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In vitro inhibition of extractives from knotwood of Scots (*Pinus sylvestris*) and black pine (*Pinus nigra*) on growth of *Schizophyllum commune, Trametes versicolor, Gloeophyllum trabeum* and *Fibroporia vaillantii*

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Abstract:

The main goal of the study was to examine and compare the inhibition effect of knotwood extractives of Scots (*Pinus sylvestris*) and black pine (*Pinus nigra*) against the two white rot fungi and two brown rot fungi. Knotwood was extracted in Soxhlet apparatus. Extracts were chemically analysed and the inhibitory effect of purified pinosylvins and crude hydrophilic extracts were tested against growth of white rot fungi Schizophyllum commune and Trametes versicolor and the brown-rot fungus Fibroporia vaillantii and Gloeophyllum trabeum with in vitro antifungal assay. Knotwood of Scots pine and black pine contained comparable amounts of total extractives. Pinosylvin, pinosylvin monomethyl ether and nortrachelogenin were characteristic compounds in knotwood extracts of both pines. Scots pine knotwood contained larger amounts of pinosylvin than black pine. The ratio between pinosylvin monomethyl ether and pinosylvin was higher in black pine knotwood. Purified pinosylvins and crude hydrophilic extracts inhibited the growth of all the white rot and brown-rot fungi tested. Pure pinosylvins better inhibited fungal growth than crude knotwood extracts, whereas efficiency was not unambiguous and relate to fungi species. Crude hydrophilic extracts of Scots pine more efficiently inhibited fungal growth than extracts of black pine. With the present investigation

hydrophilic extracts of pine knotwood were demonstrated as formulations of natural compounds with good antifungal properties. Broken tops of Scots pine, which can lie on a forest ground for a month or even more, still contain high amount of phenolic extractives and are therefore potential raw material for recovery of bioactive compounds.

1. Introduction

Extractives include a large variety of compounds that can be classified into lipophilic and hydrophilic extractives, i.e., compounds soluble in non-polar and polar solvents, respectively (Jansson and Nilvebrant 2009; Willför et al. 2006). Variability in the content of extractives is characteristic of the wood of living trees, in both radial and longitudinal directions within a tree, as well as between trees of the same species, and between species (Kai 1991; Kebbi-Benkeder et al. 2017; Morais and Pereira 2011; Partanen et al. 2011; Vek et al. 2014). It has been demonstrated that wood of living and dead knots in softwoods generally contain larger amounts of phenolic compounds, mainly flavonoids, lignans and stilbenes, than heartwood and sapwood (Holmbom 2011; Wijayanto et al. 2015; Willför et al. 2003a; Willför et al. 2004).

The most characteristic phenolic extractives in wood of the genus *Pinus* are the stilbenes, pinosylvin and pinosylvin monomethyl ether (Conde et al. 2014; Conde et al. 2013; De Angelis et al. 2018; Fang et al. 2013; Lindberg et al. 2004; Poljanšek et al. 2018; Willför et al. 2003b). Pinosylvins are considered to be the prevailing compounds determining the variation of decay resistance of pine heartwood (Belt et al. 2017; Harju and Venäläinen 2006; Jorgensen 1961) and are inducible phytoalexin-like inhibitors in living sapwood (Gottstein and Gross 1992; Jorgensen 1961). Crude extracts, pinosylvin, pinosylvin monomethyl ether and pinosylvin dimethyl ether, mixed in various combinations and concentrations, have been examined for their antimicrobial activity against gram-positive and gram-negative bacteria, yeasts and filamentous fungi (Lindberg et al. 2004; Pietarinen et al. 2006; Välimaa et al. 2007), against tip blight and canker pathogen (Sherwood and Bonello 2013), turfgrass fungi (Lee et al. 2017) and wood decay fungi (Bois and Lieutier 1997; Celimene et al. 1999; Fernandez-Costas et al. 2017; Hart and Shrimpton 1979; Lu et al. 2016). The origin and purity of pinosylvins was not always similar, sometimes purchased standards were used, or compounds were obtained from the parent laboratory

(Seppänen et al. 2004), or isolated from different tree parts of pine trees, such as pine cones (Celimene et al. 1999), heartwood (Hart and Shrimpton 1974) or knotwood (Lindberg et al. 2004; Pietarinen et al. 2006; Plumed-Ferrer et al. 2013; Välimaa et al. 2007). It has been demonstrated that pinosylvin has more substantial antibacterial and antifungal effects than pinosylvin monomethyl ether (Celimene et al. 1999; Lindberg et al. 2004; Seppänen et al. 2004; Välimaa et al. 2007). Pinosylvin and its derivatives have been proven to have numerous pharmacological properties and have potential in biomedical and food applications (Laavola et al. 2015; Plumed-Ferrer et al. 2013; Silva et al. 2014; Simard et al. 2008).

In comparison to pure pinosylvins, the antibacterial and antifungal potential of crude hydrophilic extracts has been studied far more fragmentarily. Crude extracts obtained from knotwood of a relatively limited number of pine species (*P. sylvestris*, *P. resinosa*, *P. contorta*, *P. banksiana*, *P. sibirica and P. strobus*) were tested against filamentous fungi (Lindberg et al. 2004) and papermill bacteria (Välimaa et al. 2007). Hydrophilic heartwood extract of *Pinus merkusii* possesses higher antifungal activity against wood decay fungi than knotwood extract (Wijayanto et al. 2015). The antifungal potential of crude heartwood extract *P. pinaster* and grafted extracts to wood substrate has recently been investigated against brown and white rot fungi. The study of Välimaa et al. (2007) demonstrated that crude knotwood extracts of different pine species revealed different antibacterial efficiency, suggesting that differences in the composition of extractives or relative amounts of extractive compounds influence their biological activity. A literature review showed that data on the chemical composition of extractives that occur in wood tissue of black pine (*Pinus nigra*) from different provenances (Alvarez-Nóvoa et al. 1950; Hafizoglu 1983; Ioannidis et al. 2017; Politeo et al. 2011; Ucar and Balaban 2002; Uçar and Fengel 1995; Willför et al. 2007) is far more fragmentary than for *P. sylvestris.* Data on the antimicrobial activity of wood extracts from *P. nigra* is practically non-existent. On the other hand, the antimicrobial activity of acetone and methanol extracts of leaves, resin, bark and cones of *P. nigra* against filamentous fungi (Digrak et al. 1999) and the antimicrobial activity of essential oil isolated from the needles of endemic Dalmatian black pine (*Pinus nigra* ssp. dalmatica) have been investigated (Politeo et al. 2011).

The aim of the present study was to evaluate the composition of the crude knotwood extracts of Scots pine (*Pinus sylvestris*) and black pine (*Pinus nigra*) and to examine the concentration of pinosylvin and pinosylvin monomethyl ether in the knotwood extracts of both pines. Knots,

which are considered a rich source of extractives, are an undesirable technological defect of wood. In the case of Scots pine, we had the opportunity to select trees that have been severely damaged due to the ice storm. Thus, we sampled knots in damaged stems and knots of broken away tree tops that had been lying on forest floors for nearly three months, because information on the content of extractives in coarse woody debris is missing. In view of the fact that extracts of different pine species revealed different biological activity, the influence of crude hydrophilic extracts of Scots pine (*Pinus sylvestris*) and black pine (*Pinus nigra*) knotwood, as well as isolated pure pinosylvins on growth inhibition of the white rot fungi *Trametes versicolor* and *Schizophyllum commune* and the brown-rot fungi *Gloeophyllum trabeum* and *Fibroporia vaillantii*, was investigated. We used antifungal diffusion assay in such a way that it was possible to study the growth dynamics of fungi toward extractives as well as the inhibition of fungal growth.

These wood-decaying fungi were chosen because they are most common fungi appearing on wood in use class 2 (i.e., interior wood or wood under cover) and class 3 (outdoor wood, above the ground, exposed to the weather) applications in Central Europe (Schmidt 2006). In addition, some of these fungi are considered as standard fungi for assessment of wood preservatives. Information on the inhibitory effect of pine extracts against the growth of *S. commune* and *F. vaillantii* is practically missing. *S. commune* is a prime colonizer of wood in outdoor conditions and it has been proven to be reasonably resistant against several commercial biocides (Humar et al. 2001; Schmidt 2006). Even higher resistance to biocides was proven for *F. vaillantii*. This fungus has developed tolerance predominantly against copper-based biocides (Green and Clausen 2005). It has also been shown to be able to degrade wood treated with other commercial biocides and semi-durable wood, to a greater extent than *G. trabeum* and *C. puteana* (Humar and Lesar 2008).

2. Materials and methods

2.1 Chemicals

Methanol (HPLC grade), Folin-Ciocalteu phenol reagent (2 N), formic acid (≥ 99%) and anhydrous sodium carbonate (99%) were purchased from Sigma Aldrich (Steinheim, Germany). Water and acetone, both HPLC grade, were from J.T. Baker. Cyclohexane (99%), ethyl acetate and dimethylsulphoxide were provided from Carlo Erba Reagents (Milano, Italy). All the reference compounds used for the chromatographic analysis were obtained from a commercial source. Pinosylvin (HPLC, ≥ 97%), pinosylvin monomethyl ether (HPLC, ≥ 97%), pinostilbene hydrate (HPLC, ≥ 95%), pterostilbene (HPLC, ≥ 97%), nortrachelogenin (HPLC, ≥ 95%), pinobanksin (HPLC, ≥ 95%), pinocembrin (HPLC, ≥ 95%) and gallic acid monohydrate (HPLC, ≥ 98.0%) were from Sigma Aldrich (Steinheim, Germany).

2.2 Woody biomass

Six trees of Scots pine (*Pinus sylvestris* L.) were fallen in urban forest of Rožnik in the city of Ljubljana (46°03'31.3"N 14°29'32.1"E) at the beginning of April 2014, while six black pine trees (*Pinus nigra* L.) were cut down in the Slovenian Karst near the village Dutovlje (45°46'41.6"N 13°49'19.7"E) in March of 2013. All Scots pine trees included in this investigation had tops broken away as a consequence of a massive sleet storm at the end of January 2014. The crowns of black pine trees were free of storm damage. Eight sample disks containing knotwood were taken from each fallen tree. In the case of Scots pine, four discs from stems and four discs from broken tops, which were lying on the forest ground were taken for each of the six trees. All the sampled discs were carefully examined and, thereafter, one or two samples of knotwood were dissected from each disc with a band saw. The knots were dried at 50 °C for 48 hours and grounded with a Retsch SM 2000 cutting mill (Haan, Germany) using a 1 mm sieve. Disintegrated knotwood samples were then stored in the dark at room temperatures until further processing.

2.3 Extraction of pinewood

The milled knotwood was freeze dried in a Telstar LyoQuest CC1930 lyophylizator at 4 Pa and -82 °C for 24 hours before extraction. Extraction of Scots pine and black pine knotwood was

performed in a 150 mL Soxhlet apparatus. A two-step sequential extraction procedure with cyclohexane and acetone was carried out according to Willför et al. (2003a; 2003c). Two and a half grams of each dried knotwood was first extracted with cyclohexane at 130 °C for 6 h in order to remove lipophilic extractives, and hydrophilic extractives were then extracted with 250 mL of an acetone/water mixture (95:5, v/v) at 130 °C for 6 h. Further aliquots were taken from the obtained knotwood extracts for semi-quantitative determination of total phenols and for chromatographic evaluation of individual compounds. The contents of total lipophilic and hydrophilic extractives were determined gravimetrically and expressed in milligrams of extracted matter per gram of dried knotwood (mg/g, dw). For the purposes of flash chromatography, the remaining extracts were dried in a rotary evaporator (Welch Vacuum, formerly Ilmvac, Germany) and properly stored at - 25 °C.

2.4 Characterisation of extractives and isolation of pure compounds

Chemical composition of extractives in knotwood samples of pine trees was investigated on a Thermo Scientific Accela system (Waltham, USA) for high-performance liquid chromatography (HPLC), equipped with an Accela 600 quarter pump and Accela photodiode array detector (PDA) using a pentafluorophenyl column (dimensions of 2.1 mm (i.d.) × 150 mm and 2.6 µm particle size). Sample trays in an Accela autosampler and a column oven were thermostated at 4 °C and 30 °C, respectively. Water (A) and methanol (B), both containing 0.1% formic acid (v/v), were used as the mobile phase (Li et al. 2013; Poljanšek et al. 2018; Vek et al. 2019). The flow rate of the mobile phase was set at 0.4 mL/min and the total time per run was 19.2 minutes. The elution of phenolic compounds was performed by applying a gradient from 5% to 95% of solvent B over 10 minutes. The wavelength for monitoring the phenolic compounds was set at 275 nm, and trans-stilbene isomers were further reviewed at 306 nm (Silva et al., 2014). UV spectra were recorded from 200 nm to 400 nm for peak identification. The results were expressed in milligrams of identified compound per gram of dried wood sample (mg/g dw).

Isolation of pinosylvin (PS) and pinosylvin monomethyl ether (PSMME) from hydrophilic knotwood extracts was performed by column “flash” chromatography on silica using non-chlorinated solvents as mobile phase as described by Poljanšek et al. (2018). All the targeted compounds were already relatively well purified with the first chromatographic run in this study.

The percentage recovery (w/w × 100) was estimated to be 76.00 ± 16.28%, on the basis of the gravimetrically determined yields of pure PS and PSMME obtained and the amount of crude knotwood extracts used. Purity of isolated compounds was 96%. Both crude hydrophilic extracts of knotwood and purified pinosylvins that were later used for antifungal tests were qualitatively checked with HPLC.

2.5 Wood-decay fungi used in antifungal assay

The white-rot fungus *Schizophyllum commune* (ZIM L039) and brown-rot fungus *Fibroporia vaillantii* (ZIM L037) were used for *in vitro* antifungal assay. *Trametes versicolor* (ZIM L039) and *Gloeophyllum trabeum* (ZIM L018) are generally used as representatives of white and brown rot and were therefore used as references organisms in this study. All the fungi used were stored in the culture collection of industrial microorganisms (Raspor et al. 1995) of the Biotechnical Faculty, University of Ljubljana, Slovenia. Each fungus was maintained on a previously prepared Petri dish containing potato dextrose agar (DIFCO). The white and brown rot fungi were incubated in a growth chamber at 25 °C and 85% RH for one week prior assay (Humar and Pohleven 2007).

2.6 Antifungal assay

The growth dynamics of fungi and inhibition effect of pine wood extractives on the growth of the selected white-rot (*S. commune*, *T. versicolor*) and brown-rot fungi (*F. vaillantii*, *G. trabeum*) was investigated by means of the agar diffusion test, described in the literature (Celimene et al. 1999; Chen et al. 2014; Fernandes et al. 2017; Lindberg et al. 2004; Seppänen et al. 2004; Vek et al. 2013; Zimmer and Melcher 2017).

The antifungal diffusion assay on growth media was employed as a quick and robust way of measuring the antifungal effect of extracts in order to get preliminary information on the fungistatic activity of a pure pinosylvins and crude hydrophilic knotwood extracts, taking in account drawbacks of the chosen assay method. This can be seen in the fact that the poorly water-soluble extractives must diffuse through the agar which is likely to skew the results and in

the fact that it is difficult to relate what occurs on the agar to what occurs in a woody substrate (Smith et al. 1989). Tests required for assessment of efficiency of wood preservatives are much more comprehensive. Screening tests were developed to obtain preliminary information of the interactions between fungi and particular chemicals. In addition, this type of test is predominately suitable for screening of chemicals with low water solubility. If solubility is too good, the chemicals evenly distribute in the agar; thus the effect of the respective chemical/concentration is impossible to perform, as even concentration is reached too soon.

For the antifungal assay, dimethyl sulfoxide solutions (DMSO) with concentrations of 1% (w/v) were prepared of pinosylvin (PS), pinosylvin monomethyl ether (PSMME), and a mixture of PS and PSMME (PS/PSMME) (1:1, w/w). Crude hydrophilic knotwood extracts were prepared in two concentrations, 1% and 5% (w/v, DMSO), and labelled as E1-Ps and E5-Ps for *Pinus sylvestris* (Scots pine) and, E1-Pn and E5-Pn for *Pinus nigra* (black pine). The antifungal assay was carried out in Petri dishes (d = 90 mm, h = 15 mm), each contained 20 mL of growth media with 0.8 g of potato dextrose agar (PDA, Difco). After autoclaving (p = 0.22 MPa, T = 121 °C), holes with a diameter of 8 mm were bored in the potato dextrose agar, and 100 µL of each of prepared solutions that are mentioned above were pipetted into the wells. With that each of the wells contained 1 mg of the purified compound (PS, PSMME, a PS/PSMME mixture) or, 1 mg (E1-Ps and E1-Pn) or 5 mg (E5-Ps and E5-Pn) of the crude hydrophilic extract. Pure DMSO was used in the same volume as for the control. White and brown rot fungal inoculums were placed in the centre of each Petri dish and stored in a growth chamber, in which incubation followed at 25 °C and 75% relative humidity (Humar and Pohleven 2007).

The prepared crude extracts and purified compounds were tested in 6 repetitions for each of the tested fungi. Growth of mycelium toward sites with isolated pinosylvins and crude extracts was monitored every 2 - 3 days, depending on the fungal growth. Fungal growth was determined by measuring the distance between the edge of the petri dish and the edge of the fungal mycelium in every direction. The test was conducted until the mycelium had grown to the edge of the Petri dish in at least one direction. The development of the fungal colony was measured by a digital Vernier calliper (± 0.01 mm). The results were expressed numerically as the percentage inhibition of fungal growth in a centrifugal direction (%In), i.e., as the average values of all observations (Celimene et al. 1999; Seppänen et al. 2004). Inhibition of fungal growth, %In, was calculated with following equation.

$$In = \left[\frac{R_m}{R_0}\right] \cdot 100\ [\%]$$

R_m … distance between the edge of the petri dish and the mycelia [mm]
R_0 … distance between the edge of the petri dish and the edge of inoculum [mm]

2.7 Statistics

Statgraphics software was used for basic statistical analysis of the results. The values of measurements were checked for normal distribution. Significant differences between the mean values were tested by analysis of variance (ANOVA) at a 0.95 confidence level. The results were further compared by means of a multiple range test, viz., Fisher's least significant difference (LSD) test. The results are presented as mean values with standard deviations (±SD).

3. Results and discussion

3.1 Extractives in knotwood of Scots and black pine

The knotwood samples of Scots pine and black pine gave comparable amounts of total extractives, and PSMME (Table 1). The content of lipophilic extractives was larger than that of hydrophilic extractives in both knotwood samples (Table 1). Knotwood of Scots pine was richer in lipophilic extractives, whereas a significantly larger amount of hydrophilic extractives was extracted from the knotwood of black pine (Table 1).

HPLC analysis of the two extractable fractions gave significantly different and characteristic chromatographic profiles (Fig. 1). HPLC analysis showed that lignan nortrachelogenin (NTG) and the stilbenes, pinosylvin (PS) and pinosylvin monomethyl ether (PSMME), were the most characteristic compounds in the hydrophilic knotwood extract of Scots pine (*Pinus sylvestris*) and black pine (*Pinus nigra*) (Fig. 1B and Fig. 1D). Flavonoid pinocembrin (PCB) and stilbenes

pinostilbene (PSB) and pterostilbene (PtSB), were extracted in insignificant quantities (Fig. 1), with an average content of less than 1 mg/g. Content of PS was significantly higher in knotwood of Scots pines than in knotwood of black pines (ANOVA; $p_{PS} < 0.050$) (Table 1). PSMME was the most abundant of the quantified compounds, and its content was comparable in knotwood of both pine species (Table 1). The occurrence of relatively large amounts of pinosylvin monomethyl ether in the lipophilic wood extracts of Scots and black pine can be explained by its partially non-polar character. It was reported that Scots pine knotwood can contain up to 9 mg of PS and 70 mg of PSMME per gram of wood (Fang et al. 2013; Hovelstad et al. 2006; Willför et al. 2003b) (Table 1). The PSMME/PS ratio has been reported to be higher in knotwood (from 1.1 to 4.4) than in stemwood (from 1.1 to 2.3) (Gref et al. 2000; Hovelstad et al. 2006; Willför et al. 2003b). In the case of the pine knotwood included in this investigation (Table 1), the measured PSMME/PS ratios were much higher than those reported by the Finnish and Norwegian research groups. Our results (Table 1) correlate with a report by Ioannidis et al. (2017), who found the PSMME/PS ratio in wood of Greek black pine (*Pinus nigra* Arn.) to range from 1.26 to even 8.51.

The knotwood samples of broken tree tops of Scots pine contained significantly larger amounts of PS than the knotwood of the corresponding standing tree stem (ANOVA; $p_{PS} < 0.050$, $p_{PSMME} > 0.050$) (Fig. 2). A significant difference in the content of PSMME among the knotwood samples was not found (ANOVA; $p_{PSMME} = 0.5678$). These results show the knotwood of broken tops of Scots pine, which can lie on a forest ground for a month or even more, as a relevant source of phenolic extractives. Broken treetops of Scots pine represent coarse woody debris, i.e., the material with low market value.

3.2 Antifungal potential of crude extracts and pinosylvins

The *in vitro* antifungal assay revealed growth patterns of white-rot and brown-rot fungi towards the extractives and inhibition of extractives on fungal growth. Fig. 3 and Fig. 4 show growth (mm) and growth rate (mm/day) of white-rot and brown-rot fungi towards the extractives. The fungi growth towards the control well differ significantly from fungi growth towards the wells with the extractives. Growth of *T. versicolor* towards the wells with the pure compounds and crude extractives was faster than in other fungi used in the experiment, indicating week inhibitory effect of extractives against the growth of this fungus (Fig. 3). Growth and growth rate

of *S. commune* (Fig. 3e-h) towards the control well was much faster than towards the wells with pinosylvins (Fig. 3f) and crude extracts (Fig. 3h). Fungal growth inhibition of pure compounds and crude extracts took place by day six after initiation of the experiment (Fig. 3e and 3g). It has to be considered that the diffusion of respective chemicals is slow, thus inhibition was notable when fungal mycelia reach the part of the nutrient media with sufficient concentration of chemicals that inhibits fungal growth. As the diffusion of chemicals is comparable on all of the Petri dishes, results between the fungi are comparable. *S. commune* reached the edge of a Petri dish on 10^{th} day (crude hydrophilic extracts) and 13^{th} day (purified pinosylvins) (Fig. 3). This is in line with previous observations. Knotwood extractives inhibited the growth of the brown-rot fungi *G. trabeum* (Fig. 4a-d) and *F. vaillantii* (Fig. 4e-h) as well. Inhibitory effect of pure pinosylvins and crude hydrophilic extracts was observed already on the third day in case of *G. trabeum* (Fig. 4a-c) and on the sixth day in *F. vaillantii* (Fig. 4e-g). In comparison to white rot (Fig. 3), the growth of brown-rot fungi was significantly slower, meaning that the antifungal assay was finished not until the twentieth day for *F. vaillantii* and fortieth day for *G. trabeum*, respectively (Fig. 4). This is in line with previous observations. *G. trabeum* is a fungus that is rather sensitive organisms to biocides and other bio-active compounds (Humar et al. 2001; Schmidt 2006). On the other hand, F. vaillanti is reported as one of the fungal species with the highest tolerance to bio-active chemicals (Green and Clausen 2005).

The results of the *in vitro* antifungal assay are shown in Table 2 and Table 3 as the percentage inhibition of fungal growth (%In). Pinosylvin (PS), pinosylvin monomethyl ether (PSMME) and the PS/PSMME mixture (1:1,w/w) exhibited different fungal growth inhibitions (ANOVA, $p < 0.050$) (Table 2). Table 2 showed that PS, PSMME and PS/PSMME mixture exhibited relatively weak growth inhibition of *T. versicolor* and essentially better one in case of *S. commune, G. trabeum* and *F. vaillantii.* PSMME and PS/PSMME mixture better inhibited growth of *T. versicolor* than pure PS (Table 2). All three formulations of pure compounds revealed effective and comparable growth inhibition of *S. commune* (Table 2). Formulations of pure pinosylvins effectively inhibited the growth of *G. trabeum* as well, whereas PSMME revealed somewhat better growth inhibition than PS and mixture PS/PSMME (Table 2). PS and mixture PS/PSMME had higher inhibitory effect on the growth of the brown rot fungus *F. vaillantii* than PSMME (Table 2).

Fungal growth inhibition of the analysed crude extracts towards wood decaying fungi was significantly different (ANOVA, $p < 0.050$) (Table 3). Crude extracts better inhibited growth of *S. commune*, *G. trabeum* and *F. vaillantii* than growth of *T versicolor*. Table 3 shows that 1% knotwood extract of Scots pine had better inhibition of fungal growth than 1% black pine extract (Table 3). As well, 5% crude hydrophilic extracts of Scots pine had a greater inhibition effect against wood destroying fungi than 5% crude extract of black pine. 5% Scots pine extract was a better inhibitor of *S. commune*, *G. trabeum* and *F. vaillantii* growth than 1% Scots pine extract. 5% extract of black pine revealed better inhibition of *S. commune* and *F. vaillantii* growth, than its 1% formulation. For *T. versicolor* and *G. trabeum* no differences in growth inhibition in relation to concentration of black pine extract was observed. 5% crude extracts of black pine showed significantly lower growth inhibition of *S. commune* and *G. trabeum* than did 1% Scots pine extracts.

Figs. 3 and 4 and Table 2 and Table 3 demonstrated that the growth of *S. commune*, *F. vaillantii,* and *G. trabeum* was significantly inhibited by all formulation of the crude pine knotwood extracts and pure pinosylvins. On the other hand, the white-rot fungus *T. versicolor* was most tolerant of the presence of formulations used in this study. As opposed to our observations, Celimene et al. (1999) reported that the inhibition of white and brown rot fungi was not improved by using a PS, PSMME and PSDME mixture (1:1:1, w/w/w) on agar medium. In addition, a combination of PS and PSMME has been shown to have relatively weak antifungal activity against the shoot blight and canker pathogen *Diplodia pinea* (Sherwood and Bonello 2013). The results of our investigation demonstrated PSMME to be the compound with the greatest fungal growth inhibition (Table 2) against *T. versicolor* and *G. trabeum*. The stronger inhibition effect of PSMME may also be explained by its cytotoxicity (Simard et al. 2008) and its partial lipophilicity, which may facilitate the passage of molecules through the biological membrane (Plumed-Ferrer et al. 2013). On the other hand, some research groups have found greater fungicidal and cytotoxic potential of PS (Lindberg et al. 2004; Välimaa et al. 2007).

Our results revealed that crude hydrophilic knotwood extracts of Scots and black pine were relatively strong inhibitors of fungal growth in spite of the isolated pinosylvins giving slightly better results (Table 3). Somewhat weaker fungal growth inhibition by crude hydrophilic knotwood extracts could be explained by its more complex chemical composition. In addition to

polyphenols identified (Fig. 1), hydrophilic knotwood pine extracts are reported to contain oligolignans and small amount of monomeric and dimeric sugars (Willför et al. 2003b). Crude hydrophilic extracts used in our experiment thus contain a lower concentration of PS and PSMME than formulations of pure pinosylvins. Our observations are in accordance with results of Välimaa et al. (2007) reporting that both hydrophilic knotwood extracts, as well as pure pinosylvins, were antimicrobial agents (Välimaa et al. 2007). A concentration of preservative solution is important factor in fungal growth inhibition. It has been found that the higher amount of stilbenes have a higher effect on fungal inhibition (Celimene et al. 1999; Seppänen et al. 2004).

In this investigation it was shown that pinosylvins and crude hydrophilic extracts of pine knotwood are most effective against the brown rot fungi, *G. trabeum* and *F. vaillantii* and white-rot fungus *S. commune* (Fig. 3 and 4, Table 2 and Table 3). *F. vaillantii* is known as a fungus with good tolerance to some of the copper-based wood preservatives (Humar and Lesar 2008). Our results are in accordance with Seppänen et al. (2004) reporting greater sensitivity of brown rot fungi to pinosylvins in wood block test, compared to white-rot fungi. In contrast, Celimene et al. (1999) demonstrated pinosylvins to be more toxic to white rot fungi (*Trametes versicolor* and *Phanerochaete chrysosporium*) on agar media. Of the four wood-decaying fungi tested, *T. versicolor* was the most tolerant to PS, PSMME, PS/PSMME mixture and to the hydrophilic knotwood extracts (Fig. 3, Table 2 and Table 3). The higher tolerance of white rot fungi, and predominately *T. versicolor,* can be ascribed to the presence of non-selective ligninolytic enzymes, which are able to degrade various types of phenolic and other aromatic compounds (Lekounougou et al. 2008; Mounguengui et al. 2007). The inhibition mechanisms of pinosylvins can be explained by an active response in terms of inactivation of fungal enzymes (Harju and Venäläinen 2006; Lyr 1961).

Replacement of synthetic chemicals with safer, sustainable and green products is one of the important challenges in the field of biorefining forest biomass (Holmbom 2011), where extractives from woody biomass represent chemicals with high applicable potential (González-Laredo et al. 2015; Singh and Singh 2012). In addition to wood protection (Barbero-Lopez et al. 2019; Kadir and Hale 2019; Lu et al. 2016; Tascioglu et al. 2013) and improvement of the physical properties of wood (Ermeydan et al. 2012), the novel bio-based antifungal agents have

potential in other applications, such as in-can preservatives, textile protection, packaging, or wall paints additives.

4. Conclusions

Our study confirmed knotwood and of Scots (*P. sylvestris*) and black pine (*P. nigra*) trees as a suitable source for the recovery of phenolic extractives in the form of crude hydrophilic extracts or pure pinosylvins. Broken tops of Scots pine, which can lie on a forest ground for a month or even more, still contain a high amount of phenolic extractives and are therefore potential raw material for biorefining.

Knotwood of Scots and black pine gave comparable amounts of phenolic extractives, whereas Scots pine knots contained a higher amount of pinosylvin than black pine. Pinosylvin, pinosylvin monomethyl ether, a mixture of the two pinosylvins as well as crude hydrophilic extracts of Scots and black pine knotwood effectively inhibited the growth of white-rot fungus *Schizophyllum commune* and brown rot fungi *Gloeophyllum trabeum* and *Fibroporia vaillantii* in agar diffusion test. Our study revealed that pure pinosylvins exhibited somewhat better fungal inhibition than crude knotwood extracts, whereas efficiency was not unambiguous and related to fungi species.

Finding that crude hydrophilic extracts inhibited also the growth of fungi, has further implications. We suspect that comparable inhibitory effects to pure pinosylvins could be achieved with a higher concentration of bioactive fraction in the crude extracts of Scots and black pine knotwood. Development of more selective extraction or purification method remains the challenge in future.

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