

Identification of Antimicrobial Compounds in Different Hydrophilic Larch Bark Extracts

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Larch bark is an undervalued material, but it has unique and valuable characteristics and compounds. The objective of this study was to extract and characterize molecular compounds of bark materials from different larch trees and to test these for their antimicrobial properties. The extractions were performed using methanol or water. The obtained extracts were analysed by gas chromatography coupled to mass spectrometry (GC-MS). Antimicrobial properties also were determined using different microbial strains, for example, *Staphylococcus aureus*. The GC-MS analysis showed that long chain alcohols, fatty acids, and polyphenols were present in the extracts. According to the results of the agar diffusion tests, only the methanol extract of larch bark had an inhibitory effect on the growth of *Staphylococcus aureus*. Two compounds of flavonoids and stilbenoids were shown to affect the microbial activity of the larch bark. Therefore, larch bark can be used for the extraction of compounds with specific anti-microbial properties.

Keywords: Agar-diffusion test; Gas chromatography – mass spectrometry; Solid-liquid extraction; Larch bark

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INTRODUCTION

Large amounts of bark are accumulated during timber production in the sawmilling industry. Currently, this material is used for only a few applications, such as industrial fuel, soil amendment, and ground cover. However, bark contains unique compounds that are not present in or that vary from those in wood (Dönmez *et al.* 2016; Pásztor *et al.* 2016). During the normal timber production, bark is considered as a by-product and is currently often used as combustible material for heating. During combustion, valuable components of the bark get lost.

The bark quantities and qualities vary between tree species and depend on the age and location of the tree (Pásztor *et al.* 2016). For example, the bark of larch comprises up to 16 to 24% of the total stem volume of a tree (Wagenführ 2007). Two possible applications of bark have recently been investigated: the manufacture of innovative bark materials and products; and the use of bark as a source of chemical compounds (Feng *et al.* 2013; Pásztor *et al.* 2016). For example, bark materials can be processed and used to develop insulation materials (Kain *et al.* 2013). Moreover, tannins from bark can be

extracted for their property as an adhesive and as a substitute for crude oil products (König and Roffael 2003).

Certain bark materials contain large amounts of phenolic substances, such as lignans, flavonoids, and stilbenes (Shibutani *et al.* 2004; Pietarinen *et al.* 2006; Mulat *et al.* 2014). Therefore, bark is a potentially valuable source of natural antioxidants that can be used for further applications. Condensed tannins play a role in the preservation of wood (Laks *et al.* 1988). The amount of tannin quantities varies between the tree species, whereby larch bark has the highest amount of condensed tannins when compared to other European softwood species (Bianchi *et al.* 2015). Ravber *et al.* (2015) concluded that the yield of phenolic compounds in larch bark extract amounts to 11% of dry material using pressurized hot water extraction (PHWE). However, only single phenolic compounds (*e.g.* gallic acid) and three flavonoids (vanillin, taxifolin, and quercetin) could be determined in these extracts (Ravber *et al.* 2015). Therefore, further analysis and extraction procedures are needed to understand the properties of larch bark and their possible use for novel applications.

The antimicrobial properties of several wood species, including larch, were previously tested by Laireiter *et al.* (2014) and Salem *et al.* (2016). The obtained results demonstrated that solid larch bark discs and methanol extracts affect the growth of specific microbes (Laireiter *et al.* 2014; Salem *et al.* 2016). Thus far, the characterization of the molecular compounds of the bark substances has not been performed yet and therefore, responsible compounds, which may cause these antimicrobial effects, were not identified. The presence of defined anti-microbial properties of bark extracts would allow for applications in the pharmaceutical and cosmetic industry. This would allow a reduction of waste products and introduce a new holistic value chain for the forest products industry.

Based on the existing results, the current study deals with the chemical analysis of two different larch bark extracts and the identification of possible antimicrobial activities and compounds. The molecular contents of these extracts were investigated by using gas chromatography (GC) and mass spectrometry (MS).

EXPERIMENTAL

Wood Material

For cryogenic preparation, the bark from various European larch (*Larix decidua* [Mill.]) trees was collected from a larch sawmill and ground with a cutting mill (Retsch, Haan, Germany) using solid carbon dioxide to pass a mesh of 500 µm. The powder was then dried for one week at 50 °C.

Extracting Agents

Solvents used for isolation of extractives were methanol and water. Methanol has previously been used to determine antimicrobial effects of wood compounds in several studies and is seen as the gold-standard for extraction (*e.g.*, Laireiter *et al.* 2014). Water was chosen as a blank and because it does not interfere with the bacterial growth as such. Both compounds are hydrophilic, ensuring the potential to dissolve polyphenolic compounds. Methanol (99.9%) and water were both from VWR (Padnor, PA, USA) and of a HPLC grade.

Solid-Liquid Extraction

An amount of 1 g of wood powder was weighed, placed in a 15 mL CELLSTAR® Polypropylene Tube (Greiner Bio-One, Kremsmünster, Austria) and covered with 10 mL of extracting agent, either water or methanol. The extraction process was performed for 24 h at room temperature (22 to 23 °C). After incubation, the liquid fraction was pre-filtered using filter paper. Sterilisation of the aqueous liquid was performed by filtration using a 0.22 µm Minisart® NML filter (Sartorius AG, Göttingen, Austria) to remove small particles, including bacteria and other micro-organisms.

Gravimetric Determination of Extractive Content

The different extracts were dried under vacuum at a temperature of 35 to 40 °C using a rotary evaporator. Thereafter, the gravimetric determination of the total amount of hydrophilic extractives was done and expressed as percentage (%) of the dry wood according to TAPPI T204 om-88 (1996).

GC-MS Analysis

Before GC-MS analysis, the methanol extractives were evaporated using nitrogen gas and silylated to enhance volatility. For silylation, the evaporated extractives were first dried in a vacuum oven at 40 °C (Binder, Herbertshausen, Germany) and then silylation solvents (80 µL bis-(trimethylsilyl)-trifluoroacetamide, 20 µL pyridine and 20 µL trimethylsilyl-chloride) were added. Finally, the samples were incubated at 70 °C for 45 min. Measurements were performed using Perkin Elmer Auto-System XL gas chromatograph (GC; PerkinElmer Inc., Waltham, MA, USA) and a GC-MS (HP 6890-5973 from Agilent Technologies Inc., Santa Clara, CA, USA). The GC was equipped with a HP-5 column (Length: 25 m; ID: 0.20 mm; film thickness 0.11 µm) and a flame ionization detector (FID). The carrying gas was nitrogen at a flow rate of 0.8 mL/min. Furthermore, other conditions were: internal oven 120 °C with a increasing rate at 6 °C/min to 320 °C (15 min hold); a split injection with a ratio of 25:1 and a temperature of 250 °C; the detector temperature 310 °C and injection volume of 1 µL. The data were analysed based on the mass spectra library created at the Laboratory of Wood and Paper Chemistry at Åbo Akademi University.

Test Microorganisms

The analysis of potential antimicrobial activities was performed using four representative American Type Culture Collection (ATCC®) strains that cause typical clinical diseases: *Staphylococcus aureus* (ATCC® 25923; gram-positive bacterium), *Escherichia coli* (ATCC® 25922; gram-negative bacterium), *Pseudomonas aeruginosa* (ATCC® 27853; gram-negative bacterium), and *Candida albicans* (ATCC® 10231; yeast).

Detection of Antimicrobial Effects of Bark Materials

Agar diffusion tests, as one of the most widely used antimicrobial susceptibility testing methods in routine clinical laboratories, were used to detect the antimicrobial effects of bark materials against four selected test micro-organisms. Different volumes (25 µL and 50 µL) of each undiluted extract were applied to neutral susceptibility discs (ø 5.5 mm, Thermo Scientific™ Oxoid™, Waltham, MA, USA) and then dried in an open sterile Petri dish for 24 h at 37 °C in a heating chamber (Binder, Herbertshausen, Deutschland). Microbial inoculums with an optical density of 0.53 (± 0.03) were prepared in 0.45% sterile sodium chloride solution (NaCl 0.9% Plastipur®, FRESENIUS KABI) via densitometry

(DensiCHEK, Marcy-l'Étoile, France). Inoculated agar plates (\varnothing 90 mm Mueller-Hinton Agar, Thermo Scientific™ Oxoid™) were prepared using these suspensions and the discs were applied to the surface. After an incubation for 24 h at 37 °C, the width of the uniformly circular inhibition zones was determined. As a negative control, neutral susceptibility discs with either methanol or water only were included. Each test was performed in triplicate for each test microorganism and extract on three individual days ($n = 9$).

RESULTS AND DISCUSSION

Antimicrobial Effects of Bark Materials

Each extract of the two solvents was used for the detection of antimicrobial effects without any additional treatment of the extracts. Methanol extracts from larch bark materials affected the growth of *Staphylococcus aureus*, whereas the water extracts did not show any antimicrobial activity (Table 1). *Staphylococcus aureus* (*S. aureus*) are gram-positive bacteria, which can cause human diseases, and this pathogen can easily colonize the surface and form biofilms (Ming *et al.* 2017).

Laireiter *et al.* (2014) and Salem *et al.* (2016) previously showed that larch bark, as well as larch bark extracts with methanol as solvent, had an antimicrobial effect. Therefore, the results could be confirmed in the present study. In addition, the qualitative and quantitative compositions of the two larch bark extracts with water and with methanol as solvent were tested, which has not been performed thus far. (*e.g.* Laireiter *et al.* 2014; Hubert *et al.* 2016).

Table 1. Mean Inhibition Zones (\pm SD) Caused by Exposure of Four Selected Test Microorganisms to Larch Bark Extracts

Microorganism	Inhibition Zone (mean \pm SD) (mm)	
	Water	Methanol
<i>Escherichia coli</i>	0.0 (0.00)	0.0 (0.00)
<i>Pseudomonas aeruginosa</i>	0.0 (0.00)	0.0 (0.00)
<i>Staphylococcus aureus</i>	0.0 (0.00)	8.2 (0.44)
<i>Candida albicans</i>	0.0 (0.00)	0.0 (0.00)

Chemical Characterisation of Water and Methanol Extract

Regarding the qualitative and quantitative analysis of the extracts, in a first step the amounts of solid material after drying were determined. Table 2 shows the total amounts of the dried methanol and water extractions in mg/g of oven dried larch bark powder. The total extraction yields differed in a range from 50.1 mg/g oven dried bark with methanol extraction to 22 mg/g with water extraction. Various yields have been reported for hot water extraction of larch bark. Higher values of about 91.7 mg/g and 103.6 mg/g were obtained by Bianchi *et al.* (2015) and Salem *et al.* (2016). Dissimilarity between this study and the literature references can be found in methodological alterations such as the extraction temperature, pressure and iterations, as well as in natural differences caused by tree variations. The drying and extraction methods used in the present study were chosen to be gentle in order to avoid a loss of volatile compounds by heating or a destruction of the molecules present in the extracts.

Both solvents used have a similar polarity and are used to extract polyphenols from different plant materials (Kassing *et al.* 2010), which were the target group. Nevertheless,

single sugar molecules, alcohols, and acids can also be found in methanol and water extractives (Table 2).

Table 2. Main Component Groups in Different Larch Bark Extracts Analysed by GC-MS

Component Groups	Extractives in Different Solvents (mg/g)	
	Water	Methanol
Long chain alcohols	0.00	0.59
Carboxylic acids	1.83	1.80
Single sugars	6.85	6.86
Fatty acids	0.05	0.23
Resin acids	0.01	1.38
Terpenoids	0.01	0.65
Polyphenols	0.69	4.05
Lipophilic substances	0.07	1.82

Compared to the different component groups, the non-phenolic constituents add up to a higher portion of the extractives found. Similarities in the amounts of larch bark extract compounds obtained with different solvents were determined for carboxylic acids and single sugars, whereas differences between both extracts were found for the quantitative amount of aliphatic alcohols, saturated and unsaturated fatty acids (*e.g.* lignoceric acid), resin acids (*e.g.* isopimaric acid), terpenoids, and lipophilic compound groups. For the polyphenols, the largest difference between the water and methanol extract was determined. The focus was placed on these substances, since polyphenols were shown to have antimicrobial effects towards different bacteria, yeast, and fungi (Plumed-Ferrer *et al.* 2013). For further considerations, the group of polyphenols was divided into subgroups of flavonoids, lignans, and stilbenoids (Table 3).

Table 3. Three Component Groups of Polyphenols in Different Larch Bark Extracts Analysed by GC-MS

Component Groups	Extractives in Different Solvents (mg/g)	
	Water	Methanol
Flavonoids	0.43	1.61
Lignans	0.24	0.96
Stilbenoids	0.02	1.49

The water extractives contained (+)-catechin (0.39 mg/g) as the main compound in the flavonoid substance class, as well as taxifolin. However, the taxifolin amount in the water extract (0.036 mg/g) was higher compared to the methanol extract (0.019 mg/g). The major substance of the flavonoid group in the methanol extract was (+)-catechin (1.532 mg/g). A very small amount of kaempferol (0.057 mg/g) was found *via* GC-MS analysis. Both compounds were shown to have antimicrobial activities, whereas the antimicrobial activity of the (+)-catechin without a galloyl moiety is described as very weak (Sakanaka *et al.* 1989; Kajiya *et al.* 2004). Kajiya *et al.* (2004) concluded that a high concentration of 12.8 mg/mL is required for antimicrobial activity. In addition, Rauha *et al.* (2000) showed that (+)-catechin has an antimicrobial effect towards *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which was shown by an inhibition zone between 1 and 3 mm. Based on the results from the literature, the larch bark extract with water used in the present study should have shown some antimicrobial effects. However, these results were not

found. In addition, the (+)-catechin alone cannot explain the mean inhibition zone of 8.2 ± 0.44 mm for *Staphylococcus aureus* (Table 1). Therefore, it seems that the concentration of (+)-catechin only in the water and methanol extracts was not adequate to affect the observed results. Interestingly, kaempferol is known for an antimicrobial activity against *Staphylococcus aureus* when used at a high concentration of 1 mg/ μ L of the pure phenolic compound (Rauha *et al.* 2000). However, in the present study, the amount of kaempferol in the methanol extract was low. Compared to results from Rauha *et al.* (2000), the determined kaempferol concentration was too low to induce a clear inhibition zone for *Staphylococcus aureus*.

The water extracts from the group of lignans contained lower amounts of several substances compared to the methanol extracts. The two major compounds of lignans were isolariciresinol and lariciresinol. In addition, pinoresinol could be detected with the GS/MS method used. Vainio-Kaila *et al.* (2015) showed that lignans from spruce species have an antimicrobial effect against *Streptococcus pneumoniae*. A detailed characterization of the chemical composition of these lignans was not provided (Vainio-Kaila *et al.* 2015). Therefore, the comparison between these two studies is difficult. However, since spruce wood and acetone were used for the extraction in the study of Vainio-Kaila *et al.* (2015), it can be concluded that most likely different substances were extracted in both studies. The water and methanol extracts of the present study contained mainly the same lignans, but in different quantities. However, the results from the antimicrobial analysis are quite different, and it therefore is likely that the lignans do not have an effect. This observation is confirmed by the results from Välimaa *et al.* (2007).

The stilbenoids are the final group of polyphenols determined in the two different extracts. The results of GC-MS analysis showed that the methanol extracts contain only astringin (3-O- β -glucosyl-3',4',5-trihydroxystilbene). Furthermore, a small amount of astringin was also found in water extracts (Table 3). However, this substance represents the main difference between methanol and water extracts. The concentration of astringin in the methanol extract was approximately 75 times higher than in the water extract. Therefore, it can be assumed that astringin is mainly responsible for the antimicrobial activity of the methanol extracts from larch bark against *Staphylococcus aureus*. Plumed-Ferrer *et al.* (2013) showed that astringin extracted from Norway spruce bark have some antimicrobial effects against different strains of gram-negative, gram-positive, and yeast bacteria.

To summarize the results of this study, the antimicrobial activities of flavonoids (*e.g.* (+)-catechin) towards different bacteria were not determined. Therefore, these data in combination with findings from previous studies (Sakanaka *et al.* 1989; Rauha *et al.* 2000; Kajiya *et al.* 2004) show that the compounds of kaempferol and astringin of the flavonoids and stilbenoids are responsible for the antimicrobial effects of the larch bark. Compared to the methanol extract, the water extract did not exhibit an inhibition zone against the microbes. Therefore, it can be assumed that the amount of both substance groups is too small to influence the microbial growth. Alternatively, the combined effect of several compounds in the extract acting together towards an antimicrobial effect, could have resulted in the observed results. The analysis of such mixture toxicity effects needs further investigation.

CONCLUSIONS

1. Methanol and water extracts from larch bark were evaluated for antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Two different compounds of the flavonoids and stilbenoids in larch bark were identified for the active antimicrobial effects against *Staphylococcus aureus*. The growth of the other three test microorganisms was not affected by the extracts used.
2. The two different larch bark extracts showed chemical differences in quality and quantity of the compounds tested. Water extracts had larger amounts of non-phenolic substances compared to the methanol extracts, whereas the concentration of polyphenols in the methanol solvent was higher compared to the bark extractives in water.
3. The methanol extracts show some potential in the pharmaceutical industry. Moreover, water extracts of the bark material can play an important role in the cosmetic industry as well as chemical industry. The residual material from larch bark can be used for the production of added-value products; this is advantageous for the bio-economy and for reducing the dependence on fossil fuel based raw materials. Therefore, the development of a successful production process of value-added products shows great potential.

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