Antibacterial Effects of Extracts of *Pinus sylvestris* and *Picea abies* against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Streptococcus pneumoniae*

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Pine heartwood, sapwood, and spruce extracts were tested against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), *Escherichia coli* O157:H7, and *Streptococcus pneumoniae*. The bacterial strains were cultured in a broth with and without the wood extracts. Also, the antibacterial effect of the extracts was studied by performing the antimicrobial sensitivity test method on agar plates. Both pine extracts had a clear antibacterial effect on MRSA, VRE, and *S. pneumoniae*. Only pine sapwood extract had an effect on *E. coli* and it was weaker than on other strains. Spruce showed a clear antibacterial effect on *S. pneumoniae* and a weaker effect on MRSA and VRE. The results suggest that these wood species have potential as surface materials in hospital and day care environments.

Keywords: Escherichia coli; MRSA; Streptococcus pneumoniae; VRE; Wood extracts

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INTRODUCTION

There are a number of infections that tend to be especially threatening to immunocompromised people such as very young and very old subjects. For this reason, in hospitals and day care centers, special attention to hygienic aspects is needed to avoid the spread of bacteria-causing infections in these groups of people. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) are examples of the strains with which hospitals are struggling (Hierholzer *et al.* 1995; (Dancer 2008). In day care environments, *Escherichia coli* O157:H7 has caused severe outbreaks of diarrhea (Reida *et al.* 1994; Rimhanen-Finne *et al.* 2014), and *Streptococcus pneumoniae* is the leading cause of acute otitis media among infants and young children (Bluestone *et al.* 1992; Kilpi *et al.* 2001). *S. pneumoniae* also causes pneumonia, bacteremia, and sinusitis (Van Beneden *et al.* 2000). Contaminated surfaces are one of the ways that infections are spread (Hierholzer *et al.* 1995; Dancer 2008) and with constant pressure to save money, proper surface cleaning in healthcare and daycare environments may be jeopardized. Wooden surfaces have shown antibacterial properties (Schönwälder *et al.* 2002; Milling *et al.* 2005; Vainio-Kaila *et al.* 2011, 2013), but the mechanisms behind them are still poorly known. It has been suggested that it is a combination of the drying of the surface caused by the porosity and the chemical composition of wood (Milling *et al.* 2005). Wood is composed mainly of cellulose, hemicellulose, lignin, and extractives. The extractives are a group of different chemical substances, the composition of which depends

on the wood species and the location in the trunk (Willför *et al.* 2003a,b). Several separate compounds such as pinosylvin (Välimaa *et al.* 2007; Plumed-Ferrer *et al.* 2013), its monomethyl ether (Välimaa *et al.* 2007; Plumed-Ferrer *et al.* 2013), resin acids (Söderberg *et al.* 1990; Smith *et al.* 2005) and some free fatty acids (Desbois and Smith 2010) have shown antibacterial effects mostly against Gram-positive bacterial strains. Also some lignans have been found to have antibacterial properties, but the effects are very weak (Välimaa *et al.* 2007; Al-Ani and Aziz 2013). Spruce resin has been found to have antimicrobial effects against several bacterial species including MRSA and VRE (Rautio *et al.* 2007). Knotwood extracts of Scots pine and several other wood species have been found to have both antibacterial and antifungal properties (Lindberg *et al.* 2004; Välimaa *et al.* 2007). A limited amount of literature has been published on the antibacterial effects of wood extracts as such. Laireiter *et al.* (2013) found that extracts of pine heartwood inhibited the growth of several Gram-positive bacterial strains. Extracts of Alaska cedar, western juniper, and several North American hardwoods have shown some antibacterial effect (Omar *et al.* 2000; Johnston *et al.* 2001). To investigate further the role of extractives in the antibacterial properties of wood and differences between some of the common wood species in Northern Europe, extracts of Scots pine (*Pinus sylvestris)* heartwood and sapwood and Norway spruce (*Picea abies*) were studied with *S. aureus*, *E. faecalis*, *E. coli*, and *S. pneumoniae*.

EXPERIMENTAL

Bacterial Strains

Bacterial strains used were MRSA (ATCC 43300), VRE (ATCC 51299), *S. pneumoniae* (ATCC 49619), and *E. coli* O157:H7, without *stx* genes (RHE5402). Prior to use, all strains were subcultured at least twice on sheep blood agar plates. The plates were incubated at 37 °C overnight in ambient atmosphere. *S. pneumoniae* incubation was supplemented with 5% CO_2. Bacterial colonies were moved directly from the agar to physiological NaCl solution, and their concentration was adjusted to 0.5 McFarland using a photometer (Gene-Trak Systems, Hopkinton, MA, USA). This was equivalent to the concentration of $1.5x10^8$ CFU mL^{-1}, which was further diluted to correspond to the concentrations of 1.5×10^7 and 1.5×10^5 CFU mL^{-1}.

Wood Extracts

Wood material was collected from Southern Finland. Scots pine was placed directly in the freezer and kept frozen (-20 °C) until used. The resulting pine sapwood and heartwood samples were taken based on the visible difference of sapwood and heartwood color. Spruce samples were taken near the pith. Wood material was milled to particles with a size of < 1mm and dried in 40 °C until the moisture content was *ca.* 11% (w w^{-1}). Particles were extracted with acetone using a Soxhlet apparatus for 6 h. Most of the acetone was evaporated from the extract using a rotary evaporator (Büchi; Switzerland) in a 40 °C water bath and approximately 400 mbar pressure. Extracts in the remaining acetone were poured in a small container, and the rest of the acetone was left to evaporate in ambient air. The extracts were stirred and weighed regularly until there was no observable change in weight. Extractive content was calculated based on dry wood weight. Prior to cultivations, the extracts were kept in 42 °C for approximately an hour to make them more viscous and hence easier to measure with a pipette.

Cultivations

The cultivations were carried in three parallel samples in test tubes with 900 µL FAB (fastidious anaerobe broth). For each bacterial strain there was a control, which was cultivated without extract, using only bacterial dilution in the FAB. Of the extracts, 100 µL were placed in 900 µL FAB, shaken, and were left in ambient temperature for a minimum of one hour. To the broth, 100 µL of the bacterial dilutions (1.5×10^7 CFU mL^{-1} for *S. pneumoniae* and 1.5×10^5 CFU mL^{-1} for *S. aureus*, *E. faecalis*, and *E. coli*) were then added making the final concentration in the tubes 1.5×10^6 CFU mL^{-1} for *S. pneumoniae* and 1.5×10^4 CFU mL^{-1} for other bacteria studied. The concentrations were chosen based on the results from preliminary studies. *S. pneumoniae* was incubated in an atmosphere of 5% CO_2, whereas other bacterial strains were incubated in ambient atmosphere. After incubations of 24 and 48 h at 37 °C, 50 µL of the broth was spread on a sheep blood agar that was incubated at 37 °C until the next day. Also, 20 µL of the broth was diluted in 2 mL of physiological NaCl solution. The NaCl solution was then shaken, and 50 µL was spread on a sheep blood agar that was incubated overnight at 37 °C. This gave approximately a 100-fold dilution. If the number of CFUs in the original broth could not be counted, the dilution results were used to calculate it whenever possible. The mean values and standard deviations of the three parallel samples were calculated. The control samples were estimated to have CFU > 10^8 as they grew a full mat on the agar.

The antibacterial effect of the extracts was also studied on Müller-Hinton II sensitivity agar plates, supplemented for *S. pneumoniae* with 5% of horse blood. With a pipette, 100 µL of each extract was dropped on the agar seeded with bacteria according to the EUCAST disc diffusion method (Matuschek *et al.* 2014). After incubation overnight at 37 °C, the area around the extract drops without visibly growing bacteria was measured with a ruler. The plates seeded with *S. pneumoniae* were incubated in an atmosphere of 5% CO_2, whereas other bacterial strains were incubated in ambient atmosphere.

RESULTS AND DISCUSSION

Extracts

The acetone-soluble extractive yields obtained were 9.8 (±1.8)%, 3.7(±0.2)%, and 1.4(±0.1)% for pine heartwood, pine sapwood, and spruce, respectively. The yields correlate fairly well with literature (Lindgren and Norin 1969; Martínez-Iñigo *et al.* 1999) with the exception of pine sapwood, which had a somewhat higher yield than that given in literature. This is explained by natural variation between individual trees and measurement accuracy.

The extracts are composed of a large variety of compounds. They have been extensively analyzed in earlier literature (Martínez-Iñigo *et al.* 1999; Piispanen and Saranpaa 2002; Willför *et al.* 2003a,b). The amount of components in pine heartwood and sapwood and spruce, which have shown antibacterial properties are collected in Table 1. Most antibacterially active compounds, such as resin acids, pinosylvins, and free fatty acids are most abundant in pine heartwood. Only lignans are found exclusively in spruce and their antibacterial activity has been quite minor. The main groups besides these are sugars (Saranpää and Höll 1989) and triglycerids (Saranpää and Nyberg 1987; Willför *et al.* 2003b) which are both more abundant in pine sapwood than heartwood.

Table 1. Relevant Extractives in Pine and Spruce from Literature *

	Resin acids	Lignans	Pinosylvins	Free fatty acids	Triglycerids	References
Pine heartwood	7.5-25 32.09		8.8-12	4.5-13.2 12-18 2.15	0.14-0.39 1.02	(Willför *et al.* 2003b) (Piispanen and Saranpaa 2002) (Martínez-Iñigo *et al.* 1999)
Pine sapwood	1.4-3.5 8.92		0.12-0.18	0.08-0.57 0.7 3.96	7.8-23 26 7.3	(Willför *et al.* 2003b) (Piispanen and Saranpaa 2002) (Martínez-Iñigo *et al.* 1999)
Spruce	0.8-2.4	0-2.5		1.2-2.8		(Willför *et al.* 2003a)

* Amounts are given in mg g^{-1} of dry wood

Bacterial Findings

The cultures in FAB-media showed clear differences between different wood species and bacterial strains (Fig. 1).

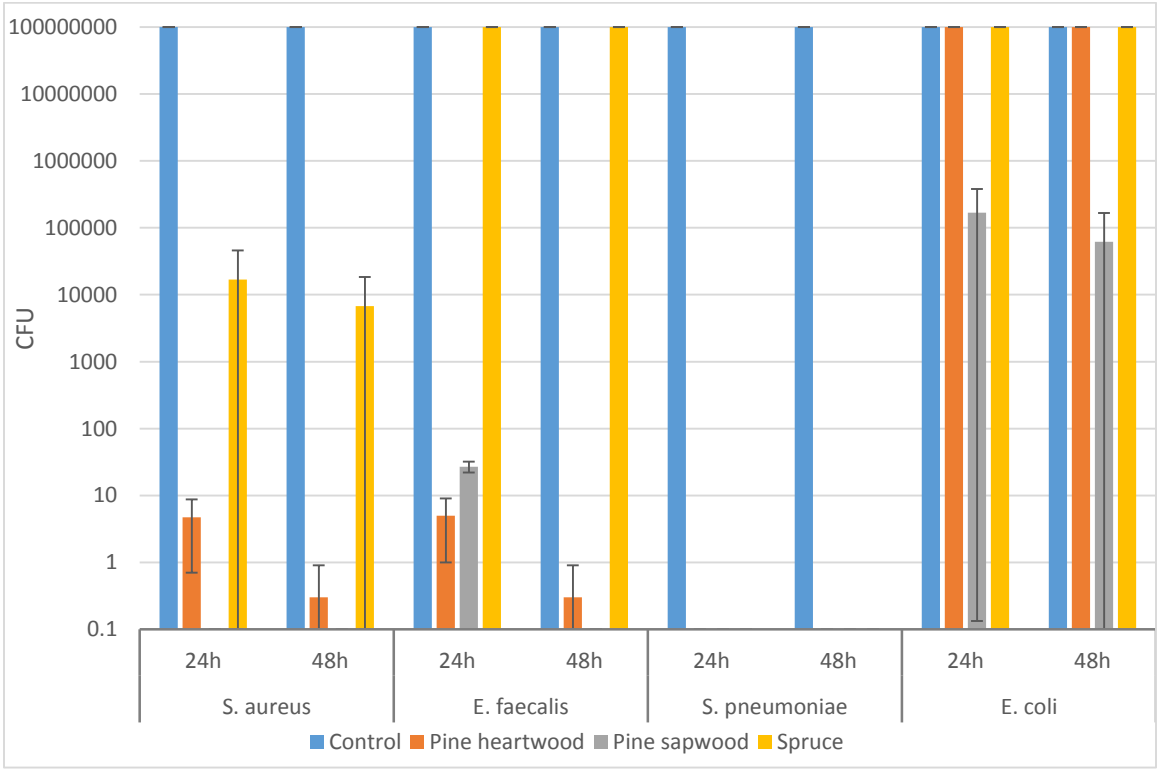

Fig. 1. The number of bacteria growing on blood agar plates after 24 and 48 h incubation in FAB media with 100 µL extracts or without extracts (control)

The growth of *S. aureus* and *E. faecalis* was inhibited by both pine extracts. With sapwood, some growth of *E. faecalis* was still observed after 24 h incubation, but after 48 h, no viable bacteria were found. Spruce had an antibacterial effect on *S. aureus* and a weak

effect on *E. faecalis*. *S. pneumoniae* was the most sensitive to the presence of extracts. There were no viable bacteria after 24 or 48 h of incubation with any of the extracts, even though the initial concentration of bacteria was higher with *S. pneumoniae* than with the other bacterial strains. Only pine sapwood had an effect on *E. coli*, but even that was weaker than was seen with other bacterial strains.

Rautio *et al.* (2007) found the genus *Streptococcus* to be the most sensitive to spruce resin in similar tests, followed by *Staphylococcus* and *Enterococcus*, which was true in the present tests. Also, they found *E. coli* unaffected by spruce resin, as our test results support, with the exception of pine sapwood.

The antibacterial effect of pine extracts was rather bactericidal than bacteriostatic since there was either no growth at all or the growth was similar to the control, as was with *E. coli*. On the other hand, the effect of spruce extract on *S. aureus* and *E. faecalis* was closer to bacteriostatic, with a constant level of bacterial growth, yet less than on the control.

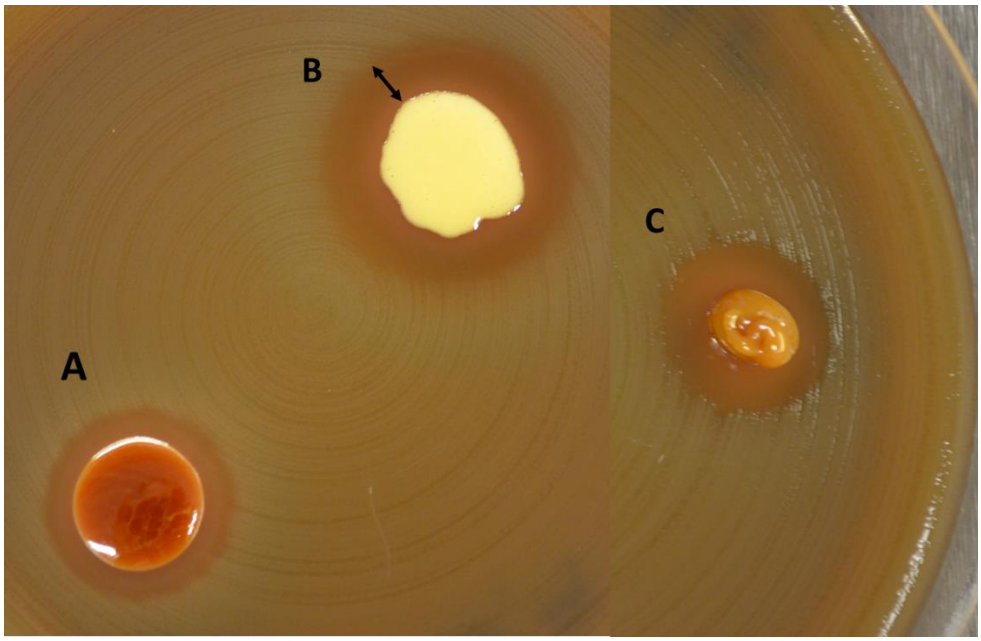

Fig. 2. Pine heartwood (A) sapwood (B) and spruce (C) extract and the areas of inhibition on *Streptococcus pneumoniae*. The arrow shows how the area of inhibition was measured.

For the diffusion test, the extractive drops were placed on agar seeded with bacteria with the help of a pipette. Based on the differing viscosity of the extracts, the resulting drops were not perfectly round or the same size. For these reasons, the results are shown as an area of inhibition (Fig. 2), where the size is only the size of the bacteria-free area, without the drop, contrary to conventional sensitivity testing. Some areas were very uneven, and in these cases the results show the range measured around the drop (Table 2).

On the plates with *S. aureus* there was an area of inhibition around all extracts. The largest areas were around both pine extracts followed by spruce. With *E. faecalis*, both pine extracts formed inhibition areas, whereas spruce did not cause any inhibition. Also in these tests, *S. pneumoniae* was the most sensitive strain. Pine sapwood had the largest area of inhibition followed by spruce. Pine heartwood had the smallest areas of inhibition with *S. pneumoniae*. None of the extracts formed any inhibition area with *E. coli*. The solubility

of the substances in agar has more of an effect on these results than the results of the FAB-test, as the test tubes in FAB-test were vortexed. This might explain why the results differed from each other. For example, both pine heartwood and spruce showed stronger antibacterial effect in the FAB-tests. Also, the extract of pine sapwood did not form any area of inhibition with *E. coli* but showed a clear antibacterial effect in the FAB-test. The FAB-test was more sensitive, showing also weaker antibacterial effects than the diffusion test.

Table 2. The Area of Inhibition (mm) around the Extractive Drops on Müller-Hinton II (*S. aureus, E. faecalis*, and *E. coli*) or Müller-Hinton II-F Horse Blood Agar (*S. pneumoniae*)

S. aureus	Pine heartwood	2
	Pine sapwood	1-3
	Spruce	0.5
E. faecalis	Pine heartwood	0.5
	Pine sapwood	0-2.5
	Spruce	-
S. pneumoniae	Pine heartwood	2
	Pine sapwood	4
	Spruce	3
E. coli	Pine heartwood	-
	Pine sapwood	-
	Spruce	-

It was somewhat surprising that pine sapwood was so effective, since the antibacterial extractives shown in Table 1 are generally more abundant in heartwood than sapwood. Yet, sapwood was the only extract showing antibacterial activity against *E. coli*. This is, however, in accordance with our earlier results with solid pine sapwood and heartwood samples, where the *E. coli* mortality rate was higher on sapwood than on heartwood (Vainio-Kaila *et al.* 2013).

Some studies have found the antibacterial effects of wood to be stronger with Gram-negative bacteria (Schönwälder *et al.* 2002; Milling *et al.* 2005) when most research on extractives and natural compounds have reached an opposite conclusion (Söderberg *et al.* 1990; Rautio *et al.* 2007; Välimaa *et al.* 2007; Laireiter *et al.* 2013). Sapwood cells, contrary to heartwood cells, are living cells and are therefore undergoing metabolic activity. This activity might produce substances that have an antibacterial effect, especially on Gram-negative strains (Conner and Kotrola 1995; Alakomi *et al.* 2000). To study this more, water-soluble extractives should be used instead of acetone extracts.

CONCLUSIONS

1. Both pine sapwood and heartwood extracts had clear antibacterial effects on MRSA, VRE, and *S. pneumoniae*.
2. Only pine sapwood was effective against *E. coli*, which was the only Gram negative strain used.
3. Also spruce had an antibacterial effect on the Gram positive strains, but it was weaker than that of pine.

4. The separate substances found to be antibacterial in the literature do not explain the results for pine sapwood.

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