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Environmentally Friendly Extraction of Bioactive Compounds from Spruce and Pine Needles to Obtain Extracts with Antioxidant and Antimicrobial Activity

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***Abstract* – Timber harvesting of coniferous trees (the most abundant species in the Northern Hemisphere) leaves significant greenery side streams – coniferous needles and branches (pine, spruce and other species). Coniferous needles have been widely used in ethnomedicine, and recent research characterizes them as rich sources of biologically active compounds with immunostimulatory and anti-inflammation activities, as well as they are traditionally used to treat inflammation of the respiratory tract, colds and flu. However, new sustainable extraction methods should be proposed to obtain coniferous needle extracts with biomedical application potential. Classical extraction methods (stirring and heating) were used in this study and compared with ultrasonic treatment, and process duration and temperature optimisation were performed. During extraction, glycerol and propylene glycol, solvents widely used in the food and pharmaceutical industries, were used to improve the extracts' stability. Obtained coniferous needle and branch extracts were characterized by their antiradical activities (17–82 % Trolox eq.) and total polyphenol concentration (6–22 % gallic acid eq.) was analysed. The extracts were tested for antibacterial and antifungal activity, which demonstrates activities comparable to synthetic drugs with potentially lower side effects caused by drugs.**

***Keywords* – Antimicrobial activity; antioxidant activity; circular bioeconomy; green chemistry; green extraction; green solvents.**

Nomenclature		
MIC	Minimum Inhibitory Concentration	mg/g extract
MBC	Minimum Bactericidal Concentration	mg/g extract
MFC	Minimum Fungicidal Concentration	mg/g extract
DPPH	2,2-Diphenyl-1-picrylhydrazyl	g/100 g Trolox eq. = %
FRAP	Ferric Reducing Antioxidant Power	g/100 g Trolox eq. = %
CUPRAC	Cupric Ion Reducing Antioxidant Capacity	g/100 g Trolox eq. = %
TPC	Total Polyphenol Content	g/100 g gallic acid eq. = %

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1. INTRODUCTION

Forestry and forest product processing is a significant sector of economy in Latvia with growing significance, considering bioeconomy development of 4.2 billion EUR annually [1]. Coniferous tree processing (pine and spruce are dominant tree species of commercial significance) leaves vast amounts of coniferous needles as waste with unrealised application possibilities. Today, there is an increasing demand for the development of environmentally friendly extraction methods, which has led to significant research developments in the direction of green chemistry [2], [3]. The processing of green biomass from conifers belongs to the basic principles of circular bioeconomy, which uses forestry waste to create new products, increasing the potential for material utilisation [4]. This study focuses on extracting bioactive compounds from spruce (*Picea abies* L.) and pine (*Pinus sylvestris* L.) branches and needles using green solvents, such as glycerol and propylene glycol, via various extraction methods. These solvents have been increasingly explored for the extraction of bioactive compounds from other plant matrices – such as Thanaka bark (*Hesperethusa crenulata*) [5], Neem leaves (*Azadirachta indica*) [6], Common Centaury (*Centaurium erythraea Rafn*) [7], and Chinese Hackberry (*Celtis sinensis*) [8] – demonstrating their effectiveness and suitability as environmentally friendly alternatives to conventional organic solvents. However, their application to forestry biomass, particularly coniferous needles, remains largely unstudied.

The green biomass of conifers contains various biologically active compounds such as polyphenols, flavanols, procyanidins and terpenes [9]. However, most existing studies have focused on their extraction using traditional organic solvents, many of which pose environmental and health risks, and require energy-intensive removal during product purification. While the bioactivity of these compounds is recognized, there is limited research on alternative extraction strategies employing non-toxic, renewable solvents, such as glycerol or propylene glycol. Thus, a key knowledge gap remains in developing efficient and environmentally sustainable extraction methods that are both effective and scalable.

This study aims to address that gap by investigating green chemistry-based extraction techniques using safe, biodegradable solvents for the recovery of biologically active compounds from spruce and pine needles and branches. Various extraction methods, including conventional, elevated temperature, and ultrasound-assisted extraction, were evaluated. Extract activity was assessed using antiradical (DPPH) and antioxidative (CUPRAC, FRAP) assays. The overarching goal is to develop a guid that can enhance forestry industry sustainability by converting coniferous side-streams into high-value bioactive extracts for potential application in pharmaceutical, cosmetic, and food sectors. This approach offers both ecological and economic benefits by integrating waste valorisation with green processing technologies.

2. MATERIALS AND METHODS

2.1. Materials

Solvents: methanol (≥99.8 %), isopropanol (≥99.8 %), glycerol (99.0–101.0 %), propylene glycol (≥99.5 %) (Sigma-Aldrich, Germany), ethanol (95 %) (Z/S "Jaunpēternieki", Latvia), acetone (≥99.5 %), acetic acid (≥99.7 %), reagents and standards bought from Sigma-Aldrich: gallic acid, 4-dimethylaminocinnamaldehyde (DMAC) (≥99 %), epicatechin (≥97.0 %), sodium acetate (≥99.0 %), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (≥99.0 %), hydrochloric acid (36 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97 %),

sodium carbonate (≥99.5 %), Folin-Ciocalteu (FC) (2 M), 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) (≥99.8 %), aluminium chloride (98 %), potassium acetate (≥99.0 %), quercetin (≥95 %), copper chloride (99 %), ammonium acetate (≥97 %), neocuproine (≥99.0 %).

2.2. Plant Material

Spruce and pine greenery were sampled from August 2023 to January 2024 in the vicinity of Aglona train station (Lat. 56.166106, Long. 26.829501) and Celmraugciems (Lat. 56.794470, Long. 23.663303), respectively. After harvesting, spruce and pine greenery were transported to the laboratory and dried at 45 ℃ (48 h). After drying, the spruce and pine greenery was split manually into two parts: needles and branches. The resulting tree parts were ground in a laboratory mill (Fritsch, Germany), sieved through a 2 mm sieve and stored at −20 ℃.

2.3. Extraction of Greenery Extractives Using Conventional Method

For the extraction of branches and needles, 2.5 g of dry material were weighed in 100 mL glass bottles with caps and mixed with individual solvents 25 g of glycerol or propylene glycol, 25 mL of methanol or ethanol, or isopropanol, then placed in a shaker for 24 h, 180 rpm (Biosan, PSU 20i, Latvia). After extraction, all extracts were filtered through a cellulose filter paper. Glycerol and propylene glycol extracts were filtrated in an oven at 60 ℃ to lower solvent viscosity and stored at room temperature. Other alcohol-based extracts were filtrated, evaporated with a rotary evaporator (Heidolph, Heizbad Hei-VAP Advantage, Germany), dried at 40 ℃ under a stream of nitrogen and stored at 4 ℃. Extraction was performed in three replicates.

2.4. Extraction of Greenery Extractives Using Ultrasound Bath

For the extraction of branch and needle extractives, 2.5 g of material were weighed in 100 mL glass bottles with caps and mixed with individual solvents – 25 g of glycerol or propylene glycol, 25 mL of methanol or ethanol, or isopropanol, then placed in an ultrasonic bath for 20 min, 400 W (Bandelin, Germany). After extraction, all extracts were filtered through a cellulose filter paper. Glycerol and propylene glycol extracts were filtrated in a heating oven at 60 ℃ to lower solvent viscosity. Other alcohol-based extracts were filtrated at room temperature. After filtration, all material was collected from filter paper and reextracted. The extraction procedure was repeated sequentially three times, and the resulting extracts were combined. After extraction, the extracts were prepared and stored as described in Section 2.3. Extraction was performed in one replicate.

2.5. Extraction of Greenery Extractives by Heating

For the extraction of branches and needles, 5 g of material were weighed in 100 mL glass bottles with caps and mixed with 50 g of glycerol and propylene glycol, then placed in a heating oven (Gallenkamp, Plus II Oven, United Kingdom) for 6 h at 60 ℃. After extraction, all extracts were filtered through a cellulose filter paper. Glycerol and propylene glycol extracts were filtrated in a heating oven at 60 ℃ to lower solvent viscosity and stored at room temperature. Extraction was performed in two replicates.

2.6. Extraction with Heating, Method Optimisation

For optimization of branch and needle extraction, 1 g of material was weighed in 20 mL chromatography headspace bottles with cap and mixed with 10 mL of glycerol and propylene glycol, then placed in a heating oven at various times (2, 6 and 10 h) and temperatures (60, 80, 100 and 120 ℃). Glycerol and propylene glycol extracts were filtrated in a heating oven at 60 ℃ to lower solvent viscosity and stored at room temperature. Extraction was performed in duplicate.

2.7. Semi-Quantitative Method for the Determination of the Concentration of Extractives in Glycerol and Propylene Glycol Extracts

10 mg of separate ethanol extracts from spruce and pine needles and branches were dissolved in 25 mL of 96 % ethanol. Calibration curves were prepared in the concentration range of 1.25–40.0 µg/mL using the resulting solutions. These standard solutions were analysed using molecular absorption (UV) spectroscopy (Shimadzu, UV-1800, Japan) in the range of 200–320 nm. A peak signal intensity at 252 nm, which corresponds to molecules conjugated double bonds, was used as the base signal for the calibration curve.

Approximately 200 mg of separate glycerol and propylene glycol extract solutions were dissolved in 25 mL of 96 % ethanol, with additional dilutions performed as needed based on UV absorbance intensity.

2.8. Preparation of Samples and Standards for Microplate Reader Analysis

10 mg of methanol, ethanol and isopropanol dry extracts or 300 mg of glycerol and propylene glycol extracts were dissolved in 15 mL 25 % ethanol and filtered through a 0.45 µL PTFE syringe filter. The prepared test and standard solutions were stored overnight in a refrigerator (4 ℃). A 1 mg/mL standard solution of gallic acid was prepared for polyphenol, antiradical and antioxidant methods.

Spectrophotometric analyses were performed using a microplate reader (Tecan Infinite 200 m nano+, Austria). Samples for analysis were prepared in a 96-well polypropylene (PP) plate, which was then covered with a polystyrene lid. Mixing of reagents and samples was ensured by the stirring function of the apparatus when the sample was placed in the apparatus and after each incubation. For all methods used, absorbance measurements were carried out under optimum conditions: 15 measurements, type circle, size 3×3, border 1000 nm. All samples and standards were measured in 4 replicates.

The Limit of Detection (*LOD*) and Limit of Quantification (*LOQ*) were determined from the calibration curve based on the standard deviation of the *y*-intercept and the slope. The standard deviation of the intercept (*SD intercept*) was calculated as Eq. (1):

$$SD\,intercept = SE\sqrt{n}, \tag{1}$$

where *SE* is the standard error of the regression and *n* is the number of calibration points.

LOD Eq. (2) and *LOQ* Eq. (3) were calculated using:

$$LOD = 3\frac{SD\,intercept}{S}, \tag{2}$$

$$LOQ = 10\frac{SD\,intercept}{S}, \tag{3}$$

where *S* is the slope of the calibration curve.

2.9. Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP working solution was prepared by mixing 0.3 M sodium acetate (pH 3.6), 10 mM TPTZ (40 mM HCl) and 20 mM $FeCl_2$ solution in a 10:1:1 ratio. The calibration solution was prepared by dissolving Trolox in 96 % ethanol to a concentration of 0.230 mg/mL. A calibration graph was prepared in the 4.16–45.7 µg/mL range, *LOD* (2.62 µg/mL), *LOQ* (8.72 µg/mL) and $R^2 = 0.9996$.

50 µL of sample, blank or standard solution was transferred to a 96-well plate, 150 µL of FRAP working solution was added and incubated for 30 min at 37.0 ℃. Absorbance measurements were made at a wavelength of 593 nm [10], [11].

2.10. Polyphenol Determination with Folin-Ciocalteu Method

The polyphenol (PF) working solution was prepared by mixing FC reagent with deionized water in a ratio of 1:9. For spectrophotometric analysis prepare 7.5 % Na_2CO_3 solution. The calibration solution was prepared by dissolving gallic acid in 96 % ethanol to a concentration of 0.500 mg/mL. A calibration graph was prepared in the 13.5–203 µg/mL range, *LOD* (13.9 µg/mL), *LOQ* (46.2 µg/mL) and $R^2 = 0.9993$.

50 µL of sample, blank or standard solution was transferred to a 96-well plate, 85 µL of FC working solution was added, incubated for 20 min, added 65 µL of 7.5 % Na_2CO_3 and incubated for 9 min. Absorbance measurements were made at a wavelength of 765 nm [10], [12].

2.11. Antiradical DPPH Method

The DPPH working solution was prepared by dissolving DPPH in 80 % methanol to a concentration of 120 µg/mL. The calibration solution was prepared by dissolving Trolox in 96 % ethanol to a concentration of 0.230 mg/mL. A calibration graph was prepared in the 2.15–109 µg/mL range, *LOD* (0.00271 µg/mL), *LOQ* (0.0903 µg/mL) and $R^2 = 0.99998$.

50 µL of sample, blank or standard solution was transferred to a 96-well plate and 150 µL of DPPH working solution was added. Absorbance measurements were made after 30 min at a wavelength of 517 nm [10], [11].

2.12. Flavonoid Determination

The flavonoid working solution was prepared by mixing 96 % ethanol, 10 % $AlCl_3$ and 1 M potassium acetate solution in a 15.78:2.63:2.63 mL ratio. The calibration solution was prepared by dissolving quercetin in 96 % ethanol to a concentration of 0.200 mg/mL. A calibration graph was prepared in the 19.0–171 µg/mL range, *LOD* (9.25 µg/mL), *LOQ* (30.8 µg/mL) and $R^2 = 0.9996$.

40 µL of sample, blank or standard solution was transferred to a 96-well plate and 160 µL of flavonoid working solution was added. Absorbance measurements were made every minute for 30 min at a wavelength of 415 nm [13], [14].

2.13. Procyanidin Determination

A 4.5 % HCl solution in 70 % ethanol was prepared to prepare the DMAC reagent. A working solution of procyanidins was prepared by dissolving 1 mg/mL DMAC in acidified ethanol. The calibration solution was prepared by dissolving epicatechin in 96 % ethanol to a 0.2 mg/mL concentration. A calibration graph was prepared in the 6.88–41.2 µg/mL range, *LOD* (4.41 µg/mL), *LOQ* (14.6 µg/mL) and $R^2 = 0.998$.

50 µL of sample, blank or standard solution was transferred to a 96-well plate and 150 µL of procyanidin working solution was added. Absorbance measurements were made after 30 min at a wavelength of 640 nm [15], [16].

2.14. Copper (II) Reduction Assay

The CUPRAC working solution was prepared by mixing 10 mM $CuCl_2$, 7.5 mM neocuproine (96 % ethanol) and 1 M ammonium acetate solution (pH 7.0) in a ratio of 5:5:6. The calibration solution was prepared by dissolving Trolox in 96 % ethanol to a concentration of 0.230 mg/mL. A calibration graph was prepared in the 6.40–230 µg/mL range, *LOD* (0.00734 µg/mL), *LOQ* (0.0245 µg/mL) and $R^2 = 0.99996$.

50 µL of sample, blank or standard solution was transferred to a 96-well plate and 150 µL of CUPRAC working solution was added. Absorbance measurements were made after 60 min at a wavelength of 450 nm [10], [11].

2.15. Microbial Cultivation and Antimicrobial Activity

Antimicrobial activity was assessed by a two-fold serial broth microdilution method against Gram-negative bacteria *Escherichia coli* MSCL 332 and *Salmonella enterica* subsp. *enterica serovar Typhimurium* MSCL 588, Gram-positive bacteria *Staphylococcus aureus* subsp. *aureus* MSCL 334, methicillin-resistant *Staphylococcus aureus* subsp. *aureus* (MRSA) MSCL 993, *Staphylococcus epidermidis* MSCL 333 and *Listeria monocytogenes* MSCL 760, and yeast *Candida albicans* MSCL 378.

Mueller-Hinton broth (Biolife, Italy) was used to cultivate bacteria, and Malt extract broth (Millipore, Germany) was used to cultivate *C. albicans*. The inoculum of microorganisms was prepared in sterile water with a density of 0.08–0.10 at A625 and diluted 100-fold in appropriate broth. The 96-well plates were then incubated at 37 °C with bacteria for 24 hours or with *C. albicans* for 48 hours. The MIC was determined to be the lowest concentration of the studied material, which showed no visible growth. From the wells without microbial growth, 4 µL of the liquid was plated on appropriate solidified media to determine MBC and MFC. Control samples included bacteria alone and bacteria treated with glycerol and propylene glycol, respectively, without plant extracts, to assess the baseline and solvent effects.

3. Results and Discussion

3.1. Extraction Efficiency

Extraction of pine and spruce greenery has been conducted using a variety of solvents and methods. Conifer biological active compounds have been extracted using mainly alcohols like methanol (MeOH) and ethanol (EtOH) [17], [18], but there is no information about plant material extraction using diols and triols like glycerol (Gly) or propylene glycol (PG). The need for environmentally friendly solvents that demand time and cost-efficient methods is growing, so it is essential to find new solvents prospective for industrial applications. Glycerol and propylene glycol is used in pharmaceutical, food, beverage, cosmetical and personal care products as sweeteners, preservatives and carriers for flavourings, colours and biologically active compounds [19]–[22].

Based on the literature study on solvents suitable for use in biologically active compound extraction, three classical solvents were chosen: MeOH, EtOH, and isopropanol (i-PrOH), as well as glycerol and propylene glycol, and the yields were calculated in grams of extractant

per 100 g of dry biomass (%). Comparing the results, a trend was observed where the yield decreased as the number of carbons in the simple alcohols increased. The conventional stirring method showed surprisingly high results compared with ultrasound-assisted extraction. Spruce material tends to have higher (15.43–27.28 %) yield than pine material (16.28–22.11 %). The spruce samples' yield was higher than the pine samples. For the simple alcohols, the ultrasound assisted extraction yield for spruce samples was observed to be 18.08–27.60 % and for pine samples 16.97–26.28 %. The average yield of the spruce samples using ultrasonic extraction was 1.82 % higher than those of the pine samples, however, this difference was not statistically significant ($p > 0.05$) (see Table 1).

TABLE 1. EXTRACTION YIELD (%) FOR EXTRACTION METHODS AND SOLVENTS

Solvent / biomass	**Spruce**			**Pine**		
	US[3]	**Conventional**	**Heating**	**US**	**Conventional**	**Heating**
MeOH	27.60	27.28		26.28	22.11	
EtOH	23.48	18.22		20.45	17.83	
i-PrOH	18.08	15.43		16.97	16.28	
Gly[1]	5.10	3.14	42.47	5.03	2.69	42.12
PG[2]	30.17	8.09	50.10	21.77	7.17	60.01

[1]Gly – glycerol, [2]PG – propylene glycol, [3]US – ultrasound

Glycerol and propylene glycol are well-known solvents for maintaining the stability of bioactive compounds over a longer period, as they provide an inert environment that prevents microbiological growth. These solvents are chemically inert compared to methanol, ethanol and isopropanol, which ensures the fidelity of the compounds obtained. The glycerol and propylene glycol extracts obtained can be used immediately after extraction, as they do not require additional processing steps, such as solvent removal, which would reduce the energy cost needed to produce the product. Based on this information, several food, pharmaceutical and cosmetic companies use glycerol and propylene glycol as fillers in their products [23]–[25].

Comparing glycerol and propylene glycol extracts using the conventional extraction method, they produce a lower yield (2.69–8.09 %) than the simple alcohols used. One of the differences between these solvents and simple alcohols is their viscosity, which reduces the extraction efficiency. As seen with other extraction methods such as US and heating, even a small increase in temperature to the extraction solutions can significantly increase the extraction yield. During US extraction, mechanical work is carried out, contributing to cavitation and heating of the solution by about 5 °C during the 20 min extraction. These conditions can increase the extraction efficiency by a factor of 1.6–1.9 for glycerol (5.03–5.10 %) and 3.0–3.7 for propylene glycol (21.77–30.17 %) in a short period of time. This ensures a decrease in the viscosity of the solvents used, which allows more efficient penetration of the solvent into the sample material, thus increasing the extraction efficiency. The heating method resulted in 13.5–15.6 times higher yield than the conventional extraction method for glycerol (42.12–42.47 %) and 6.1–8.4 times for propylene glycol (50.10–60.01 %).

3.2. Coniferous Needle/Greenery Extraction Yield Depending on Solvents Used in the Extraction

During the experiments, many parameters were obtained to characterise the extracts. Statistical analysis of the data provides an opportunity to assess correlations between the different parameters of the samples. Aggregating similar data or accepting a single parameter value facilitates interpretation of complex data, allowing only the most critical parameters to be used.

The extracts were analysed using six spectrophotometric methods to identify various substance groups, including total polyphenols, procyanidins, and flavanols. The antiradical and antioxidative capacities of the extracts were assessed using the FRAP, DPPH, and CUPRAC assays. Pearson's correlation coefficient *R* (as depicted in Fig. 1) was employed to evaluate the relationships between these methods across all extracts. The strongest correlation (0.925***) was observed between the CUPRAC and FRAP antioxidant assays, likely due to their shared ability to prevent metal-catalysed oxidative processes in biological systems. Given this high correlation, the CUPRAC results were prioritised for further evaluation. Similarly, strong correlations were found between the DPPH assay and the other antioxidant methods: CUPRAC (0.760***) and FRAP (0.697***).

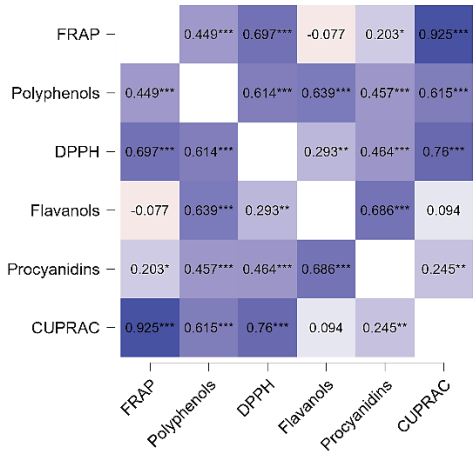

Fig. 1. Pearson's *R* correlation for the extracts obtained, analysing substance groups, antioxidant and antiradical properties; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Blue indicates a positive correlation between parameters, white indicates no correlation between parameters.

When comparing the polyphenolic subclasses of flavanols and procyanidins in the extracts, weaker correlations were observed with the antiradical and oxidative methods. For flavanols, no significant correlation was found with the CUPRAC (0.094) and FRAP (−0.077) assays, though a moderate correlation was noted with the DPPH assay (0.293***). For procyanidins, low correlations were observed with CUPRAC (0.245**) and FRAP (0.203*), but a stronger correlation was evident with DPPH (0.464***). Both procyanidins (0.457***) and flavanols (0.639***) exhibited strong correlations with total polyphenols. Therefore, total polyphenol content was used for further statistical analyses (see Fig. 1).

3.3. Extraction Using Conventional Extraction Method

Conventional extraction is one of the most primitive and ancient methods. The process does not require a large amount of energy but is time-consuming. Five solvents were used to obtain biologically active extracts from spruce and pine needles and branches containing polyphenols with antiradical and antioxidant properties.

Differences in polyphenol concentrations were observed between the extracts obtained depending on the antiradical (DPPH) and antioxidant (CUPRAC) activity. The highest of these parameters are observed in all spruce needle extracts, and they decrease in a particular order: spruce branches, pine needles, and pine branches. The concentrations of polyphenols (22.16–24.71 %), DPPH (12.70–15.70 %) and CUPRAC (46.19–61.63 %) in spruce needle alcohol extracts decrease with increasing number of carbon atoms in the alcohol molecule. The glycerol extract is comparable to the simple alcohol extracts in both polyphenolic and bioactivity results. In contrast, the concentration of polyphenols (7.49 %) in PG extract is low, but the results of antiradical (17.25 %) and antioxidant (37.73 %) activities are equally high in other pine needle extracts (see Figs. 2–3).

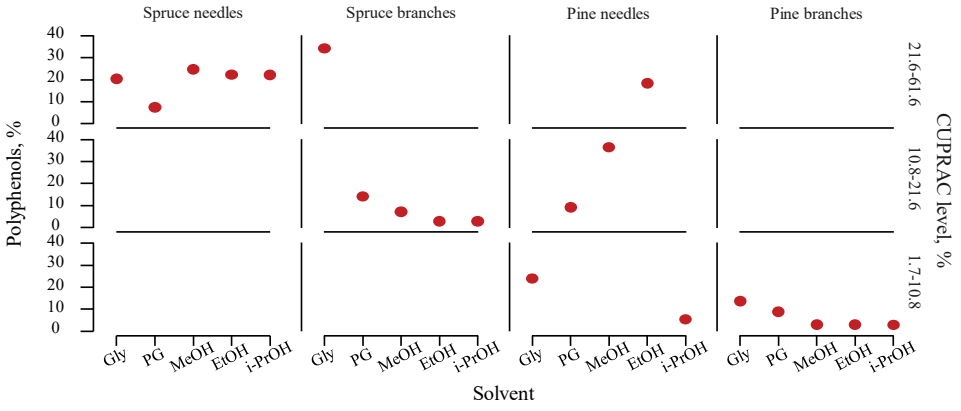

Fig. 2. The concentration of polyphenols in the spruce and pine extracts obtained by a conventional method, divided into three CUPRAC antioxidant activity groups; low (1.7–10.8 %), medium (10.8–21.6 %), and high (21.6–61.6 %) activity levels.

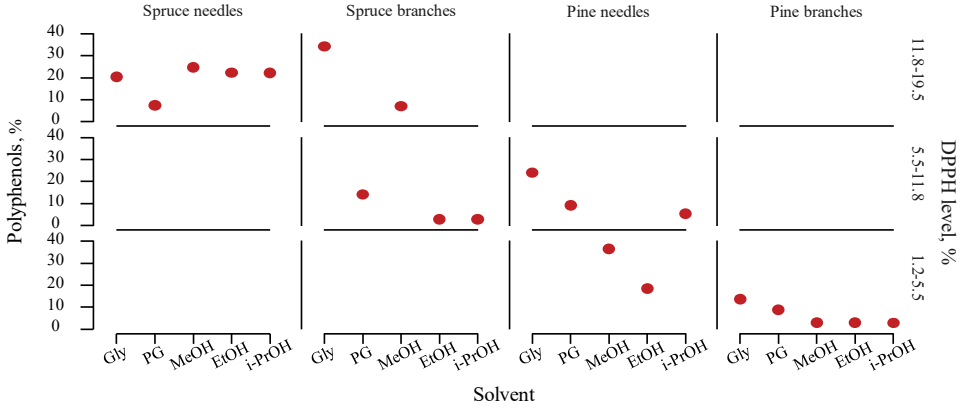

Fig. 3. The concentration of polyphenols in the spruce and pine extracts obtained by a conventional method, divided into three DPPH antiradical activity groups; low (1.2–5.5 %), medium (5.5–11.8 %), and high (11.8–19.5 %) activity levels.

3.4. Extraction Using Ultrasound

Ultrasound-assisted extraction is one of the advanced methods, requiring a short processing time and being suitable for the extraction of thermolabile compounds. Five solvents were used to obtain a potentially bioactive fraction, with spruce, pine needles, and branches as the extraction material.

Ultrasound-assisted extraction tends to resemble the mixing method based on trends in polyphenol content, antiradical, and antioxidant activity, with higher values observed in spruce needles, spruce branches, pine needles, and pine branches, respectively. Glycerol extracts from all materials exhibited the highest polyphenol and CUPRAC values compared to the other solvents used. Propylene glycol was the second most effective solvent, but notable variations were observed depending on the material, with needle extracts showing lower values than branch extracts. Conventional solvents (MeOH, EtOH, and i-PrOH) yielded extracts with higher polyphenol and CUPRAC content from spruce needles compared to extracts from other materials (see Fig. 4).

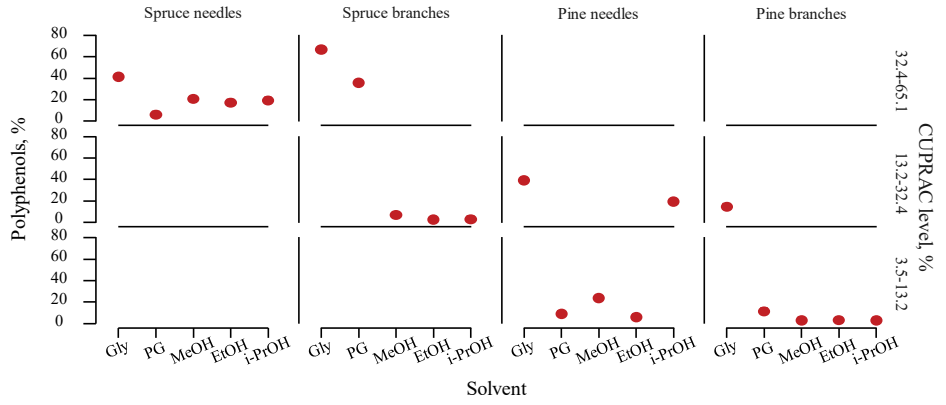

Fig. 4. The concentration of polyphenols in the spruce and pine extracts obtained using ultrasound-assisted method, divided into three CUPRAC antiradical activity groups; low (3.5–13.2 %), medium (13.2–32.4 %), and high (32.4–65.1 %) activity levels.

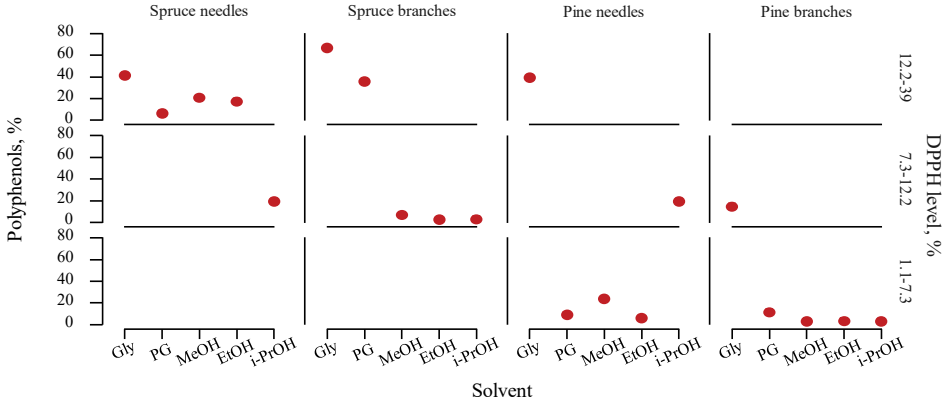

Fig. 5. The concentration of polyphenols in the spruce and pine extracts obtained using ultrasound-assisted method, divided into three DPPH antiradical activity groups; low (1.1–7.3 %), medium (7.3–12.2 %), and high (12.2–39.0 %) activity levels.

As illustrated in Fig. 5, for DPPH the trend is the same as in the CUPRAC graph, with the only differences observed in the isopropanol extract of spruce needles and the glycerol extract of pine needles. Comparing CUPRAC and DPPH levels, the antiradical activity of the isopropanol extract is at a moderate level relative to its antioxidant capacity, whereas the antiradical activity of the glycerol extract from pine needles is higher compared to its antioxidant activity.

3.5. Extraction Using Heating

Extraction by heat is one of the oldest methods used to extract various biologically active compounds from herbs used in folk medicine. Decoctions of spruce and pine are still used today for colds and respiratory tract infections. At home, decoctions are typically made in aqueous media; however, improper storage and contamination can promote bacterial growth, which can be avoided by using glycerol or propylene glycol for extraction.

Spruce and pine needles and branches were heated in Gly and PG solutions for 6 h at 60 °C in three replicates during the experiment. After extraction, the solutions were filtered at elevated temperature (60 °C) to maintain the low viscosity of the solvents, which increases the filtration rate. Fig. 6 shows the concentrations of polyphenols, CUPRAC and DPPH activity found in extracts depending on the biomass material and solvent used. There are noticeable differences between the solvent and the material used. In all cases, the concentrations of total polyphenols, antiradical activity and antioxidative power are higher in glycerol solutions. Extracts of coniferous materials yield similar results for spruce and pine, whereas extracts from pine branches yield approximately twice the results.

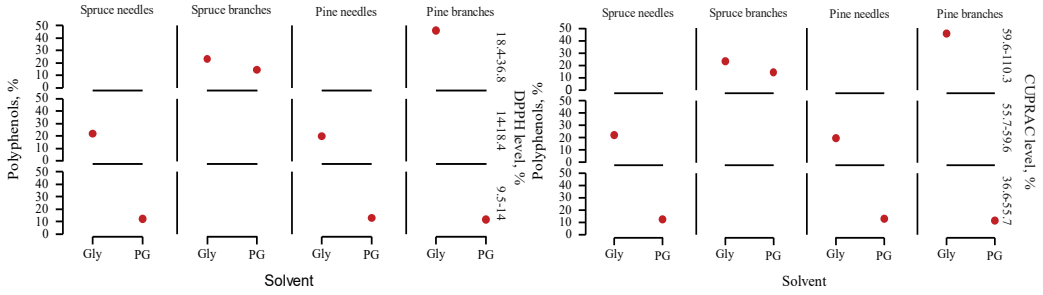

Fig. 6. The concentration of polyphenols in the spruce and pine extracts obtained via heating method, divided into three CUPRAC antioxidant activity groups; low (36.6–55.7 %), medium (55.7–59.6 %), and high (59.6–110.3 %) and three DPPH antiradical activity groups; low (9.5–14.0 %), medium (14.0–18.4 %), and high (18.4–36.8 %) activity levels.

Previously, conventional (Fig. 2 and Fig. 3) and US extraction (Fig. 4 and Fig. 5) showed higher concentrations of polyphenols and activities in conifer needle extracts compared to branch extracts. Still, the trend is reversed, with branch extracts having higher activity than needle extracts. Glycerol is more polar than propylene glycol, which enhances its ability to dissolve polyphenols, particularly those with higher hydrophilicity. Propylene glycol may be less effective than glycerol in breaking down the plant matrix and releasing bound phenolic compounds, as glycerol more effectively disrupts lignocellulose, facilitating the release of phenolic molecules. This could be due to the presence of tannins in the bark. Tannins are highly polymerised molecules based on different flavonoid basic structures. At elevated temperatures and in the presence of water, these basic structures are hydrolysed to form smaller polymerisation flavonoid structures, thus increasing the concentration of free polyphenols and activities.

3.6. Heating Extraction Method Optimisation

As described above, the conventional solvents (MeOH, EtOH, and i-PrOH) give lower extraction yields than Gly and PG. From an industrial perspective, conventional extraction solvents increase production costs, as evaporation and drying techniques are required after extraction to achieve solvent-free final products.

Compared to the conventional and US methods, the heating method gives higher total polyphenolic, antiradical scavenging, and antioxidative power results. Additionally, the method is robust enough for scale-up, as it does not require complex engineering solutions. Only Gly and PG were used to optimise the heating method, which was studied at different temperatures (60, 80, 100, and 120 °C) and times (2, 6, and 10 h) at 3 replicates.

Fig. 7 shows the results of the optimisation of the heating method, where the different extraction materials (spruce, pine, branches, needles) were not separated out of schedule. The results obtained confirmed the study hypothesis of a decrease in polyphenol concentration and radical scavenging activity with increasing temperature. The previously observed yields obtained using Gly and PG have the same trends as previously observed, with Gly being the optimal solvent for extraction of biologically active compounds from the materials used. Experimental results indicate that the concentration of total polyphenols at 60 °C varies with the duration of heating, with polyphenol concentrations ranging from 6–22 % in the 2 h and 6 h intervals and 20–34 % in the 10 h period. A similar situation is observed in the antioxidant activity measurements, where the CUPRAC concentration varies between 17 % and 61 % at 2 h and 6 h, while at 10 h the results increase to 43–82 %. At higher temperatures (80–120 °C), a decrease in polyphenol and antioxidant concentrations is observed over the whole interval, possibly due to polyphenol degradation.

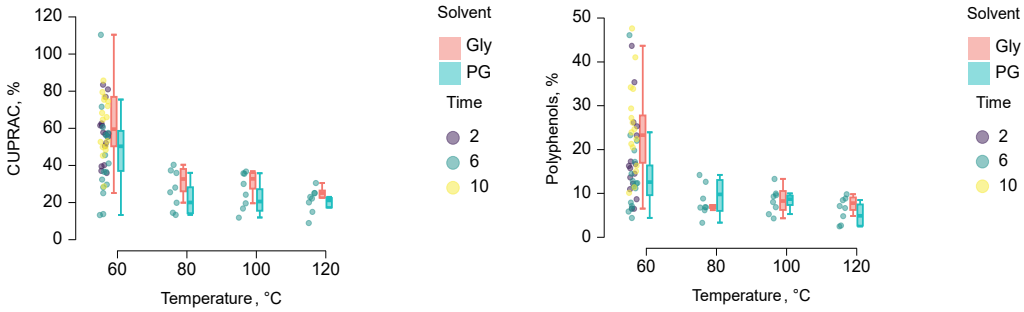

Fig. 7. Optimization of heating methods for polyphenol extraction in propylene glycol (PG) and glycerol (Gly) solvents: influence of temperature and time for all spruce and pine extracts.

3.7. Antimicrobial Activity of Extracts

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) define the lowest concentration at which the resulting extract kills ≥99.9 % of the bacteria and fungi used. The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration at which an extract inhibits the growth of microorganisms. MBC and MFC are usually higher than MIC; higher concentrations are required to kill microorganisms [26].

The MBC and MFC values in the experiment differ from the MIC values, which provides additional information on the obtained pine and spruce extracts. The glycerol and propylene glycol extracts used in the experiment were obtained by the heating method (6 h, 60 °C).

Glycerol and propylene glycol extracts have a wide range of applications, as they can act against both Gram-positive and Gram-negative bacteria, as well as fungi (Table 2).

Pine branch extracts exhibit the most potent antimicrobial activity across tested microorganisms. The lowest MBC and MIC values were observed for *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), with values as low as 0.07–0.33 mg/mL and 0.16–0.29 mg/mL, respectively, demonstrating a strong inhibitory and bactericidal effect. Similarly, spruce branch extracts demonstrated high effectiveness, particularly against Gram-positive bacteria, with MIC and MBC values ranging from 0.18 to 1.43 mg/mL.

On the other hand, glycerol-based extracts generally exhibited weaker antimicrobial activity compared to PG extracts. However, pine branch extracts (Gly) still showed notable effectiveness, with MIC and MBC values as low as 0.16 mg/mL for MRSA. This suggests that pine branches contain potent antimicrobial compounds [27], [28] that remain effective even in a more polar solvent, such as glycerol. Spruce branches (Gly) were also relatively active, with MIC values for *S. aureus* and MRSA reaching 1.43 mg/mL, although slightly weaker than those of PG extracts.

The extracts were more effective against Gram-positive bacteria, especially *S. aureus*, MRSA, and *Listeria monocytogenes*, likely due to their simpler cell wall structure, which is more susceptible to bioactive compounds from conifer extracts. Pine and spruce branch extracts in PG had the lowest MIC values (≤0.46 mg/mL) for these bacteria, confirming their effective antibacterial properties.

In contrast, Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica*) were generally more resistant, requiring higher MIC and MBC values (≥0.94 mg/mL). This is expected, as Gram-negative bacteria have an outer membrane that limits the penetration of antimicrobial compounds. However, pine branch extracts (PG and Gly) still demonstrated moderate activity, with MIC values as low as 1.15 mg/mL for *E. coli*.

Glycerol and propylene glycol extracts have antibacterial and antifungal properties. Propylene glycol extracts exhibit varying antimicrobial properties, where one microbe can be effectively countered while another requires 100–200 times higher minimum bactericidal concentration (MBC) concentrations. Glycerol extracts have similar MBC concentrations between microbes, meaning that the resulting extract is equally effective against different microbes.

Among the tested extracts, those from pine and spruce branches in PG exhibited the highest antimicrobial activity, particularly against Gram-positive bacteria. The most substantial extract was pine branches (PG), followed by spruce branches (PG), both of which had consistently low MIC and MBC values. Glycerol-based extracts were generally weaker but still retained moderate activity, particularly in the case of pine branch extracts. The least effective extracts were spruce needles (Gly), which had higher MIC values than most microorganisms. These results highlight the potential of pine and spruce branch extracts, especially in PG, as natural antimicrobial agents with promising applications in pharmaceuticals and biotechnology.

Against Gram-positive bacteria such as *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), pine branch propylene glycol extracts exhibited MIC values ranging from 70 to 290 µg/mL. In comparison, vancomycin, a last-resort antibiotic for Gram-positive infections, typically has MIC values of 0.5–2 µg/mL, with MRSA strains exhibiting MICs of 0.5–4 µg/mL [29], [30]. Penicillin, particularly against resistant strains, has MIC values of greater than 128 µg/mL, making pine extracts comparable in some cases, but generally less potent than conventional antibiotics.

For Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica*, pine branch propylene glycol extracts exhibited MICs of 940–1430 µg/mL, which are significantly higher than those of standard antibiotics. Ciprofloxacin, a fluoroquinolone used against *E. coli* and *S. enterica*, typically has MIC values ranging from 0.015 to 0.5 µg/mL [31], [32]. Similarly, gentamicin, an aminoglycoside antibiotic, has MICs between 64–256 µg/mL against *E. coli* [33]. The higher MICs observed for pine extracts suggest reduced efficacy against Gram-negative bacteria, likely due to the protective outer membrane acting as a barrier to antimicrobial compounds [34], [35].

Against *Candida albicans*, MICs for pine branch propylene glycol extracts ranged from 460 to 1290 µg/mL. In contrast, fluconazole, a widely used azole antifungal compound, typically has MIC values between 0.25 and 4 µg/mL against *C. albicans* [36]. Amphotericin B, a polyene antifungal, exhibits MIC values between 0.03 and 1 µg/mL [37]. These findings indicate that while pine extracts have antifungal properties, their activity is significantly lower than that of conventional antifungals – as seen in Table 2.

TABLE 2. ANTIMICROBIAL ACTIVITY OF GREEN SOLVENT EXTRACTS FROM CONIFER NEEDLES AND BRANCHES. CONCENTRATIONS EXPRESSED IN MG/ML

		Gram-positive								**Gram-negative**				**Fungus**	
		MSCL 333 (*Staphylococcus epidermidis*)		MSCL 334 (*Staphylococcus aureus*)		MSCL 993 (methicillin-resistant *Staphylococcus aureus* subsp. *aureus*)		MSCL 760 (*Listeria monocytogenes*)		MSCL 332 (*Escherichia coli*)		MSCL 588 (*Salmonella enterica* subsp. *enterica* serovar *Typhimurium*)		MSCL 378 (*Candida albicans*)	
Material	Solvent	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC
Pine needles	PG	1.84	1.84	0.23	0.23	0.90	0.90	3.65	7.30	3.65	3.65	3.65	3.65	0.90	0.90
Pine branches	PG	0.58	0.58	0.07	0.07	0.29	0.29	1.15	2.31	1.15	1.15	1.15	1.15	0.58	0.58
Spruce needles	PG	3.60	3.60	0.46	0.89	1.82	1.82	3.60	7.21	3.60	3.60	3.60	3.60	0.46	1.82
Spruce branches	PG	1.86	3.71	0.24	0.46	0.46	0.46	1.86	3.71	1.86	0.94	0.94	0.94	1.82	0.46
Pine needles	Gly	0.47	0.47	0.96	1.90	3.80	3.80	1.90	3.80	1.90	3.80	3.80	3.80	3.80	3.80
Pine branches	Gly	0.33	0.65	0.16	0.33	0.16	0.33	0.65	1.29	0.65	1.29	1.29	1.29	1.43	1.29
Spruce needles	Gly	0.31	2.47	0.62	1.24	2.47	2.47	1.24	2.47	1.24	2.47	2.47	2.47	1.29	2.47
Spruce branches	Gly	0.18	1.43	0.36	1.43	1.43	1.43	0.71	1.43	0.71	1.43	1.43	1.43	2.47	1.43

Blue indicates the necessity for low concentrations of extractants to affect microbial growth, white indicates the necessity for medium concentrations, red indicates the necessity for high concentrations.

4. CONCLUSIONS

This study demonstrates that coniferous greenery, serves as a rich source of bioactive compounds with significant antioxidant and antimicrobial activity. The application of green solvents, such as glycerol and propylene glycol, in combination with various extraction techniques, successfully produced biologically active extracts while maintaining environmentally sustainable practices.

Among the extraction methods studied, ultrasound-assisted and heating extraction showed enhanced efficiency compared to conventional method. The heating method, in particular, significantly improved polyphenol recovery and antioxidant activity, suggesting its potential for large-scale applications in bioactive compound production. Additionally, glycerol extracts exhibited higher total polyphenol content and antioxidant activity, while propylene glycol extracts demonstrated superior antimicrobial efficacy, particularly against Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*.

These findings highlight the potential of coniferous needle extracts as natural antioxidants and antimicrobials, offering promising applications in pharmaceuticals, cosmetics, and food industries. Further research should focus on optimizing extraction conditions and evaluating the stability and bioavailability of these extracts in real-world applications.

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