Characterization of extracts from wood waste of cembran pine (Pinus cembra L.)– chemical composition, antioxidant activity and antimicrobial potential

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Abstract

Wood residues of furniture production from cembran pine (*Pinus cembra* L.) were used in supercritical CO_2 extraction at high and medium extraction pressure. The Gas chromatography – mass spectrometry (GC/MS) analysis of extracts was performed, and the concentration of volatiles compared to the reference, commercially available (reference R) cembran pine oil. The reference sample had a much higher TVOC (Total Volatile Organic Compounds) content than the samples extracted with supercritical carbon dioxide (CO_2). α-phellandrene, o-cymene and tricyclen were detected in the samples, extracted with the high (HP) and medium pressure (MP), with the highest TE (Toluene D8-Equivalents) value of o-cymene. To determine the antioxidant activity of the samples, DPPH (2,2-diphenyl-1-picrylhydratyl), ABTS (2, 2'-azino-bis(3- ethylbenzothiazoline-6-sulfonic) acid) and FRAP (ferric reducing antioxidant) assays were performed. In all three tests, our extracts showed stronger antioxidant activity than the reference, which showed significantly lower antioxidant activity, although the TVOC value was 75 times higher. The antimicrobial activity of the cembran pine extracts was determined as MIC (minimal inhibitory concentration) and MBC (minimal bacterial concentration) and MFC (minimal fungicidal concentration) values for Gram-positive, Gram-negative bacteria and fungi. The best antimicrobial activity was found in sample MP with the lowest achieved MICs followed by sample HP. For the sample R, the MIC could only be determined for *C. albicans*. Cembran pine wood waste extracts have shown good effect against *C. jejuni* intercellular signalling as well as against *C. jejuni* adhesion, indicating that cembran pine wood waste extracts have potential against complex bacterial properties that could be important for biofilm formation.

1. Introduction

Pinus spp. is one of the most economically important tree groups worldwide and represents one of the largest forest formations in Europe (Ferreira-Santos et al. 2020). Pine wood is used in all major industries such as sawmilling, furniture manufacturing, packaging, pulp and paper industries where wood processing residues are generated. It is important to implement a biorefinery concept for the valorization of pine wood. This is a sustainable process for the use of biomass with the aim of moving towards more environmentally friendly processes and reducing the use of chemicals, efficient use of energy and reduction of waste in order to fully utilise the wood material (Ferreira-Santos et al. 2020; Herrero and Ibañez 2018).

An important part of the biorefinery approach is the extraction of value-adding components from pine wood residues via material recycling/reuse networks. In this way, bioactive compounds can be obtained that are used in pharmaceutical and cosmetic products, food fortification and preservation products, food supplements and nutraceuticals (Mármol et al. 2019). It is important to use environmentally friendly and sustainable processes, focusing on the use of supercritical fluids for the extraction of high value-added products from natural sources (Herrero and Ibañez 2018). Here, carbon dioxide (CO_2) is most commonly used as it is non-toxic, safe and the critical temperature is low (Ferreira-Santos et al. 2020). Therefore, supercritical CO_2 extraction is suitable for temperature-sensitive materials and the result is a

rapidly available, high-quality end product. CO_2 extraction has already been successfully applied to *Pinus gerardiana* and *Pinus niruri* (Amani et al. 2021).

Cembran pine (*Pinus cembra* L., *Pinaceae*) is a coniferous species that grows at the alpine timberline at altitudes of 1500–2200 m in the Central European Alps (Austria, Switzerland, south-eastern France and northern Italy) and the Carpathian Mountains (Ukraine, Slovakia, Romania and Poland) (Apetrei et al. 2011; Lis et al. 2017). It is a regionally important pine tree that is gaining in economic importance and accounts for around 2.3% of the trees that can be used for forestry in the Austrian province Tyrol (Chizzola and Müllner 2020). The wood is used in Central Europe for the construction and manufacture of furniture. It is highly valued for its long-lasting odor, which has been shown to have a positive effect on sleep and reduce stress in humans (Chizzola and Müllner 2020; Ghadiriasli et al. 2020). Previous studies (Apetrei et al. 2013; Lis et al. 2017; Chizzola and Müllner 2020) have investigated the composition of essential oils and extracts from different morphological parts of *P. cembra* L., suggesting that the different parts have different compositions. These studies have shown that α-pinene, limonene, β-phellandrene and β-pinene are present in the wood, but it is not known whether the content of these compounds is comparable in wood waste. The chemical composition of extracts obtained from wood waste of *P. cembra* L. is not well known, but it is crucial for the valorization of these materials.

The aim of this study was: i) to extract aromas and essential oils from the *P. cembra* L. wood waste (wood shavings) by supercritical CO_2 extraction using two different extraction pressures; ii) to determine the main constituents of the extracts obtained by GC-MS analysis and compare them with commercial steam-distilled cembran pine oil; iii) to evaluate the antioxidant activity of the extracts obtained; and iv) to evaluate the antimicrobial potential of the extracts obtained, focusing on different levels of antimicrobial activity.

2. Materials and methods

2.1 Wood waste material

Wood shavings from the heartwood of *P. cembra* L. were used as raw material for the extraction. These wood shavings were residues from furniture production (Zirbenherz GmbH, Gewerbestraße 12, 9330 Althofen, Austria). The fine wood shaves had a moisture content of 6.6%, were then packed in a plastic bag and send for extraction.

2.2 Supercritical CO_2 extraction

The extraction experiments were performed in a lab-scale extraction plant of the type HDL 4 from NATECO$_2$ (Hopfenveredlung St. Johann GmbH, Auenstraße 18–20, 85283 Wolnzach, Germany). The extractions at NATECO$_2$ are carried out in a batch process, as the extractor can only be emptied and refilled at atmospheric pressure. The main components of the extraction plant are the extractor with an

internal volume of 5 L and the separation unit. Only CO_2 was used as a solvent for the extractions. The system is designed for a maximum extraction pressure of 1000 bar and a temperature of up to 95°C.

First, supercritical CO_2 was produced by pressurizing liquid CO_2 with a pump and heating it in a heat exchanger. The supercritical solvent then flowed through the extractor, which was charged with wood shavings. After extraction, the homogeneous CO_2-extract mixture was separated into a gaseous CO_2 phase and an extract phase by pressure reduction. The extract can be removed from the process while the gaseous CO_2 is recycled. If required, the extract can be fractionated by gradually reducing the pressure.

Experiments were performed with high (60 MPa) and medium extraction pressure (30 MPa) to produce two different extracts (Schuster et al. 2018). The names of the experiments, the extraction conditions and the codes of the samples obtained are listed in Table 1. Commercially available steam-distilled cembran pine oil was used as a reference for all further experiments.

Table 1
List of samples with extraction conditions and designations of obtained samples

Extraction conditions	Sample
steam-distilled cembran pine oil (reference; commercially available; BIO-Zirbenöl, Sägewerk Greiler, Sirnitz, Austria)	R
CO_2 extracted cembran pine oil at high pressure	HP
CO_2 extracted cembran pine oil at medium pressure	MP

2.3 GC-MS Analysis of extracts

GC-MS (gas chromatography-mass spectrometry) analysis (GC-MS Agilent 7890A/5975C, United States) was performed on the samples listed in Table 1. In addition to the identification of the volatiles, their relative concentration compared to an internal standard and the total volatile organic compounds (TVOC) value were determined.

The volatiles were determined at 23°C in the microchamber. For this purpose, 100 µL of the respective sample was dripped onto a watch glass for each microchamber cell and conditioned for 1 hour with an air flow of 50 mL/min. The emissions were measured by adsorption of the volatiles on Tenax tubes (desorption tubes). The sampling time was 5 minutes in each case. Before sampling, 1 µL of a toluene D8 standard (Sigma-Aldrich Handels GmbH, Vienna, Austria) dissolved in methanol (Sigma-Aldrich Handels GmbH) was applied to each desorption tube. The analytes were thermally desorbed, separated and analyzed by GC-MS. The following GC-MS temperature program was used for the Tenax measurement: start temperature at 35°C, hold time: 3 min; heating rate / ramp: 10°C / min up to 160°C, hold time: 0 min; heating rate / ramp: 20°C / min up to a final temperature of 260°C, hold time: 10 min. The identification of the individual compounds was performed using commercially available spectra

libraries and retention indices. Quantification was performed in toluene D8 equivalents (TE). Two parallel samples of each variant were measured.

2.4 Evaluation of the antioxidant activity

The antioxidant activity of the samples was determined using the radical scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) assay and the ferric reducing antioxidant (FRAP) assay.

2.4.1 DPPH assay

The DPPH assay was performed according to Vladimir-Knežević et al. (2014) with adaptations. 0.6 mM DPPH (Sigma Aldrich, USA) solution was prepared in absolute methanol (Merck, Germany). 60 µL of the sample at a selected concentration from 0.0625 mg/mL to 10 mg/mL and 20 µL of the DPPH solution were added to the wells of a 96-well microtiter plate, mixed (600 rpm, 1 min; Thermomixer Comfort, Eppendorf, Germany) and incubated for 30 min in the dark at room temperature. After incubation, the absorbance was measured at 517 nm (Multiskan GO, Thermo Fisher Scientific, USA). The results were expressed as EC_{50}.

2.4.2 ABTS assay

The ABTS assay was performed according to Xiao et al. (2020) with adaptations. For the ABTS assay, ABTS stock solution (7 mM; Sigma Aldrich) and potassium persulfate solution (2.45 mM; $K_2S_2O_8$; Merck,) were mixed in a 1:1 ratio and kept in the dark for 16 h to obtain an ABTS reaction solution ($ABTS^{\bullet}$). After incubation, $ABTS^{\bullet}$ was diluted with potassium phosphate buffer (0.1 M, pH 7.4; prepared with potassium dihydrogen phosphate (Merck) and dipotassium hydrogen phosphate (Kemika, Zagreb, Croatia) to obtain an ABTS working solution ($dABTS^{\bullet}$) with an absorbance at 734 nm of 0.70 ± 0.01. 10.0 µL of the sample and 200 µL of $dABTS^{\bullet}$ were added to a well of a 96-well microtiter plate, shaken (600 rpm, 1 min; Thermomixer Comfort, Eppendorf) and kept in the dark at room temperature for 6 min. Then the absorbance was measured at 734 nm (Varioskan LUX, Thermo Fisher Scientific, USA). The results were expressed as µmol gallic acid equivalents (GAE) per g sample.

2.4.3 FRAP assay

The FRAP assay was performed according to Xiao et al. (2020) with adaptations. For the FRAP reagent, acetic buffer (300 mM, pH 3.6; Merck), 2,4,6-tri(2-pyridyl)-s-triazine (10 mM; Sigma Aldrich) in hydrochloric acid (40 mM; Merck) and iron (III) chloride hexahydrate (20 mM, pH 6.6; Merck) were mixed in a ratio of 10:1:1. 180 µL of the reagent and 20 µL of the sample were added to a well of a 96-well microtiter plate and mixed (1 min, 600 rpm; Thermomixer Comfort, Eppendorf). After 10 min incubation at 37°C, the absorbance was measured at 593 nm (Multiskan Go, Thermo Fisher Scientific). The results were expressed as µmol gallic acid equivalents (GAE) per g sample.

2.5 Antimicrobial analyses

The antimicrobial activity of samples R, HP and MP was determined as minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) for different groups of microorganisms. As an additional effect on microorganisms, the effect on bacterial intercellular signalling and the effect on adhesion formation were analysed on a model bacterium *Campylobacter jejuni*.

2.5.1 Determining minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC)

The antimicrobial activity of the essential oils was determined using the broth microdilution method (Klančnik et al. 2010). Stock solutions of samples were prepared in dimethyl sulfoxide (DMSO; Merck) and used to prepare working solutions (20 mg/mL) in selected growth media. DMSO had no effect on the growth of the microorganisms at the final concentrations used. Two-fold serial dilutions (from 10 to 0.005 mg/mL) were prepared in 96-well microtiter plates in 50 µL growth media (Mueller Hinton broth (MHB, Oxoid, Hampshire, UK) for bacteria and malt extract broth (Merck) for fungi). To each well, 50 µL of the prepared inoculum of the selected microorganism was added. Representatives of Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Listeria innocua* ŽM39), Gram-negative bacteria (*Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 and *Campylobacter jejuni* NCTC 11168) and fungi (*Candida albicans* ATCC 10261 and *Aspergillus flavus* ATCC 16875) were selected. All bacteria, except *C. jejuni*, were incubated at 37°C for 24 hours; *C. jejuni* at 42°C for 24 hours under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2); *C. albicans* at 30°C for 24 hours; *A. flavus* at 25°C for 5 days. After incubation, microbial growth was evaluated. For Gram-positive bacteria, *E. coli*, *P. aeruginosa* and *C. albicans*, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride was used as an indicator of microbial growth and for *C. jejuni* resazurin was used. The growth of *A. flavus* was evaluated visually. The minimal inhibitory concentration (MIC) was determined as the first concentration at which no microbial growth was observed in the wells of the microtiter plate. The contents of the wells with no visible microbial growth were inoculated onto the solid medium and further incubated under the conditions described in this paragraph. The minimal bactericidal/fungicidal concentration (MBC/MFC) was determined as the first concentration at which no microbial growth was observed after subsequent inoculation onto specific solid growth media.

2.5.2 Determining effect of cembran pine essential oils on *Campylobacter jejuni* intercellular signalling and adhesion

The subinhibitory concentration (0.25× MIC) was used to determine the effect of the samples on intercellular signalling and adhesion of the model microorganism *C. jejuni* NCTC 11168.

2.5.2.1 Autoinducer-2 bioassay

To determine the effect of the samples on the intercellular signalling of *C. jejuni*, an autoinducer-2 (AI-2) bioassay was performed to detect changes in the bioluminescence response of the reporter strain *Vibrio harveyi* MM30 (Bassler et al. 1997). The bioassay was performed as described in Šimunović et al. (2020) with minor modifications. Briefly, standardized inocula of *C. jejuni* NCTC 11168 and *C. jejuni* 11168 Δ*luxS* were prepared in 5 mL MHB. *C. jejuni* 11168 Δ*luxS* was used as a negative control in which AI-2 was not present. For the treatment with the samples, 0.25× MIC was used, the first concentration that had no effect on the growth of the two strains (Šimunović et al., 2020a). The prepared cultures were incubated under microaerobic conditions at 42°C for 24 h before cell-free supernatants (CFSs) were obtained by filter sterilization (0.2 µm filter, Sartorius, Göttingen, Germany). The CFSs were added to the prepared inoculum of *V. harveyi* in autoinducer bioassay (AB) medium (17 g/L NaCl (Merck), 12.3 g/L $MgSO_4$ (Merck), 2 g/L casamino acids (BD Bacto, Fisher Scientific, USA), 1 mM K_2HPO_4 (Kemika), 0.1 mM L-arginine (Sigma Aldrich) and 1% v/v glycerol (Kemika)) to obtain a final concentration of 10% v/v in each well of the white transparent-bottomed microtiter plate (Greiner Bio-One, Kremsmünster, Austria). The bioluminescence signals and the OD_{600} were measured kinetically (Varioskan LUX, Thermo Scientific) at 30 min intervals during the15 h incubation for at 30°C in an aerobic atmosphere. The reduction in bioluminescence was calculated at the point where no further growth of bioluminescence was observed.

2.5.2.2 Evaluation of *Campylobacter jejuni* adhesion

The effect of the samples on the adhesion of *C. jejuni* was performed by determining the viable adhered cells using the plate count method as described in Kurinčič et al. (2016). The 0.25× MIC was used to treat *C. jejuni* with the samples. 200 µL of the control and treated cultures were added to each well of the microtiter plate and incubated under microaerobic conditions at 42°C for 4 h before the supernatant was removed and each well was rinsed three times with phosphate-buffered solution (PBS, Oxoid). 200 µL of PBS was added and sonicated to detach the adhered cells and to obtain cell supernatants, which were used to determine viable adhered cells as colony forming units (CFU)/mL.

2.6 Data analysis

The results of the ABTS and FRAP assays and the assays with the model bacterium *C. jejuni* were normally distributed and homogeneity of variance was met. The results were compared using SPSS Statistics V23 using analysis of variance and Tukey's post-hoc test. The results for intercellular signalling and adhesion of *C. jejuni* were correlated with Pearson´s correlation test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 *Pinus cembra* essential oils

During the tests it was found that the CO_2 extracted cembran pine oil could not be obtained anhydrous at high pressure (HP). In order to obtain pure oil extracts, a subsequent oil separation must be carried out in a separating funnel. In this way, about 39.5% water could be separated from the extract. The CO_2

extracted cembran pine oil at medium pressure (MP) could be obtained almost anhydrous. Here, only about 5% of the water was separated via the separating funnel. When evaluating the relative extraction yield, an average of 3.9% was determined for the HP sample and an average of 3.7% for the MP sample, i.e. the achievable yields are comparable. The distinctive characteristics of supercritical CO_2 (Sc-CO_2), which has a high density like a liquid, diffusivity like a gas, and viscosity between gas-liquid, have gained much attention for its use as a solvent in the extraction of valuable bioactive compounds from plant biomass matrices. Usually, density is considered to determine the solvating power of solvents. In this process, the CO_2 first diffuses throughout the plant matrix and then dissolves the valuable phytochemicals using its solvent density properties. Some key advantages of the supercritical phenomenon, including solvating strength, diffusion coefficient, and lower viscosity, favor more efficient extraction of constituents compared to the subcritical process (Arumugham et al. 2021).

3.2 Identified compounds by GC-MS analysis

The detected and identified compounds, the values relative to the internal standard toluene D8, and the total volatile organic compound (TVOC) values for all three samples are listed in Table 2. The steam-distilled sample (R) has a much higher TVOC content than the two oils extracted with CO_2 (HP, MP). The compounds furan, cyclopentadiene, toluol, 3-hexen-1-ol, santene, β-phellandrene, fenchyl alcohol, camphor, endo-borneol, terpin-4-ol, α-terpineol and 1-naphtalen carboxylic acid were only determined in sample R, with the highest TE value for β-phellandrene. Tricyclene, o-cymene, benzene and α-phellandrene were detected in the HP and MP samples but not in R, with o-cymene having the highest TE value in both CO_2 extracted samples. Hexanal was only detected in the HP sample.

Table 2

Relative contents in Toluene D8-Equivalents (TE in µg/m^3) and the Tital Volatile Organic Compounds (TVOC in µg/m^3) of the steam-distilled reference oil sample (R) and of the extracted oil samples at medium (MP) and at high (HP) pressure

Compound	Retention time (min)	TE (µg/m^3)		
		Sample R	Sample HP	Sample MP
Furan	10.301	655.3	0.0	0.0
Cyclopentadiene	11.08	635.3	0.0	0.0
Toluol	11.819	8250.7	0.0	0.0
Hexanal	12.207	0.0	0.0	83.1
3-Hexen-1-ol	13.448	3607.6	0.0	0.0
Santene	14.449	26823.9	0.0	0.0
Tricyclene	15.302	0.0	74.7	29.8
Alpha pinene	15.717	3682916.3	3960.4	5357.0
Camphene	16.026	670200.3	610.1	348.4
Beta pinene	16.45	1052841.6	406.0	508.6
Beta phellandrene	16.656	88799.7	0.0	0.0
o-Cymene	16.796	0.0	1988.1	668.1
3-Carene	16.817	357695.4	1919.9	2802.6
Alpha phellandrene	16.99	0.0	441.7	291.3
Limonene	17.192	2231702.1	723.0	402.5
Gamma terpinene	17.395	21488.5	141.9	85.8
Benzene	17.698	0.0	323.8	162.4
Alpha terpinolene	17.825	67194.9	450.3	290.7
Fenchyl alcohol	18.188	45620.2	0.0	0.0
Camphor	18.524	10589.6	0.0	0.00
2-Bornanone	18.582	857.9	0.0	0.0
Endo-Borneol	18.827	20667.5	0.0	0.0
Terpin-4-ol	18.912	18208.7	0.0	0.0
Alpha terpineol	19.013	9985.0	0.0	0.0

Compound	Retention time (min)	TE (µg/m^3)		
		Sample R	Sample HP	Sample MP
Anisole	19.36	12491.2	45.0	34.1
Bornyl acetate	20.046	7746.5	43.6	42.8
1-Naphtalen carboxylic acid	23.346	38880.5	0.0	0.0
TVOC		8377858.7	11128.5	11107.1
	Equates to	837.78 mg/m^3	11.12 mg/m^3	11.10 mg/m^3

All detected compounds are typical for coniferous species and the compounds responsible for the typical odor are α-pinene β-pinene,3-carene, limonene and α-terpinolene (Piccand et al. 2019). All of these compounds were responsible for the largest proportion of TVOCs in our samples, with α-pinene, camphene, β-pinene, 3-carene, limonene, γ-terpinene, α-terpinolene, anisole and bornyl acetate present in samples R, HP and MP, respectively, and α-pinene being the most abundant in all samples.

The essential oil composition of different parts of *P. cembra* L. has previously been investigated (Apetrei et al. 2013; Chizzola and Müllner 2020, Lis et al. 2017) and in all three studies α-pinene was singled out for its abundance. Apetrei et al. (2013) evaluated the chemical composition of essential oils extracted from needles and twigs. In both essential oil samples α-pinene and limonene + β-phellandrene were the most abundant. Chizzola and Müllner (2020) found that α-pinene was present in all samples, with the highest content in cones (49.3%) and the lowest in sapwood (0.7%). Twig tips with needles from various parts of the crown had a similar essential oil composition and contained α-pinene (43.9–48.3.%), β-phellandrene (13.1–17.2%), β-pinene (6.6–9.3%), germacrene D (5.1–6.8%) and limonene (4.1–6.1%). Lis et al. (2017) identified over 130 compounds in the essential oils from needles, twigs, wood, bark and cones using GC-FID, GC/MS and 1H-NMR spectroscopy. They found that the main components of the oil from twigs with needles were α-pinene, limonene and β-phellandrene. In both, wood oil and cone oil, the main components were α-pinene and β-pinene. Although in these studies (Apetrei et al. 2013; Chizzola and Müllner 2020, Lis et al. 2017) the samples were obtained by hydrodistillation and not by supercritical extraction as in our study, the results are comparable to our study and confirm the presence of similar main components in the essential oils from various parts of *P. cembra* L. and its waste material researched in our study. However, there is a difference in the isomeric form of phellandrene – when obtained by hydrodistillation it is β-phellandrene, but in supercritical CO_2 extraction it is α-phellandrene.

α-pinene and β-pinene are bicyclic monoterpenes commonly found in various species of the genus *Pinus* (Borges et al. 2018; Dziedziński et al. 2021). These phytochemicals are known to have diverse biological activity that contributes to their various applications. The use of α-pinene and β-pinene goes beyond therapeutic and nutritional applications. As versatile compounds, they are also used in polymer

synthesis (Dziedziński et al. 2021). α-Pinene has long been known as an odour-forming component of pine wood (Schreiner et al. 2018). Antonelli et al. (2020) reported that inhaling forest volatile organic compounds like pinene and limonene can lead to beneficial antioxidant and anti-inflammatory effects on the respiratory tract. Plant extracts obtained through supercritical CO_2 extraction are enriched with a wide range of phytochemicals and exhibit antioxidant properties along with various other therapeutic properties, such as antimicrobial, anti-inflammatory, anti-diabetic, and anti-cancerous activities (Arumugham et al. 2021).

3.3 Antioxidant activity

Extracts from waste materials such as knotwood and bark of coniferous trees, including those from the *Pinaceae* family are rich sources of potent antioxidants (Ferreira-Santos et al. 2020; Pietarinen et al. 2006). In our work, the samples obtained by supercritical CO_2 extraction of *P. cembra* L. wood shavings were analysed for their antioxidant activity using DPPH, ABTS and FRAP assays. The results are presented in Table 3. In all three assays, samples HP and MP showed stronger antioxidant activity than sample R and there was no significant difference between samples HP and MP, despite the better antioxidant activity of sample HP as determined by DPPH. These two samples also had comparable TVOC values. On the other hand, sample R showed significantly lower antioxidant activity compared to samples HP and MP, although the TVOC value was 75 times higher. Supercritical CO_2 extraction is a fast and efficient approach to obtain bioactives, and although it has shown to be less effective than the conventional Soxhlet method in recovery of quercetin, rutin and kaepferol from pinecone of *Pinus brutia* (Nuralın et al. 2021), it has its advantages as environmentally friendly method that leaves no harmful residues and is less-time consuming.

Table 3
Antioxidant activity of commercially available steam-distilled cembran pine oil (sample R), CO_2 extracted cembran pine oil at high pressure (sample HP), and CO_2 extracted cembran pine oil at medium pressure (sample MP) expressed as EC_{50} (mg/mL) of scavenging activity for the DPPH assay and as mean ± standard deviation of gallic acid equivalents (μmol GAE) per 1 g of sample for the ABTS and FRAP assays. Different superscript letter in column means significant difference

Sample	DPPH EC_{50} (mg/mL)	ABTS (μmol GAE/g)	FRAP (μmol GAE/g)
R	> 10	0.177 ± 0.09^a	18.43 ± 1.26^a
HP	0.368	223.54 ± 21.6^b	55.14 ± 0.51^b
MP	0.468	201.88 ± 15.6^b	57.30 ± 2.13^b

The differences in the better antioxidant potential of the HP and MP samples compared to the commercial R sample can probably also be attributed to the extraction method. Essential oils of

Echinophora platyloba DC obtained by supercritical CO_2 extraction showed higher antioxidant activity than essential oils obtained by hydrodistillation. This was attributed to the absence of light and oxygen and the use of milder temperatures, which reduced the degradation of sensitive compounds in the essential oil obtained with supercritical CO_2 extraction (Sodeifian and Sajadian 2017).

Only a few studies investigated the antioxidant potential of differently obtained extracts and essential oils of *P. cembra* L. (Apetrei et al. 2011, 2013; Garzoli et al. 2021). In hydromethanolic extracts from the bark and needles of *P. cembra* L., the bark extract proved to be more active as a free radical scavenger and reducing agent compared to the needle extract. Their results showed lower EC_{50} values for both the bark extract (71.1 ± 0.5 µg/mL) and the needle extract (186.1 ± 1.7 µg/mL) compared to our results. However, both extracts had weaker DPPH scavenging activity than gallic acid (1.56 ± 0.05 µg/mL) and (+)-catechin (5.56 ± 0.05 µg/mL) (Apetrei et al. 2011). Essential oil from needles and twigs showed weak DPPH activity (19.93 ± 0.75 mg/mL and 18.66 ± 0.70 mg/mL, respectively) compared to BHA (3.3 ± 0.1 µg/mL) and hydromethanolic extracts (Apetrei et al. 2013). A comparable DPPH scavenging activity (EC_{50} 13.01 ± 0.86 µg/mL) was also found for the essential oil obtained by steam distillation from the needles of *P. cembra* L. (Garzoli et al. 2021). Compared to these results, our samples obtained from wood shavings of *P. cembra* L. were less active. However, the differences in the protocols used to determine the antioxidant capacity and the variations in chemical composition make it challenging to directly compare our results with those from the literature.

3.4 Antimicrobial potential

The antimicrobial activity of the samples was determined as MIC and MBC/MFC values for different groups of microorganisms (Gram-positive and Gram-negative bacteria, fungi). The results are presented in Table 4. Differences were found between the samples and between the microorganisms. The best antimicrobial activity with the lowest MICs achieved was found in sample MP, followed by sample HP. Based on the MIC values obtained, the susceptibility could also be divided by groups of microorganisms, with the highest susceptibility found in Gram-positive bacteria, followed by Gram-negative bacteria and finally fungi. This is consistent with literature data on the susceptibility of individual groups of microorganisms to natural antimicrobial agents (Klančnik et al. 2010), as it was previously observed when methanolic extracts from *P. cembra* bark and needles and essential oil of needles and twigs were tested (Apetrei et al. 2011, 2013). Comparable antimicrobial activity was observed in supercritical CO_2 extracts and solvent extracts from *Picea abies* and *Pinus sylvestris* (Selivanova et al. 2020).

Table 4
Antimicrobial activity of commercially available steam-distilled cembran pine oil (sample R), CO_2 extracted cembran pine oil at high pressure (sample HP), and CO_2 extracted cembran pine oil at medium pressure (sample MP) expressed as minimal inhibitory concentration (MIC, mg/mL) and minimal bactericidal/fungicidal concentration (MBC/MFC, mg/mL) for Gram-positive bacteria, Gram-negative bacteria and fungi. Results were consistent in 3 independent experiments carried out in triplicate

Strain	Sample R		Sample HP		Sample MP	
	MIC (mg/mL)	MBC/MFC (mg/mL)	MIC (mg/mL)	MBC/MFC (mg/mL)	MIC (mg/mL)	MBC/MFC (mg/mL)
Gram-positive bacteria						
Staphylococcus aureus	nd	nd	1.25	1.25	0.625	0.625
Listeria innocua	nd	nd	1.25	1.25	0.625	0.625
Gram-negative bacteria						
Escherichia coli	nd	nd	nd	nd	nd	nd
Pseudomonas aeruginosa	nd	nd	2.5	2.5	1.25	1.25
Campylobacter jejuni	2.5	5.0	0.625	1.25	1.25	2.5
Fungi						
Candida albicans	2.5	5.0	2.5	5.0	2.5	2.5
Aspergillus flavus	nd	nd	nd	nd	nd	nd
nd – MIC could not be determined, > 10 mg/mL						

Samples MP and HP showed better antimicrobial activity compared to sample R, where MIC could only be determined for *C. jejuni* and *C. albicans*. The weak antimicrobial activity of essential oils of different *Pinus* spp., despite the presence of well-known individual antimicrobial components, was also indicated by the results of Kim et al. (2019), where they found, that the antimicrobial effect is better against Gram-negative bacteria compared to Gram-positive, which is also consistent with our results for sample R, where for bacteria we could only show activity against Gram-negative *C. jejuni*.

Studies on different *Pinus* spp. showed that oxygenated monoterpenes and sesquiterpenes are responsible for the antimicrobial activity (Kurti et al. 2019), including α- and β-pinene, 3-carene, limonene, γ-terpinene and α-terpinolene, which were found in all samples. Samples MP and HP showed better antimicrobial activity compared to sample R, where MIC could only be determined for *C. jejuni* and *C. albicans*. There are differences in the terpene composition of the three samples. Tricyclene, α-phellandrene and o-cymene were identified in the HP and MP samples but not sample R, where santene, β-phellandrene and camphor were found. The differences in terpene composition could contribute to the

differences in antimicrobial activity. However, these differences could also be due to different extraction procedures. To the best of our knowledge, there is no comparison of the antimicrobial activity of supercritical extract and essential oil of *Pinus* spp. However, supercritical CO_2 extraction has already been described to yield materials with higher antimicrobial activity from different plant materials compared to essential oil obtained by hydrodistillation (Kokoska et al. 2008, Korpinen et al. 2021, Mesomo et al. 2013). In addition, supercritical CO_2 extraction also yields lipophilic compounds (Conde et al. 2013), including resinic acids, which are known to have antimicrobial activity (Savluchinske-Feio et al. 2006) and could contribute to the overall antimicrobial effect.

The effect on bacterial adhesion and intercellular signalling were evaluated as additional antimicrobial properties. Adhesion to surfaces is an important microbial feature and the first stage of biofilm formation. Microbial adhesion is strongly associated with microbial intercellular signalling (Püning et al. 2021) and this can be influenced with natural compounds (Šimunović et al. 2020a) by modulating microbial intercellular signalling. To evaluate this effect of our samples, we selected the model bacterium *C. jejuni* NCTC 11168, which is a good model bacterium for studying the effect of natural compounds on intercellular signalling (Šimunović et al. 2020a).

The effect of cembran pine preparations in subinhibitory concentrations on the intercellular signalling of *C. jejuni* was indirectly verified by measuring the emitted bioluminescence of the biosensor strain *V. harveyi* MM30 (Ramić et al. 2022). This showed that all samples significantly reduced intercellular signalling (Fig. 1a), although the differences between the samples were not significant. Adhesion (Fig. 1b) was also modulated by the samples and a reduced number of *C. jejuni* was observed, with a significant decrease only in the MP sample. The trend for both parameters shows that the activity of the cembran pine materials increases from sample R to MP, which is also confirmed by the correlation test ($p = 0.028$). Thus, the MP sample was the most effective and statistically significantly reduced intercellular signalling and adhesion of *C. jejuni* by 72% and 90%, respectively. *P. cembra* has not been previously evaluated for these two antibacterial characteristics. However, *Pinus nigra* essential oil has been shown to downregulate genes involved in intercellular signalling of *P. aeruginosa* (Visan et al. 2024), and essential oil of *Pinus sylvestris* has been shown to have high capacity to disperse *Neisseria gonorrhoeae* biofilm (Jurado et al. 2023). Natural products are known for their potential in the regulation of the intercellular signalling and adhesion (Šimunović et al. 2020a). In *C. jejuni*, α-pinene has been known for its reduction of intercellular signalling (Šimunović et al. 2020b). α-Pinene was one of the most important compounds determined in our samples, with the highest values in sample R. However, the lowest antimicrobial potential was evaluated for sample R, further contributing to the likelihood that extraction itself has exceptional importance for the overall antimicrobial activity of *P. cembra* wood waste.

4. Conclusion

Supercritical CO_2 extraction of wood waste from *P. cembra* L. has been shown to be an effective method for extracting highly bioactive compounds compared to commercial essential oils. The study

demonstrates the importance of using sustainable and environmentally friendly methods to extract value-added products from biomass and to valorize *P. cembra* L. wood waste. Although the extraction pressure has no significant influence on the yield, it does affect the chemical composition and bioactivity of the extracts. Interestingly, the extracts obtained at medium and high pressure showed stronger antioxidant activity compared to the steam-distilled reference oil, although they had a lower TVOC content. The antioxidant and antimicrobial activity obtained with supercritical CO_2 extraction from *P. cembra* L. wood waste is good. The additionally evaluated property of modulating microbial intercellular signalling and adhesion further expands the possibilities of its use for the prevention and treatment of microbial surface colonisation.

Declarations

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Competing interests

The authors declare that they have no potential competing interests.

Author Contribution

Vanja Štolcer: conceptualization, formal analysis and investigation, writing – original draft preparation; Christoph Jocham: formal analysis and investigation, resources, writing – review and editing; Judith Sinic: formal analysis and investigation, resources, writing - review and editing; Valentina Malin: formal analysis and investigation, writing - review and editing; Dina Jug: formal analysis and investigation, writing – review and editing; Sonja Smole Možina: conceptualization, funding acquisition, writing - review and editing; Meta Sterniša: formal analysis and investigation, methodology, writing – original draft preparation.

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Figures

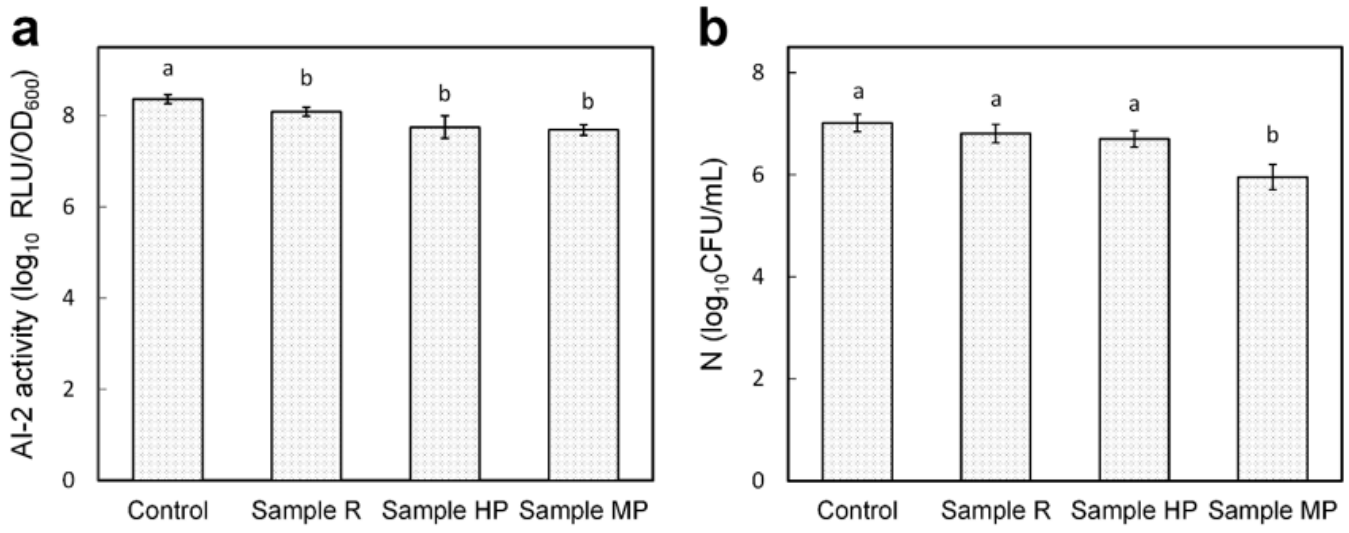

Figure 1

Effect of commercially available steam-distilled cembran pine oil (sample R), CO_2 extracted cembran pine oil at high pressure (sample HP), and CO_2 extracted cembran pine oil at medium pressure (sample MP) on *Campylobacter jejuni* NCTC11168 intercellular signalling (Fig. 1a) and adhesion (Fig. 1b). Different letter designates statistically significant difference ($P < 0.05$)

Figure 2

Pictures of the samples HP and MP prepared for GC-MS analysis