-	-	-	-
			0.593 $\pm$ 0.125	-	-	-	-	-	-
ESBL <i>Escherichia coli</i> ATCC 35218	Negative control	Positive control	Subinhibitory concentrations of EOs (μL/mL)						
	0.245 $\pm$ 0.218	3.624 $\pm$ 0.261	7.81	3.90	1.95	0.97	0.48	0.24	0.12
			0.125 $\pm$ 0.013*	0.250 $\pm$ 0.047*	0.367 $\pm$ 0.069*	0.485 $\pm$ 0.095*	0.526 $\pm$ 0.060*	0.657 $\pm$ 0.045*	0.671 $\pm$ 0.137*
			0.06	0.03	0.01	0.007	0.003	0.001	0.0005
			0.685 $\pm$ 0.192*	0.664 $\pm$ 0.167*	0.630 $\pm$ 0.264*	0.679 $\pm$ 0.215*	0.645 $\pm$ 0.184*	0.679 $\pm$ 0.049*	0.712 $\pm$ 0.137*
			0.00025	0.00012	0.00006	-	-	-	-
			0.703 $\pm$ 0.070*	0.776 $\pm$ 0.061*	0.807 $\pm$ 0.078*	-	-	-	-

*Biofilm elimination.

Table 6. Absorbance values (mean $\pm$ STDEV) in evaluating the impact of *P. sylvestris* EO on the bacterial biofilmsTabela 6. Vrijednosti absorbanci (mean $\pm$ STDEV) u evaluaciji uticaja eteričnog ulja *P. sylvestris* na bakterijske biofilmove

Bacteria	Negative control	Positive control	Subinhibitory concentrations of EOs (μ L/mL)						
<i>Staphylococcus aureus</i> ATCC 6538	0.068 $\pm$ 0.006	0.191 $\pm$ 0.026	0.97	0.48	0.24	0.12	0.06	0.03	0.01
			0.258 $\pm$ 0.100	0.335 $\pm$ 0.011	0.319 $\pm$ 0.038	0.442 $\pm$ 0.036	0.516 $\pm$ 0.084	0.442 $\pm$ 0.249	0.398 $\pm$ 0.136
			0.007	0.003	0.001	0.0005	0.00025	0.00012	0.00006
			0.414 $\pm$ 0.062	0.488 $\pm$ 0.103	0.444 $\pm$ 0.102	0.453 $\pm$ 0.068	0.404 $\pm$ 0.041	0.509 $\pm$ 0.081	0.425 $\pm$ 0.032
ESBL <i>Escherichia coli</i> ATCC 35218	Negative control	Positive control	Subinhibitory concentrations of EOs (μL/mL)						
	0.086 $\pm$ 0.043	0.848 $\pm$ 0.069	1.95	0.97	0.48	0.24	0.12	0.06	0.03
			0.105 $\pm$ 0.011*	0.159 $\pm$ 0.052*	0.235 $\pm$ 0.084**	0.275 $\pm$ 0.063**	0.323 $\pm$ 0.112**	0.259 $\pm$ 0.162**	0.286 $\pm$ 0.174**
			0.01	0.007	0.003	0.001	0.0005	0.00025	0.00012
			0.298 $\pm$ 0.169**	0.276 $\pm$ 0.155**	0.340 $\pm$ 0.174**	0.480 $\pm$ 0.124***	0.571 $\pm$ 0.102***	0.588 $\pm$ 0.101***	0.589 $\pm$ 0.104***
			0.00006	-	-	-	-	-	-
			0.675 $\pm$ 0.069***	-	-	-	-	-	-

*Biofilm elimination.

**Decreasing for two biofilm-forming categories.

***Decreasing for one biofilm-forming category.

al., 2013; Li et al., 2021), where biofilm formation was triggered in vitro by certain plant compounds, probably used by bacteria as a sugar source during the synthesis of the exopolysaccharide matrix. Furthermore, there are reports indicating that the application of plant compounds increases biofilm formation in certain bacteria (Abedini et al., 2020), which may be a specific bacterial response to stressful conditions.

CONCLUSION - Zaključak

Chemical analysis revealed that α -pinene was the major constituent, representing 88.91% in *Pinus nigra* and 63.42% in *P. sylvestris*. These findings highlight the therapeutic potential of pine essential oils, particularly in combating multidrug-resistant microorganisms. This study demonstrates that essential oils from *P. nigra* and *P. sylvestris* exhibit moderate antioxidant activity and dose-dependent antimicrobial effect. The impact of the EOs on bacterial biofilms was also noted. Further in vivo studies, toxicological assessments, and standardization of oil composition are recommended to validate clinical applications. Integrating traditional knowledge with modern research provides a promising framework for developing new antimicrobial and antioxidant agents.

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SAŽETAK

Ovo istraživanje imalo je za cilj ispitati hemijski sastav, antioksidativna i antibakterijska svojstva eteričnih ulja crnog bora (*Pinus nigra*) i bijelog bora (*Pinus sylvestris*), te evaluirati uticaj ulja na bakterijske biofilmove. Eterična ulja su dobijena metodom hidrodestilacije i analizirana pomoću gasne hromatografije-masene spektrometrije (GC-MS). Kao dominantni hemokonstituenti istraživanih eteričnih ulja identificirani su monoterpeni i seskviterpeni (Tabela 1), koji su prepoznati po svojoj bioaktivnosti. Antioksidativna aktivnost eteričnih ulja je procijenjena DPPH (2,2-diphenyl-1-picrylhydrazyl) metodom, a dobijeni rezultati sugeriraju efekat zavisn od koncentracije (Tabela 2). Antibakterijska aktivnost je testirana na jednu Gram-pozitivnu bakteriju: *Staphylococcus aureus* i jednu Gram-negativnu bakteriju: *Escherichia coli* koja proizvodi prošireni spektar beta-laktamaza (ESBL), koristeći metodu difuzije u agaru i mikrodilucijsku tehniku. Dobijeni rezultati ukazuju na varijabilan antibakterijski potencijal ispitivanih eteričnih ulja u zavisnosti od vrste ulja i vrste testirane bakterije (Tabela 3). Minimalne inhibitorne koncentracije (MIK) eteričnih ulja određene su mikrodilucijskom metodom, čime je omogućena kvantitativna procjena njihove antibakterijske efektivnosti. Utvrđene MIK vrijednosti razlikovale su se između ispitivanih eteričnih ulja, kao i između Gram-pozitivne i Gram-negativne bakterije, što upućuje na različitu osjetljivost testnih sojeva (Tabela 4). Tokom determinacije MIK vrijednosti, praćene su promjene optičke gustine (OD), a prosječne OD vrijednosti kvadrupleta sa standardnim devijacijama prikazane su grafički pružajući uvid u dinamiku inhibicije rasta bakterija pri različitim koncentracijama eteričnih ulja (Slika 1). Uticaj eteričnih ulja na bakterijske biofilmove je evaluiran metodom kulture tkiva na ploči. Rezultati agar difuzije su sugerirali jače antibakterijsko djelovanje bijelog bora u odnosu na crni bor, što je u korelaciji sa rezultatima mikrodilucijskog testa. Soj *S. aureus* korišten u ovoj studiji je pokazao kapacitet za formiranje umjereno jakog biofilma, dok je ESBL soj *E. coli* formirao jaki biofilm (Tabele 5 i 6). Istraživana eterična ulja su ispoljila potencijal promjene kategorije biofilma na način koji je specijski-specifičan i koncentracijski ovisan.

Received: Decembar, 30, 2025; **Accepted:** Februar, 2, 2026; **Published:** July, 31, 2026

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.



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