Aalto University
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WOOD PRESERVATIVE POTENTIAL OF SCOTS PINE BARK AND KNOT EXTRACTIVES

Master's thesis for the degree of Master of Science in Technology submitted for inspection, Espoo, 20 November, 2013.

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Abstract of master's thesis

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Abstract

The purpose of this investigation was to assess the potential of Scots pine (*Pinus sylvestris* L.) bark and knot extractives to function as preservative chemicals. Scots pine was chosen to due to its importance in Finland; bark and knots were chosen due to their high extractives content and lack of industrial applications. The bark and knots of Scots pine were extracted with acetone and the extraction yields calculated. The chemical composition of the extracts was determined using gas chromatography-mass spectrometry, Fourier transform infrared spectroscopy, and UV Raman spectroscopy. Their antifungal activity was studied using a wood-free agar plate method.

The bark and knots of Scots pine were found to contain large amounts of extractives, 16.8% and 24.6%, respectively. The bark extract consisted primarily of monosaccharides and resin acids; the knot extract of resin acids and pinosylvins. Both extracts also contained smaller amounts of fatty acids and other compounds. The bark extract was found to have no significant antifungal activity: at a low concentration the extract promoted the growth of fungi, while at a high concentration it had no statistically significant effect on growth. The lack of activity was concluded to be due to the chemical composition of the extract. None of the main compounds present in the bark extract are significantly antifungal, and the saccharides most likely acted as an additional food source, leading to increased growth at the lower concentration. The knot extract inhibited the growth of fungi at the higher concentration, and the activity of the extract was concluded to be due to the presence of large amounts of pinosylvins, which are known for their antifungal properties.

The results of this investigation showed that Scots pine knot extractives may have potential as wood preservative chemicals. The knot extract showed significant antifungal character at a moderate concentration, and the extractives were obtainable in high yields. However, more study is needed before definitive conclusions can be drawn. Most importantly, the preservative potential of the extractives should be determined using tests that involve impregnation of wood. The use of extractives in wood impregnation is also subject to a number of general problems that should be studied before conclusions can be drawn.

Keywords Scots pine, extractives, pinosylvin, bark, knots, decay, antifungal

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Abstract of master's thesis

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Työn nimi Männyn kuoren ja sisäoksien kyllästepotentiaali		
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Tiivistelmä

Tämän työn tarkoituksena oli tutkia männyn (*Pinus sylvestris* L.) kuoresta ja sisäoksista saatavien uuteaineiden potentiaalia toimia lahonsuoja-aineina. Mänty valittiin sen teollisen merkittävyyden johdosta; kuori ja oksat niiden korkean uuteainepitoisuuden ja teollisten sovellusten puutteen vuoksi. Lahonsuojapotentiaalin selvittämiseksi männyn kuorta ja oksia uutettiin asetonilla ja uuteaineiden saannot laskettiin. Uutteiden kemiallinen koostumus määritettiin kaasukromatografia-massaspektrometrian, Fourier-muunnos infrapunaspektroskopian, sekä UV Raman spektroskopian avulla. Uutteiden antifungaalisia ominaisuuksia tutkittiin puuvapaan agarmaljamenetelmän avulla.

Männyn kuoren ja oksien havaittiin sisältävän merkittäviä määriä uuteaineita, 16.8% kuoressa ja 24.6% oksissa. Kuoriuute koostui pääosin monosakkarideista ja hartsihapoista, oksauute taas hartsihapoista ja pinosylviineistä. Molemmat uutteet sisälsivät myös pienempiä määriä rasvahappoja ja muita aineita. Kuoriuutteella ei havaittu olevan merkittäviä antifungaalisia ominaisuuksia: matalalla konsentraatiolla uute lisäsi sienten kasvua, korkealla konsentraatiolla uutteella ei taas ollut tilastollisesti merkittävää vaikutusta kasvuun. Antifungaalisten ominaisuuksien puutteen todettiin johtuvan kuoriuutteen kemiallisesta koostumuksesta. Mikään kuoriuutteen pääkomponenteista ei ole merkittävästi antifungaalinen, ja sakkaridit puolestaan saattoivat toimia ylimääräisenä ravinnonlähteenä, johtaen lisääntyneeseen kasvuun. Kuoriuute hidasti sienten kasvua merkittävästi korkealla konsentraatiolla, ja uutteen antifungaalisten ominaisuuksien todettiin olevan seurausta uutteessa suurissa määrin esiintyvistä pinosylviineistä. Pinosylviinien antifungaaliset ominaisuudet ovat hyvin tunnettuja.

Tämän työn tulokset osoittavat, että männyn sisäoksien uuteaineilla saattaa olla potentiaalia toimia lahonsuoja-aineina. Oksauutteella havaittiin olevan merkittäviä antifungaalisia ominaisuuksia, ja lisäksi uuteaineiden saanto oksista oli korkea. Tutkimusta tulee kuitenkin jatkaa ennen kuin oksauutteiden lahonsuojapotentiaalista voidaan vetää varmoja johtopäätöksiä. Ensisijaisen tärkeää on määrittää uutteiden lahonsuojapotentiaali kokeissa, joissa tarkastellaan uutteilla kyllästettyä puuta. Uuteaineiden käyttöön kyllästyksessä myös liittyy lukuisia yleisen tason ongelmia joihin täytyy perehtyä ennen kuin johtopäätöksiä voidaan tehdä.

Avainsanat Mänty, uuteaineet, pinosylviini, kuori, sisäoksat, laho, antifungaalinen

FOREWORD

I would like to give my sincerest thanks to the instructor of my thesis, Lauri Rautkari, for his invaluable help and encouragement during this project. I would also like to thank Professor Mark Hughes, the supervisor of my thesis, for his comments and support, and Professor Tapani Vuorinen for his assistance with all things chemical.

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Last but not least, I would like to thank James and my family for their support.

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CONTENTS

1 INTRODUCTION

1.1 Background

Wood is a natural and biodegradable material, and as such it can be digested and degraded by a variety of insects and microorganisms. Among the most important wood deteriorating organisms are the decay fungi. Decay fungi are beneficial in nature as they break down dead tissue, but in wood products decay is a significant problem, as it can dramatically shorten the service life of products.

The susceptibility of wood to decay varies greatly: some species are highly resistant, while some others have virtually no resistance at all. The differences in resistance are largely due to the presence of extractives, i.e. small compounds that cannot be classified as cellulose, hemicellulose, lignin, or their components. Highly durable wood species are characterised by a high content of extractives, while nondurable species contain little extractives by comparison. The wood of Finnish species tends to have fairly little resistance; the heartwood of Scots pine, the most common wood species in Finland, is moderately resistant to decay, but the sapwood has virtually no resistance at all. Unsurprisingly, the extractives content of Scots pine heartwood is known to be much higher than that of sapwood.

Many commercially important wood species have little resistance to decay, and many treatments have therefore been developed to improve this property. Methods of improving durability include both preservative treatments and wood modification methods. Preservatives have been very popular in the past, especially the highly efficient CCA (chromated copper arsenate) preservative, but recently new environmental regulations have placed strict limitations on the use of CCA. The loss of CCA and the new environmental regulations have created a great need to develop new environmentally friendly wood preservatives, and as natural durability enhancing products the wood extractives may be a solution to this need.

Wood extractives can improve the durability of wood, because the extractives have antifungal properties. Although the most potent extractives mixtures are usually found in tropical hardwoods, even the extractives of Scots pine can protect wood from decay and are therefore worth studying as potential wood preservation chemicals. Scots pine

heartwood is known to be rich in extractives, but the use of heartwood as a source of chemicals is wasteful, as the wood can be used in other applications. Scots pine bark and knots, on the other hand, make an ideal source of extractives, as neither is used in any high-value applications, but both are known to contain notable amounts of extractives.

1.2 Objectives

The objective of this thesis was to investigate the wood preservative potential of Scots pine bark and knot extractives. To achieve this objective, this work sought to determine the quantities of extractives extractable from bark and knots, their chemical composition, and their antifungal activity. To obtain additional information on the antifungal properties of Scots pine extractives, the effects of extractives concentration and fungal species on antifungal activity were also studied.

The antifungal properties of extractives were studied using a simple wood-free agar plate method. The agar plate test was chosen over a standardised decay test because of time limitations, and due to the wood-free nature of the method, the results should not be considered directly applicable to extractives impregnated wood. The compounds present in the extracts were qualitatively analysed using gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectroscopy with photoacoustic detection (FTIR-PAS), and UV Raman spectroscopy but no quantification was performed. The analysis was limited to compounds that could by studied by GC-MS; IR and Raman spectroscopies were merely used to provide supporting information. Factors such as tree age, height, or growth site were not taken into account in the study of chemical composition or antifungal activity.

2 WOOD EXTRACTIVES

2.1 Chemistry of extractives

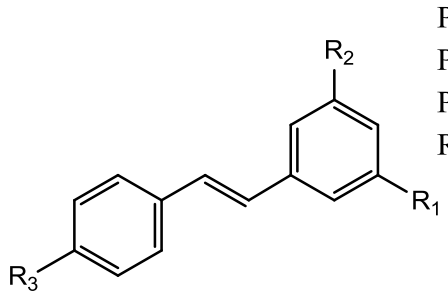
Wood extractives are a large and varied group of chemical compounds found in virtually all tissues of trees. Extractives are concentrated in resin ducts and ray parenchyma cells but smaller quantities can also be found in the middle lamella, tracheid/ fibre cell walls, and intercellular spaces (Fengel and Wegener 1989, p. 183). The term extractives can be defined in a handful of different ways, but in this work, the term is used to describe any small compounds that are extractable by organic solvents or, in some cases, water or inorganic solvents such as sodium hydroxide (NaOH). Defined in this way, the wood extractives can be divided into three groups: phenolic compounds, terpenes and terpenoids, and aliphatic compounds.

2.1.1 Phenolic extractives

The phenolic extractives are a large and diverse group of compounds. The phenolics can be divided into subgroups based on their structure, and while differences in classification exist, most classifications include the stilbenes, the lignans, the flavonoids, and the tannins. Other phenolic compounds, such as simple phenolics and phenolic acids, can also be found in wood. (Fengel and Wegener 1989 p. 194-198, Sjöström 1981 p. 92-95, Hillis 1987 p. 99-103)

The stilbenes are phenolic compounds with two aromatic rings connected by a conjugated double bond (Sjöström 1981, p. 95). Stilbenes are typically encountered in conifers, especially in pines, where they are present primarily in the heartwood and knots (Hillis 1962 p. 95, Fengel and Wegener p. 197, Willför et al. 2003a, Hovelstad et al. 2006, Pietarinen et al. 2006, Conde et al. 2013b). However, small quantities of stilbenes can also be found in the sapwood, bark, and cones (Celimene et al. 1999, Willför et al. 2009, Valentin et al. 2010, Conde et al. 2013b). Stilbenes typically found in pines include pinosylvin (PS), pinosylvin monomethyl ether (PSM), and pinosylvin dimethyl ether (PSD): PS and PSM are typically the dominant stilbenes and are present in far higher quantities than PSD (Willför et al. 2003a, Hovelstad et al. 2006, Willför et

al. 2009). The structures of PS, PSM, PSD, and a fourth stilbene, resveratrol, are depicted in Figure 1.



PS: R_1=R_2=OH, R_3=H
PSM: R_1=O-CH_3, R_2=OH, R_3=H
PSD: R_1=R_2=O-CH_3, R_3=H
Resveratrol: R_1=R_2=R_3=OH

Figure 1. Chemical structures of the stilbenes pinosylvin (PS), pinosylvin monomethyl ether (PSM), pinosylvin dimethyl ether (PSD), and resveratrol

Lignans are also phenolic compounds with two aromatic rings. Lignans, however, are structurally more varied than the stilbenes: the two phenyl propane units of lignans can be linked in a variety of different ways. (Fengel and Wegener 1989 p. 194). Lignans are widespread extractives and can be found in the sapwood, heartwood, bark, and roots of trees, but they are particularly concentrated in knots (Hathway 1962, p. 160, Holmbom et al. 2003, Willför et al. 2003a). Lignans found in pines include secoisolariciresinol, matairesinol, nortrachelogenin and liovil (Willför et al. 2003a). The structure of the lignan nortrachelogenin is shown in Figure 2.

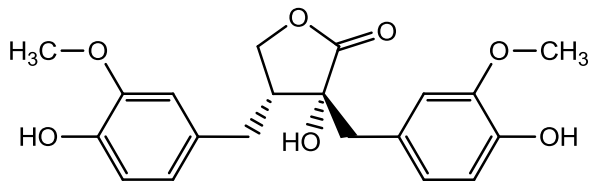


Figure 2. Chemical structure of the lignan nortrachelogenin

Flavonoids are a group of polyphenolic compounds that comprise several chemically distinct subgroups, including flavones, flavanes, flavanones, and isoflavanones. All flavonoids have a $C_6C_3C_6$ carbon skeleton and contain two aromatic rings. Although

many flavonoids occur independently in wood, some flavonoids, primarily catechins and leucoanthocyanidins, are also the building blocks of condensed tannins. (Sjöström 1981 p. 93, Fengel and Wegener 1989 p. 198). Flavonoids are also related to the stilbenes, with which they co-occur (Bate-Smith 1962 p. 145). A large number of different flavonoids have been identified in the different tissues of different wood species. In softwoods, however, pinocembrin, pinobanksin, taxifolin, and catechin are among the most common compounds and are present in a number of tissues, including heartwood, sapwood, knots, and bark (Fengel and Wegener 1989 p. 198, Karonen et al. 2004, Conde et al. 2013a, Conde et al. 2013b). The chemical structures of these compounds are depicted in Figure 3.

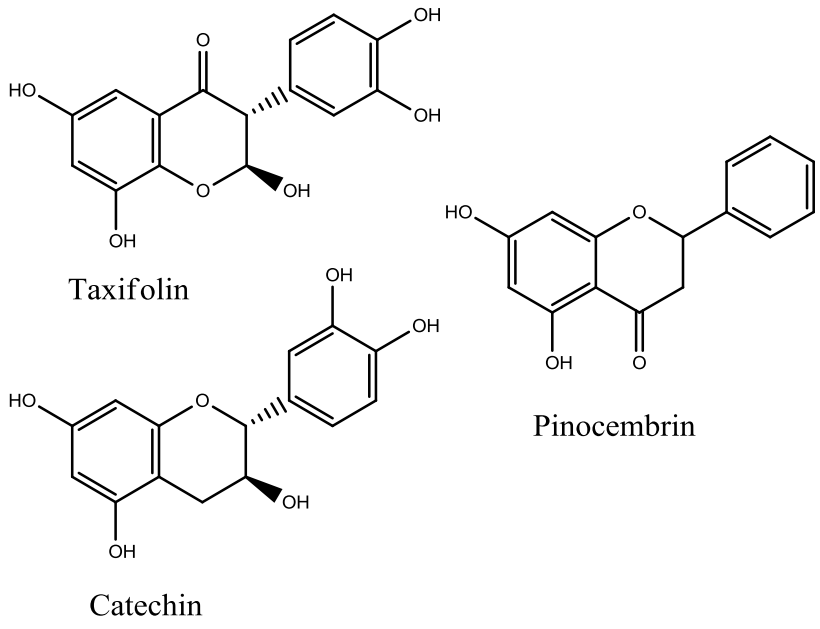


Figure 3. Chemical structures of the flavonoids taxifolin, catechin, and pinocembrin

Tannins are polyphenolic compounds characterised by their ability to convert hides into leather. As a consequence of this industrially important property, tannins have been studied extensively. As a group of chemical compounds, tannins can be divided into two subgroups, the hydrolysable tannins and the condensed tannins. The hydrolysable tannins are esters of a sugar with one or more polyphenolic carboxylic acids, and due to

the nature of the ester linkage, they are readily hydrolysed by acids, alkalis, enzymes, or even warm water. (Jurd 1962 p. 229-230, Fengel and Wegener 1989 p. 207). Hydrolysable tannins are relatively scarce in wood (Sjöström 1981 p. 93).

Condensed tannins, on the other hand, are fairly common in wood. Condensed tannins are polymers of the flavanol-type flavonoids, and their formation is initialised by the condensation of two flavonoid units to form a biflavonoid (a proanthocyanidin). The proanthocyanidins then polymerise further to form condensed tannins. True condensed tannins contain 3 to 8 flavonoid units, but larger compounds have also been isolated from wood. (Fengel and Wegener 1989 p. 209, 211). Tannins occur in a variety of wood tissues, including heartwood, sapwood, and cones (Hillis 1962 p. 66, Anttila et al. 2013) but are often particularly concentrated in bark. Proanthocyanidins and condensed tannins of different degrees of polymerisation have been isolated from the bark of pines (Pan and Lundgren 1996, Matthews et al. 1997, Willför et al. 2009). Figure 4 shows an example of the chemical structure of tannins.

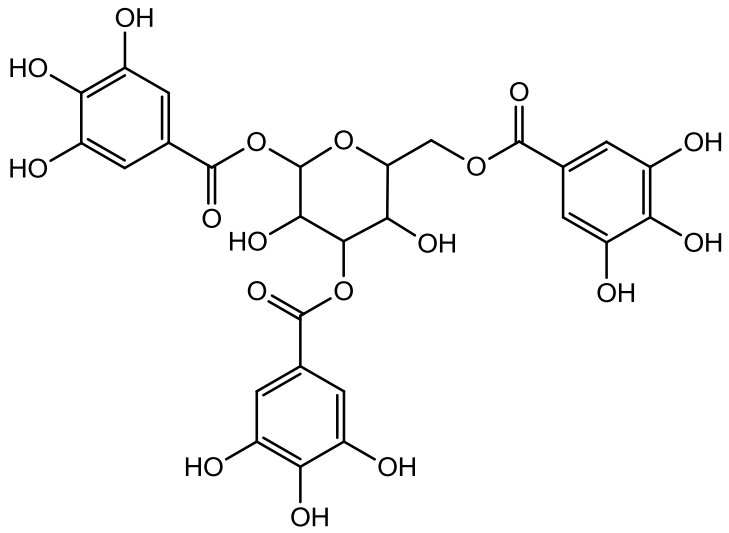


Figure 4. An example of tannin structure

In addition to stilbenes, lignans, flavonoids, and tannins, trees also contain other phenolic extractives such as phenolic acids, aldehydes, and alcohols (Bate-Smith 1962

p. 134, Fengel and Wegener 1989 p. 194). Phenolic acids include the C9-acids, such as coumaric, cinnamic, and caffeic acids, and smaller acids such as benzoic acids. C9 acids that bear a hydroxyl group at the ortho position can react intramolecularly to form coumarins. (Bate-Smith 1962 p. 134-135, 147). A number of phenolic acids and aldehydes have been identified in pines (Willför et al. 2009, Valentín et al. 2010).

2.1.2 Terpenes and terpenoids

Terpenes and terpenoids are a large group of compounds found in wood resin. Terpenes are derived from isoprene, and depending on the number of isoprene units in the terpene molecule, they are classified as monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), triterpenes (6 units), and so on. Terpenes are pure hydrocarbons, while terpenoids bear functional groups such as COOH, OH, or C=O. Resin acids, an important class of terpenoids, are diterpenes bearing a carboxylic acid group (COOH). Resin acids are present only in softwoods. The terpenes are often linked intramolecularly, forming one or more 6-membered rings; monoterpenes typically contain one ring, sesquiterpenes two, and diterpenes three. Terpene derivatives with a 7-membered ring are called tropolones and can be found in the family Cupressaeae. (Fengel and Wegener 1989 p. 184-188).

Terpenes and terpenoids are widely distributed in wood. Monoterpenes are primarily found in turpentine, the volatile fraction of wood resin, whereas diterpenes and resin acids are mainly a component of the non-volatile fraction (Sjöström 1981 p. 89, Fengel and Wegener 1989 p. 185). Terpenes have been extracted from the heartwood, sapwood, knots, bark, leaves, and cones of trees, and a very large number of compounds have been identified. In pines among the most common monoterpenes are α-pinene, β-pinene, limonene, and Δ^3-carene. The most common resin acids include abietic, neoabietic, dehydroabietic, pimaric, and palustric acids. (Norin and Winell 1972, Hafizôglu 1983, Krauze-Baranowska et al. 2002, Willför et al. 2003a, Hovelstad et al. 2006, Valentín et al. 2010, Kilic et al. 2011, Conde et al. 2013b). Examples of the structures of monoterpenes, resin acids, and tropolones are shown in Figure 5.

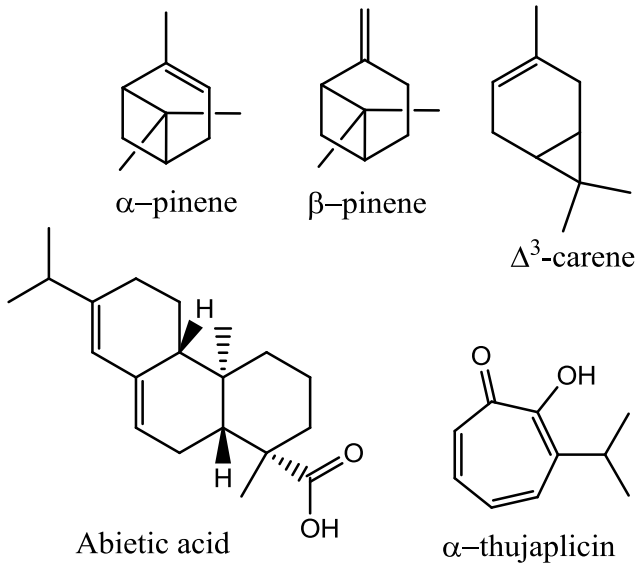


Figure 5. Chemical structures of the monoterpenes α-pinene, β-pinene, and Δ^3-carene, the resin acid abietic acid, and the tropolone α-thujaplicin

2.1.3 Aliphatic compounds

In addition to the phenolics and terpenes, wood extractives also constitute fatty acids, fatty alcohols, fats, and waxes. Fatty acids and alcohols are long-chain carboxylic acids and alcohols, respectively, and they are the components of fats and waxes. Fats are esters of fatty acids with glycerol whereas waxes are esters of fatty acids with fatty alcohols. (Fengel and Wegener 1989 p. 192). The abundance of fats and fatty acids is much higher than that of waxes and fatty alcohols (Sjöström 1981 p. 89, Fengel and Wegener 1989 p. 192).

2.1.4 Solubility and extraction

The solubility of extractives varies greatly from one solvent to another. Extractives are typically removed from wood by solvent extraction, and the choice of solvent is a critical factor that determines the chemical composition and yield of the extract. Although significant differences can exist between specific extractives, hydrophilic extractives (phenolics) generally have better solubility in more polar solvents, while lipophilic extractives (terpenoids and aliphatic compounds) have better solubility in less polar solvents. Acetone is an exception to this general trend, as it is known to dissolve

both hydrophilic and lipophilic extractives with great efficiency. (Harkin and Rowe 1971, Holmbom 1999 p. 126). As for inorganic solvents, aqueous alkali is highly effective at dissolving otherwise insoluble components, mainly some phenolic acids and the insoluble fraction of condensed tannins, while water is a moderately effective solvent for most phenolics (Harkin and Rowe 1971, Fengel and Wegener 1989 p. 254-255, Conde et al. 2013a). Supercritical CO_2, on the other hand, can extract all extractives in moderate yields (Conde et al. 2013b).

2.2 Extractives in Scots pine bark and knots

2.2.1 Bark

The bark is the outermost tissue of the tree trunk and accounts for 10-20% of its volume. The bark is composed of several morphological fractions but can be divided into two main fractions, the inner bark (phloem) and the outer bark (rhytidome). The tissues of the outer bark are dead and serve to protect the tree from abiotic stress and microbial invaders. (Sjöström 1981 p. 100, Fengel and Wegener 1989 p. 240)

The chemical compositions of barks from different wood species have been studied extensively, and even the bark of Scots pine has been the subject of some investigations. The different morphological fractions of bark vary in composition (Sjöström 1981 p. 100), but as a whole, the bark of Scots pine is characterised by a higher extractives and lignin content than the wood. The total extractives content is often measured to be some 20%, although, as always, it should be remembered that the yield of extractives is strongly dependent on the extraction method. Bark extractives have good solubility in water and polar organic solvents, but large amounts of bark chemicals can also be obtained by extractions with 1% NaOH. (Fengel and Wegener 1989 p. 244-245, 259, Valentín et al. 2010, Miranda et al. 2012)

The chemical composition of Scots pine bark has not been studied extensively, but it is known to contain a wide variety of phenolic, terpenoid, and aliphatic compounds. The phenolics have been studied by several researchers and have been found to include proanthocyanidins (the components of condensed tannins), lignans, flavonoids (primarily catechin and taxifolin and their derivatives), phenolic acids, and simple

phenols and aromatics (Pan and Lundgren 1996, Karonen et al. 2004, Yesil-Celiktas et al. 2009, Valentín et al. 2010). Proanthocyanidins are an important component of Scots pine bark, comprising 34% of the extract of Karonen et al. (2004) and accounting for 1% of the dry weight of bark in the work of Matthews et al. (1997). However, most of the proanthocyanidins in Scots pine bark are non-extractable (Matthews et al. 1997). In the work of Karonen et al. (2004) catechin and catechin derivatives accounted for 33% of the extract, but in the extract of Valentín et al. (2010) phenolics were present only in small quantities: the content of phenolic acids was 0.045% of the weight of dry bark and other phenolics were present in even smaller quantities.

Lipophilic extractives (aliphatics and terpenoids) have been accounted for by Norin and Winell (1972) and Valentín et al. (2010). Valentine et al. (2010) found the acetone extract of Scots pine bark to contain resin acids (0.5% of the dry weight of bark), sterols (0.28%), and fatty acids (0.25%). Norin and Winell (1972), who extracted only lipophilic compounds, found the bark to contain fatty compounds (fatty acids and alcohols), resin acids, monoterpenes (α- and β-pinene, Δ^3-carene, and *p*-cymene), sesquiterpenes, diterpenes, and triterpenes.

2.2.2 Knots

Knots are branch bases that have become embedded in the trunk of a tree (see Figure 6). The chemical composition of knots differs from that of the surrounding wood, primarily due to their high concentration of extractives. Extractives accumulate in knots after the branch has begun to decay: without the presence of antimicrobial extractives the decaying branch would serve as an easy point of entry for the decay organisms. Recently knots have become a topic of interest due to their high concentrations bioactive compounds, and several investigations detailing the extractives composition of knots have been undertaken. The knots of several species have been studied, and especially the pines have been found to contain exceptionally large quantities of extractives, often amounting to 20-30% of the dry weight of knots and far exceeding the extractives concentration of heartwood (Hillis 1987 p. 167).

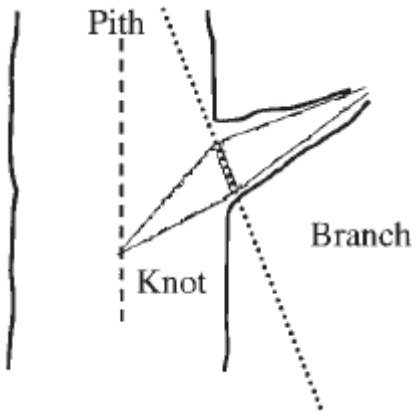


Figure 6. Knot (after Willför et al. 2003a)

The dominant group of extractives in the knots of Scots pine is the resin acids, although large variations exist in reported quantities. Willför et al. (2003a) found the concentration of resin acids in the dead knots of Scots pine to be 10-30% of the dry weight of knots and 2.5-20% in living knots, while Hovelstad et al. (2006) measured the concentration to be 5-10% without distinction between dead and living knots. The resin acids identified by Willför et al. (2003a) were pimaric, sandaracopimaric, isopimaric, levopimaric, palustric, abietic, neoabietic, and dehydroabietic acids. Abietic acid was the dominant compound, followed by neobietic and palustric acids. Hovelstad et al. (2006), on the other hand, identified neoabietic, abietic, dehydroabietic, levopimaric, and palustric acids, with abietic acid again being the dominant compound. While Willför et al. (2003a) and Hovelstad et al. (2006) recorded high concentrations, Lindberg et al. (2004) measured the concentration of resin acids in Scots pine knots to be fairly low.

The knots of Scots pine are also known to contain exceptionally high amounts of phenolic extractives, mainly stilbenes and lignans. The concentration of stilbenes has been found to be in the region of 2-8% (Willför et al. 2003a, Hovelstad et al. 2006, Pietarinen et al. 2006). Lindberg et al. (2004) did not record concentrations based on the dry mass of knots, but their results show that stilbenes were the dominant group of extractives in the Scots pine sample. The concentrations of PS and PSM are fairly similar in knots, while the concentration of PSD is significantly lower (Willför et al.

2003a, Lindberg et al. 2004, Hovelstad et al. 2006, Pietarinen et al. 2006). Lignans are found in Scots pine knots in amounts of 0.5-3%, with nortrachelogenin comprising over 90% of the lignans (Willför et al. 2003a, Pietarinen et al. 2006). Other lignans, such as liovil and mataisoresinol, are present only in trace amounts (Willför et al. 2003a). In addition to stilbenes and lignans, Willför et al. (2003a) also isolated trace amounts of the flavonoid pinocembrin.

3 EXTRACTIVES AS ANTIFUNGAL AGENTS IN PRESERVATIVES

3.1 Role of extractives in preventing decay

3.1.1 How rot fungi function

Wood material can be decayed by three types of rot fungi: brown rot, white rot, and soft rot. Brown rot and white rot fungi are Basidiomycetes, while soft rot fungi belong to either Ascomycetes or Fungi imperfecti. Brown rot fungi degrade primarily the polysaccharide components of wood, causing massive strength losses and turning the wood brown and brittle. Some chemical changes and degradation occur in lignin as well. White rots degrade lignin as well as polysaccharides, with degradation starting with lignin and advancing to cellulose and hemicelluloses. The degradation of lignin loosens the wood fibres, leaving the wood white and fibrous. Finally, soft rots are a varied group of fungi that digest both polysaccharides and lignin. With soft rots the rates of degradation and the strength losses imparted on wood depend heavily on the rot species in question. (Fengel and Wegener 1989 p. 374, 384)

Brown rot, white rot, and soft rot fungi all degrade wood by enzymatic action. Brown rot fungi employ a vast array of enzymes capable of degrading cellulose and the hemicelluloses, while white rot fungi use a host of ligninolytic enzymes and some polysaccharide degrading enzymes. The enzymes of rot fungi are highly specialised, and the successful degradation of wood components requires synergistic action by multiple enzymes. (Fengel and Wegener 1989 p. 375,-381, 384).

In addition to enzymes, wood degradation also involves the production of free radicals. Even the smallest wood degrading enzymes are too large to penetrate sound wood, and smaller degradation agents are therefore needed to initiate decay. Rot fungi have been found to use free radicals for this purpose, and several fungi have been discovered to employ an extracellular Fenton's system (Fe^{2+} H_2O_2). Organic acids have also been associated with brown rot decay. (Green and Highley 1997). The degradation of wood by rot fungi is therefore characterised by a need for iron, free radical production, and intense enzymatic activity.

3.1.2 How extractives defend against fungal attack

The mechanisms by which extractives impart decay resistance to wood are not fully understood, but the evidence suggests that extractives can function as free radical scavengers, metal chelators, and enzyme inhibitors. Free radical scavenging and metal chelation can both prevent the initiation of decay: free radical scavenging results in the removal of radicals needed for the initiation of decay, while metal chelation results in the removal of iron (Schultz and Nicholas 2000). Many extractives have indeed been found to possess notable free radical scavenging, antioxidant, and metal chelation capacity, including tannins, tropolones, lignans and flavonoids (Scalbert 1991, Diouf et al. 2002, Willför et al. 2003b, Pietarinen et al. 2006). Many wood extractives are also effective enzyme inhibitors, although no extractives compound has been found capable of preventing the production of a large number of different enzymes (Highley and Micales 1990, Scalbert 1991).

Wood extractives defend against fungal attack also by physical means. The presence of some extractives has been found to correlate with a reduction in the equilibrium moisture content of wood (Hernández 2007), and wood resin can form mechanical barriers that prevent the entry of invading organisms. The resin-based defences are activated when the wood becomes exposed: the volatile component of resin evaporates, leaving behind the resin acids that oxidatively polymerise to form a solid mass that traps the invaders (Phillips and Croteau 1999).

Extractives work as both constitutive and induced defences in trees. Large amounts of extractives are accumulated in susceptible wood tissues, and the natural durability of wood has been found to correlate with the concentration of many extractives (Hart and Shrimpton 1979, Harju et al. 2003, Venäläinen et al. 2004, Chong et al. 2009). Large amounts of extractives are accumulated in heartwood, which is dead tissue and cannot therefore mount an active defence, and in knots, which become pathways to microbial invasion when the branch begins to decay (Hart 1989 p. 867, Hillis 1987 p. 167). As induced defences the extractives are produced in response to stress or attack. Abiotic stress, wounding, and attack by microorganisms have all been found to result in an increase in the concentration of particular extractives. Tree individuals where the induced defences have been activated have been found to possess higher resistance to

further microbial attack than intact individuals. (Christiansen et al. 1999, Evensen et al. 2000, Cvikrova et al. 2006, Chong et al. 2009)

3.2 Antifungal activity of extractives

3.2.1 Problems in analysis

Extractives have a role in protecting the tissues of living trees from microbial invaders, and the transfer of this protective function to wood products is of great interest. Wood extractives are of interest both as isolated compounds and as natural mixtures of chemicals obtained by extraction (extracts). The wood preservative potential of extracts and extractives has been studied by a range of different methods, including both simple wood-free screening methods and methods that involve the impregnation of wood. The wood-free tests, such as agar plate and nutrient solution tests, involve the measurement of fungal growth on a medium that contains extractives, while tests that include impregnation measure the mass loss due to fungi of extractives impregnated wood.

The variability of research methods and the nature of extractives cause significant problems in analysis. The results are affected by a large number of factors, and obtaining a true assessment of the antifungal properties of extractives can prove difficult. One of the most important factors affecting the results is the choice of test method. Agar plate methods are easy, fast, and popular, but they tend to provide different results than tests involving impregnation, often exaggerating the antifungal potency of the extractives. The reason for this is believed to be that the extractives are free to interact with the fungi in agar solution but are, in some way, bound to wood in its presence. (Hart and Shrimpton 1979, Hart 1989 p. 871). Significant differences exist even between different wood-free methods and impregnation tests (Hart 1989 p. 871).

Another important factor is the selection of fungi used in the test. Most wood extractives are not equally effective towards all fungi, and some can show excellent activity towards one fungus and absolutely no activity towards another (e.g. Seppänen et al. 2004, Kofujita et al. 2006). Results are also affected by the duration of the test and the concentration of extractives. In the case of extracts, comparability is also complicated by variations in the chemical nature of the extracts. The chemical

composition of extracts depends strongly on the extraction method but also on the age of the tree, growth site, genetic characteristics, the vertical and radial location of the tissue in the tree trunk, and time of felling (Hafizôglu 1983, Fengel and Wegener 1989 p. 183, Hovelstad et al. 2006). Care should therefore be taken when drawing conclusions based on single tests.

3.2.2 Studies on the antifungal properties of extractives

Due to their speed and ease of use, wood-free methods such as agar plate tests have been used to study the antifungal properties of many wood-derived compounds. In the case of extracts, those from the heartwood of naturally durable tropical species have raised the most interest and have often proven highly successful (Reyes-Chilpa et al. 1997, Reyes-Chilpa et al. 1998, Mihara et al. 2005, Amusant et al. 2007). Barks from several species have also been studied, with the results showing that all of them are capable of reducing the growth of fungi to different extents (Harun and Labosky 1985, Alfredsen et al. 2008). Extracts from different tissues of Japanese cedar have also shown good activity (Cheng et al. 2005).

Simple wood-free methods have also been used to study many isolated extractives, including tannins, lignans, terpenes, stilbenes, tropolones, flavonoids, and simple phenolics. The tropolones have been found extremely toxic to fungi and are often considered the most toxic extractives found in wood (Hart 1989 p. 870, Yen et al. 2008). The stilbenes are also considered relatively toxic (Hart 1989 p. 869, Seppänen et al. 2004), although some researchers have found them moderately toxic at best (Celimene et al. 1999). Condensed tannins and terpenes have shown moderate activity, as have some lignans, although the toxicity of lignans is usually low (Hart 1989 p. 868, Wu et al. 2005, Céspedes et al. 2006, Anttila et al. 2013). Some flavonoids have also shown mild antifungal activity but most are nontoxic (Loman 1970, Malterud et al. 1985, Hart 1989 p. 869).

A number of extracts and extractives have also been used in experiments that involve impregnation of wood with said compounds. In the case of extracts, the experiments that have been performed have been dominated by extracts from the heartwood of

naturally durable tropical hardwood species. Some of such experiments have yielded excellent results, with susceptible wood showing little to no mass loss when impregnated with extractives (Smith et al. 1989, Kamdem 1994, Onuorah 2000, Onuorah 2002). However, some other heartwood extracts, such as those from walnut, were found to have almost no effect on durability (Hashemi and Latibari 2011, Feraydoni and Hosseinihashemi 2012). A few extracts not originating from the heartwood of tropical species have been studied as well. Mimosa bark, quebracho bark, and eucalyptus extracts have been found effective, while *Pinus brutia* bark and juniper sawdust extracts showed only moderate efficacy at best (Hart and Hillis 1974, Eller et al. 2010, Tascioglu et al. 2013).

In the case of isolated extractives, impregnation research has mainly been focused on the use of condensed tannins. The research has shown that although tannins can reduce mass loss due to fungi, the improvement is moderate at best and non-existent at worst (Laks and McKaig 1988, Eberhardt and Young 1994, Yamaguchi and Okuda 1998, Yamaguchi and Yoshino 2001, Yamaguchi et al. 2002). Chemically modified tannins have shown improved preservative potential, but even these have not been able to eliminate mass loss completely (Yamaguchi and Okuda 1998, Yamaguchi and Yoshino 2001, Yamaguchi et al. 2002). Although the toxicity of tannins is insufficient for preservative applications, their metal chelating properties make them an excellent fixing agent for other preservatives. The use of tannins has been found to significantly improve the stability of preservative agents such as copper, which alone is leachable. (Laks and McKaig 1988, Scalbert et al. 1998, Yamaguchi and Okuda 1998, Yamaguchi and Yoshino 2001, Yamaguchi et al. 2002)

Other extractives studied in wood impregnation include the stilbenes, tropolones, resin acids, vanillin, and some tropical hardwood phenolics. The stilbenes have been studied by Celimene et al. (1999) and Seppänen et al. (2004), and while Seppänen et al. (2004) found the treatments successful, with a combination of PS and resveratrol eliminating mass loss completely, Celimene et al. (1999) found that the stilbenes had relatively little effect on mass loss. Tropolones were found to eliminate mass loss completely, and impregnation with vanillin and two of the phenolics also resulted in low mass losses

(Eslyn et al. 1981, Diouf et al. 2002, Rättö et al. 2005). Impregnation with resin acids, on the other hand, had very little effect on mass loss (Eberhardt et al. 1994).

Data on the antifungal activities of different isolated extractives are summarised in Table 1 below.

Table 1. Summary of the antifungal activities of different isolated extractives (++++ excellent activity, +++ good activity, ++ moderate activity, + poor or no activity, - not tested)

Compound	Toxicity
Phenolics	
Stilbenes	+++
Flavonoids	+
Lignans	+
Tannins	++
Other phenolics	+++
Terpenoids	
Monoterpenes	-
Resin acids	+
Tropolones	++++
Other terpenes	++
Aliphatics	-

3.3 Potential and challenges

The theoretical potential of extractives as a source of preservative chemicals is great. Many extractives are highly effective towards wood rot fungi, and they are derived from fully renewable resources. In Finland among the most interesting extractives are those from Scots pine. Bark and knots are particularly interesting as sources of extractives, because both contain large amounts of extractives and neither is currently used in any high-value industrial applications. Bark is mainly burned for fuel, while knots are considered a material defect and discarded where possible. The separation of these materials from wood should be possible on an industrial scale, since bark is already separated from wood in debarking, and methods have been developed for the separation of knot tissue from pulp wood chips (Holmbom et al. 2003).

Despite the great theoretical potential of extractives, a number of challenges and problems still exist. One of such challenges is, naturally, the toxicity of the extractives. Many extractives have high antifungal activity, but most of these originate from the heartwood of tropical hardwoods or other sources that have limited availability. Scots pine bark and knots are interesting sources of extractives in Finland, but their antifungal potency may prove insufficient. None of the isolated extractives found in bark (aliphatics, tannins, lignans, flavonoids, and resin acids) are known for substantial antifungal activity, and when tested on agar the bark extract was found to reduce the growth of fungi only by 20-50% depending on the fungus (Hart 1989 p. 868-869, Eberhardt et al. 1994, Scalbert et al. 1998, Alfredsen et al. 2008, Anttila et al. 2013). Knots contain large amounts of the antifungal stilbenes, but even their toxicity might prove insufficient: stilbenes are highly toxic to fungi on agar but are known to lose toxicity when brought into contact with wood (Loman 1970, Hart and Shrimpton 1979, Hart 1989 p. 871).

Unfortunately, the problems associated with extractives are not limited to toxicity. One of the most significant problems is that many wood extractives, unlike inorganic biocides, are not equally effective towards all fungi. One extractives compound can completely inhibit the growth of one fungus and at the same time promote the growth of another (e.g. Seppänen et al. 2004). Rot fungi are also capable of degrading extractives: several researchers have detected significant degradation in extended exposure (Bois et al. 1999, Martínez-Inigo et al. 1999, Sallé et al. 2005, Valentín et al. 2010).

Extractives also suffer from leachability when impregnated into wood. Extractives are not “locked” in the wood cell wall and can therefore be leached by water. Consequently, when extractives impregnated wood is soaked prior to durability testing, the durability of wood is reduced significantly (Yamaguchi et al. 2002, Rättö et al. 2005). Some modifications have been attempted to improve the stability of extractives (Rättö et al. 2005), but no solution that can completely eliminate leaching has yet been found.

4 MATERIALS AND METHODS

4.1 Material information

The wood material used in these experiments was Scots pine (*Pinus sylvestris* L.) grown in southern Finland. The materials were provided by Koskisen Oy and included six green Scots pine logs: three larger bottom logs and three smaller top logs. The bottom logs were used to study bark extractives and the top logs to study knot extractives. The trees were felled during late spring or early summer, but no detailed information about the time of felling or the age or growth site of the trees is available.

The fungi chosen for the agar plate tests were *Trametes versicolor*, *Rhodonia placenta* (previously known as *Poria placenta*), and *Coniophora puteana*. *T. versicolor* is a white rot fungus, while *R. placenta* and *C. puteana* are brown rot fungi. All three fungi were provided by the University of Helsinki. The brown rots are Finnish strains but the white rot is of foreign origin. The fungi were cultured on malt extract agar containing 2% (w/w) malt extract and 2% (w/w) agar. The original cultures were stored in a refrigerator and new cultures were prepared from these for use in agar plate tests. The test cultures were cultured at room temperature for 12 days before use in the experiment.

4.2 Wood material processing

The wood material was processed immediately after delivery. All the logs were brushed to remove lichen and grit and then manually debarked to remove both the inner and outer bark. Bark from each bottom log was collected into a separate container and placed in a conditioning chamber (RH 35%, 20 °C) to dry. The barks were allowed to dry for one week, after which they were manually splintered and ground to a fine powder (1 mm mesh) in a Wiley mill.

The debarked top logs were sawn into 3-5 cm thick discs with a band saw. The knots were removed from the discs by means of a plug and dowel cutter (inner diameter 25 mm) installed onto an upright drill, producing cylinders containing knot tissue. The knot cylinders were trimmed where necessary with a handsaw to remove sapwood from

the ends of the cylinders. However, no complete removal of sapwood was attempted. The knot cylinders from each log were collected into separate containers and placed into a conditioning chamber (RH 35%, 20 °C) to dry. The cylinders were allowed to dry for two weeks, after which they were ground to a fine powder (1 mm mesh) in a Wiley mill.

Both the bark and knot powders were stored at room temperature, in separate closed containers to protect them from moisture and sunlight until extraction.

4.3 Extraction

The bark and knot powders were Soxhlet extracted with acetone according to the standard SCAN-CM 49:03. The samples (approx. 10 g of each) were extracted with 300 ml of solvent for six hours. Bark and knot powders from different logs were extracted separately, except those that were to be used for chemical analysis. The dry matter content (c) of the samples was determined using a moisture balance (Precisa HA 300).

After extraction the solvents were evaporated by means of a rotary evaporator, with the exception of a portion of each chemical analysis sample. The samples were evaporated to a very small volume in the boiling flask, after which they were moved to smaller flasks and evaporated to dryness. The flasks were weighed and the extraction yields calculated according to Equation 1, where m_e is the combined weight of the extract and the small flask, m_f the weight of the empty small flask, c the dry matter content of the original powder, and m_p the weight of the original powder.

$$Yield\ (\%) = \frac{m_e - m_f}{c \cdot m_p} \cdot 100\% \qquad (1)$$

For agar plate tests the extracts were re-dissolved in acetone and solutions with concentrations of 2 and 20 mg/g were produced. The solutions were stirred with a magnetic stirrer until the solid extractives had dissolved.

4.4 Chemical analysis

4.4.1 IR and Raman spectroscopy

IR and Raman spectra were recorded for the bark and knot samples to study the functional groups and bonds present in them. Fully dried samples were used in spectroscopic measurements. The IR spectra were recorded using a FT-IR spectrometer (Bio-Rad FTS 6000) equipped with a photoacoustic cell (Gasera PA301) for detection. The spectra were collected in the 400-4000 cm^{-1} range and 200 scans were performed before Fourier transformation.

The Raman spectra were recorded using a UV Raman instrument (Renishaw 1000). The instrument was equipped with a UV-coated CCD camera for detection and an Innova 90C FreD frequency-doubled Ar^{+} ion laser, which was tuned to 224 nm for excitation. The spectra were collected through a Leica DMLM microscope (40× objective) using an acquisition time of 60 seconds. The samples were spun during measurement to avoid burning. The spectra were collected in the 220-2400 cm^{-1} range.

4.4.2 GC-MS

Individual components present in the bark and knot extracts were identified using gas chromatography-mass spectrometry (GC-MS). A Thermo Scientific ISQ series single quadrupole mass spectrometer was used, coupled with a Trace 1300 gas chromatograph. The extractives were pertrimethylsilylated (< 1 mg dry sample mixed with 0.5 ml dry pyridine and 0.25 ml BSTFA with 5 % TMCS) and analysed with a 30 m x 0.25 mm i.d. column coated with trifluoropropylmethyl polysiloxane (TraceGOLD TG-200MS, 0.25 µm film thickness). The oven temperature program was 2 min at 100 °C, 4 °C /min to 280 °C and 20 min at 280 °C. Helium was used as the carrier gas (1.2 ml/min). Mass spectra were recorded in the 50-700 (m/z) range at an ionization energy of 70 eV.

For compound identification the mass spectra were searched for selective ions corresponding to the molar masses of the expected trimethylsilylated compounds, or, where appropriate, molar masses of expected compounds minus the molar mass of a methyl ($-CH_3$) group. Resin acids were distinguished from one another based on their elution order and on their expected relative abundances.

4.5 Agar plate tests

Agar plate tests were performed on malt extract agar plates amended with extract solutions. The plates were produced by mixing deionised water, malt extract (2% w/w), and agar (2% w/w) to produce solutions. Extractives solutions (section 4.3) were then added to produce final extractives concentrations of 1 mg/g, 0.1 mg/g, and 0 mg/g (control). Acetone was added to the 1 mg/g and control plates to bring the final acetone concentration to 4.9% (w/w) on all plates. The solutions were sterilised in an autoclave, poured onto sterile plastic petri plates, and allowed to solidify.

For the determination of antifungal activity the plates were inoculated with small plugs (approximately 10 x 10 mm^2 in size) of fungal mycelium. The plugs were removed from pure cultures by means of a sterile surgical blade and placed on the plates, one plug per plate. The plugs contained both the fungal mycelium and the agar on which the fungus had grown. Five replicates were produced per type of plate. Inoculated plates were sealed with parafilm and incubated at 25 °C for 10 days. After incubation the plates were photographed, and the surface area of fungal mycelium determined using the area measurement tools in Adobe Photoshop (version CS5). The steps involved in image processing are illustrated in Figure 7. Finally, the antifungal activity (AFA) of each extract was calculated according to Equation 2, where *REF* is the average area of fungal mycelium on the relevant reference plates and *A* the area of fungal growth on each test plate.

$$AFA\ (\%) = \frac{(REF - A)}{REF} \cdot 100\% \qquad (2)$$

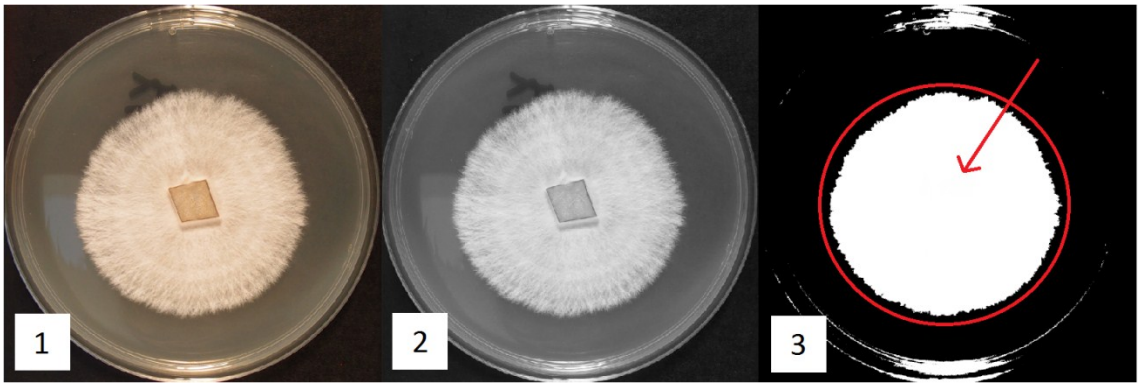


Figure 7. Photograph processing in Photoshop: original (1), greyscale (2), black and white (3). The surface area of fungal mycelium was determined from the black and white image

4.6 Statistical analysis

The results of the agar plate tests were analysed using Tukey's range test. The test was used to determine if the areas of fungal growth measured on the test plates differed significantly ($p<0.05$) from the areas on the appropriate reference plates.

5 RESULTS AND DISCUSSION

5.1 Extraction yields

The yields from the acetone extraction of Scots pine bark and knots are given in Table 2. Although the acetone soluble extractives content of Scots pine bark has not been reported, the 16.8% recorded here is similar to the 14-29% of acetone soluble extractives reported for other pine species (Hafizoğlu 1989, Willför et al. 2009). The acetone soluble extractives yield of Scots pine knots has not been reported either, but Hillis (1987 p. 167) states that the knots of pine species contain typically 20-30% extractives, although Willför et al. (2003a) have shown that in Scots pine even the content of resin acids alone can reach 30%. As resin acid contents of 30% were reached only in samples consisting solely of dead knots, the yield recorded here (24.6%) can be assumed to in the normal range for samples consisting of both live and dead knots.

Table 2. Yield of extracts from the bark and knots of different logs

Tissue	Log	Yield (%)
Bark	1	16.0
	2	16.5
	3	18.1
	Average	**16.8**
Knots	1	22.8
	2	25.0
	3	26.1
	Average	**24.6**

No large differences in yield were seen between different logs for bark or knot samples. The small differences may be due to random variations, but no definitive conclusions can be drawn without more information on the origin of the logs and more samples. The logs are, however, likely to have some differences in chemical composition: extract solutions from different logs differed slightly in terms of colour and, in the case of bark, also in cloudiness.

The extraction yields show that both the bark and knots of Scots pine are good sources of extractives, at least in terms of quantity. For comparison, the yield of acetone soluble

extractives from Scots pine heartwood is some 10%, while the yield from sapwood is only in the region of 5% (Harju et al. 2003, Rautkari et al. 2012). The total amount of extractives in Scots pine bark has not been determined, but the barks of most species have been found to contain some 20% of extractives soluble in organic solvents (Fengel and Wegener 1989 p. 244-245), showing that acetone is an effective solvent for Scots pine bark. Taking the extractives content of knots to be 20-30%, acetone can be concluded to be an effective solvent for knots as well.

5.2 Chemical analysis

5.2.1 IR and Raman spectroscopy

The FTIR-PAS spectra of the bark and knot extracts are shown in Figure 8. The spectra are similar, with both extracts showing a broad shouldered peak around 2970 cm^{-1} and strong peaks at ~1740, ~1367, and ~1220 cm^{-1}. The bands in the region of 2970 cm^{-1} most likely correspond to methylene groups (-CH_2) and the O-H bonds of carboxylic acids (Sun and Sun 2001, Nuopponen et al. 2005). The carboxylic acid bands could originate from any acids, but they most likely belong to resin acids, as these have been found to be the dominant compounds in both bark and knots (Willför et al. 2003a, Hovelstad et al. 2006, Valentín et al. 2010). The acid O-H vibrations were much stronger in the knot spectrum than the bark spectrum, and the knot spectrum also contained the characteristic band of resin acids, 1697cm^{-1} (Nuopponen et al. 2003), which the bark spectrum did not contain. The absence of the resin acid band in the bark spectrum is slightly surprising, since resin acids are known to be abundant in Scots pine bark as well (Valentín et al. 2010).

The peaks at 1740 cm^{-1}, in turn, correspond to esterified fatty acids (Holmgren et al. 1999, Nuopponen et al. 2003). Fatty acid esters are the components of fats and waxes: fats are esters of fatty acids with glycerol, while waxes are esters of fatty acids with alcohols. Particularly the fats are an important component of wood extractives, and their identification in the extracts was therefore not a surprising finding. Wood extractives also constitute free fatty acids, but these could not be identified in the IR spectra. (Fengel and Wegener 1989 p. 192)

The two spectra also contain a series of sharp bands in the region of 3900-3600 cm^{-1} and 1700-1400 cm^{-1}. Sharp bands at such wavenumbers are characteristic of water vapour, indicating that the samples may not have been fully dry at the time of measurement or that they may have been contaminated. In the knot spectrum the water vapour bands at 1700-1400 cm^{-1} are very pronounced and may be masking other bands. For example, both Sun and Sun (2001) and Nuopponen et al. (2005) have identified the aromatic ring stretch of phenolic compounds at 1520 cm^{-1}. The knot spectrum also contains a wide shouldered band at approximately 2300 cm^{-1}; a signal at 2300 cm^{-1} is typical of CO_2 and indicates contamination. Contamination of the sample is possible, but unfortunately its extent is not known.

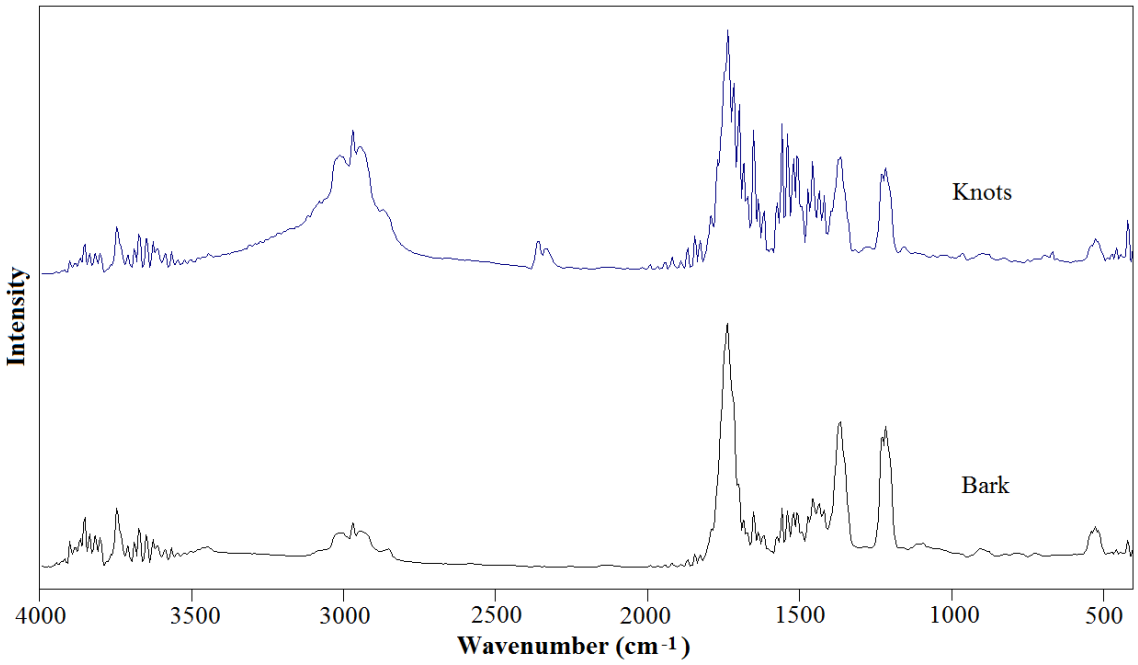


Figure 8. FTIR-PAS spectra of bark and knot extracts

The UV Raman spectra of bark and knot extracts are shown in Figure 9. The spectra of the two extracts are very similar, with two partially overlapping main bands at 1648 cm^{-1} and 1609 (bark) or 1604 (knots) cm^{-1}. Nuopponen et al. (2004a,b) and Holmgren et al. (1999) have studied the Raman spectra of Scots pine and suggest that

the bands originate from alkene stretching and aromatic ring stretching, respectively. Resin acids and fatty acids are the major contributors to the alkene stretching (Nuopponen et al. 2004a,b), but the aromatic ring stretch could originate from any phenolic compounds or dehydroabietic acid. Holmgren et al. (1999) suggest that the ring stretch corresponds to pinosylvins, but this is an unlikely explanation in the case of the bark sample, as bark is known to contain minor amounts of pinosylvins at best (Valentín et al. 2010).

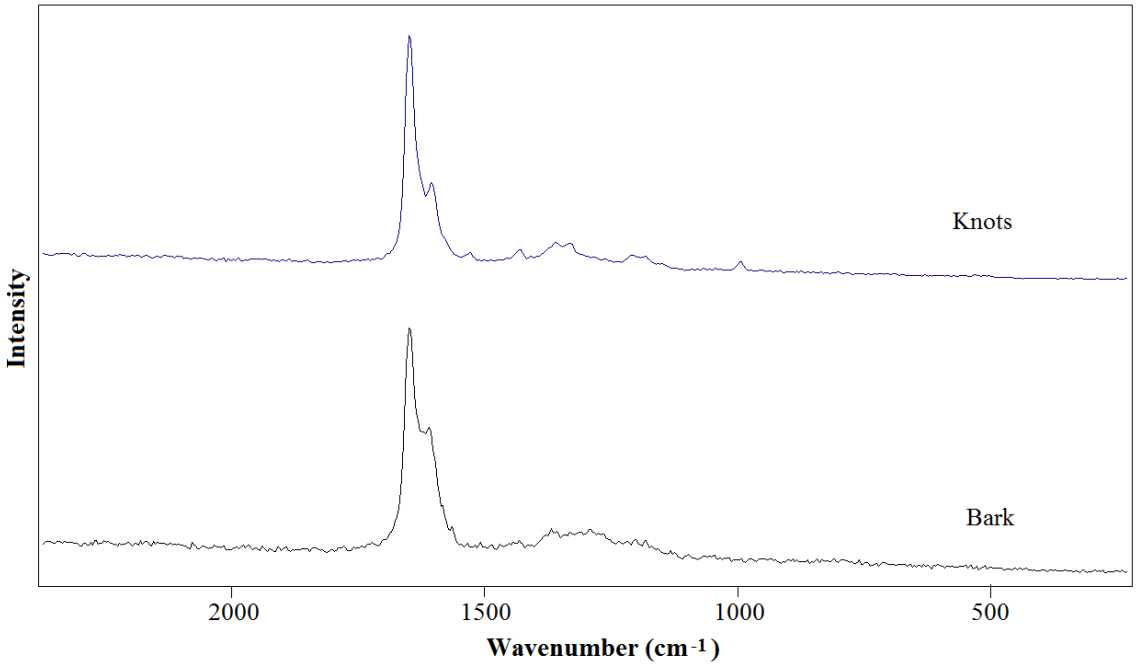


Figure 9. UV Raman spectra of bark and knot extracts

The identification of resin acids, fatty acids, and aromatic compounds in both extracts is not surprising. Resin acids and fatty acids are the dominant compounds in Scots pine bark (Valentín et al. 2010) and therefore an expected finding. In bark the aromatic compounds can be accounted for by the presence of small amounts of phenolic compounds, mainly proanthocyanidins and flavonoids, and the resin acid dehydroabietic acid, which is the dominant resin acid in Scots pine bark and contains an aromatic ring (Karonen et al. 2004, Valentín et al. 2010). Resin acids are also the dominant

compounds in knots, along with the phenolic stilbenes and lignans (Willför et al. 2003a, Hovelstad et al. 2006). Scots pine knots also contain small amounts of fatty acids (Willför et al. 2003a).

In addition to the main bands a series of fingerprint bands can be identified in the spectrum of the knot extract. The bands at 1360, 1331, 1208, and 1182 cm^{-1} are most likely attributable to resin acids. The 1360 cm^{-1} band corresponds to conjugated resin acids (abietic, neoabietic, palustric, levopimaric, and dehydroabietic acid), while the bands at 1208 and 1182 cm^{-1} are characteristic of abietic and neoabietic acids (Nuopponen et al. 2004b). The presence of the 1360 cm^{-1} band and the 1208 and 1182 cm^{-1} bands is not surprising, as abietic, neoabietic, and palustric acids are the dominant compounds in Scots pine knots (Willför et al. 2003a). The band at 995 cm^{-1}, on the other hand, is known to correspond to the 1,3,5-substituted aromatic ring of pinosylvins (Nuopponen et al. 2004b). The absence of this peak in the bark spectrum shows that pinosylvins are not present in the bark sample.

5.2.2 GC-MS

The gas chromatogram of the bark extract is shown in Figure 10. The extract was found to contain a large number of different compounds, including monosaccharides, resin acids, fatty acids, sterols, and phenolics. Pertrimethylsilylated monosaccharides were found at the lowest retention times (6.54 and 6.84 min). The monosaccharides could not be conclusively identified, but their mass fragment composition (m/z 191, 204, 217, and 540) suggests that they are hexopyranose sugars, possibly the two anomers of glucose. The m/z 191, 204, and 217 mass fragments are characteristic of glucose and other hexopyranose sugars (DeJongh et al. 1969), while the m/z 540 ion corresponds to their molar mass. Glucose has been previously identified in the acetone extracts of Scots pine bark (Valentín et al. 2010), and significant amounts of unspecified sugars have been found in the bark extracts of a number of different pine species (Willför et al. 2009). In this work the sugars were found to be a major contributor to the bark extract, having the second highest abundance, while Valentín et al. (2010) found sugars to be present only in small amounts.

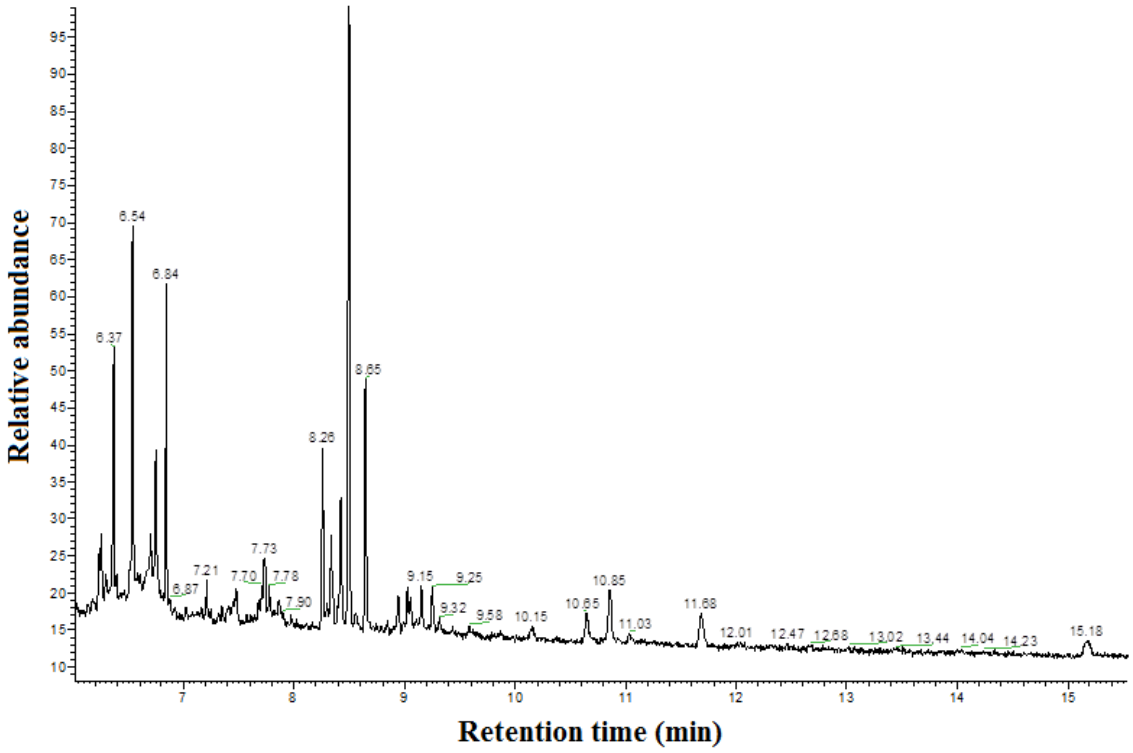


Figure 10. Gas chromatogram of bark extract (retention times 6-15.5 min)

In addition to monosaccharides the bark extract also contained large amounts of resin acids. Five resin acids (retention times 8.26, 8.33, 8.42, 8.65, and 9.02 min) were identified based on m/z 374 mass fragment, which corresponds to the molar mass of most trimethylsilylated resin acids. A sixth resin acid was identified based on the m/z 372 mass fragment. The molar mass of the sixth resin acid differs from those of the other resin acids by 2: the acid contains an additional double bond and can therefore be conclusively identified as dehydroabietic acid. The identities of the other five resin acids were obtained based on the known elution order of resin acids (Ekeberg et al. 2006, Valentín et al. 2010) and on comparisons of measured resin acid abundances in the bark and knot extracts to those presented in the literature (Willför et al. 2003a, Hovelstad et al. 2006, Valentín et al. 2010). The resin acids could not be conclusively identified without the use of reference compounds, but based on the elution order and the abundances, the most likely matches are pimaric, isopimaric, palustric, abietic, and

neoabietic acids (retention times 8.26, 8.33, 8.42, 8.65, and 9.02 min, respectively). The structures of the six resin acids are shown in Figure 11.

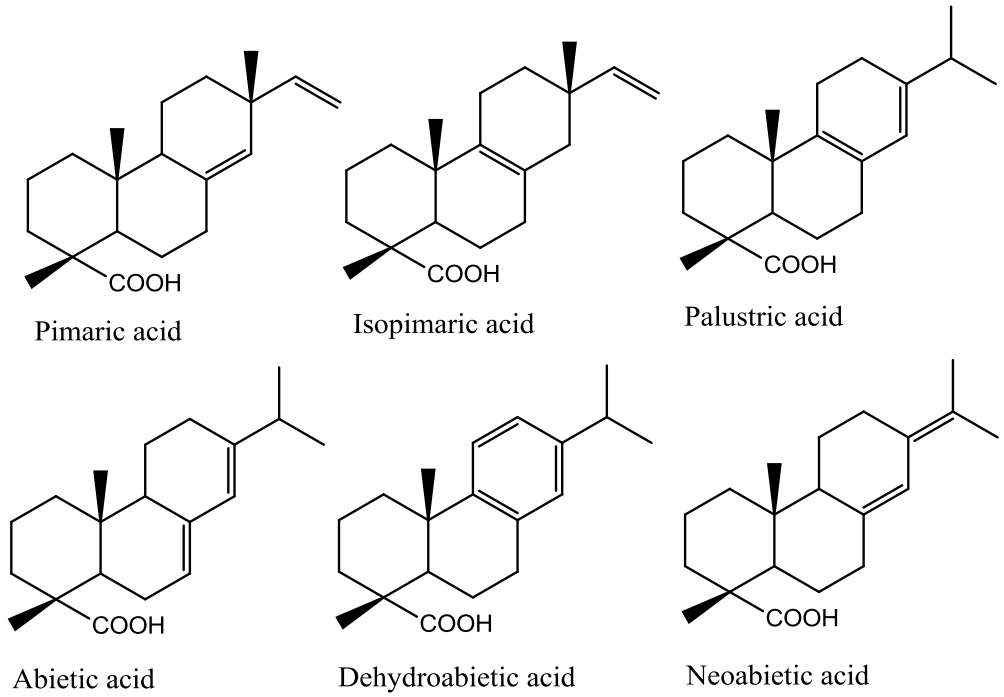


Figure 11. Chemical structures of the resin acids identified in this experiment

The resin acid dehydroabietic acid was the dominant compound in the bark extract, having an abundance far higher than any other compound. Pimaric and abietic acids were also relatively abundant, although their concentration was not as high as that of the monosaccharides. The results are very similar to those of Valentín et al. (2010), who also found dehydroabietic acid to be the dominant compound, followed by pimaric and abietic acids. Other acids were lower in abundance, and much like in this work, neoabietic acid was found only in minute amounts.

The bark extract was also found to contain smaller amounts of other lipophilic compounds as well as some phenolics. The lipophilic compounds included fatty acids, fatty alcohols, oxidised resin acids, and sterols. The fatty acids identified were oleic (C18:1), linoleic (C18:2), stearic (C18), and behenic (C22) acids. These four fatty acids

have been previously identified in Scots pine bark by Valentín et al. (2010): oleic, linoleic, and behenic acids were the dominant fatty acids, while stearic acid was present only in minute amounts. The fatty alcohols, on the other hand, included only one compound, behenyl alcohol (C22). Willför et al. (2009) have found this and other fatty alcohols in the barks of several pine species, but the fatty alcohols of Scots pine have not been previously reported. As for the sterols and oxidised resin acids, the compounds sitosterol, hydroxydehydroabietic acid, and a compound similar to hydroxydehydroabietic acid were identified on the basis of the molar masses of their trimethylsilyl derivatives. These compounds have previously been identified by Valentín et al. (2010), although in their work sitosterol was found to be present in much higher amounts. The chemical structures of some of these compounds are given in Figure 12.

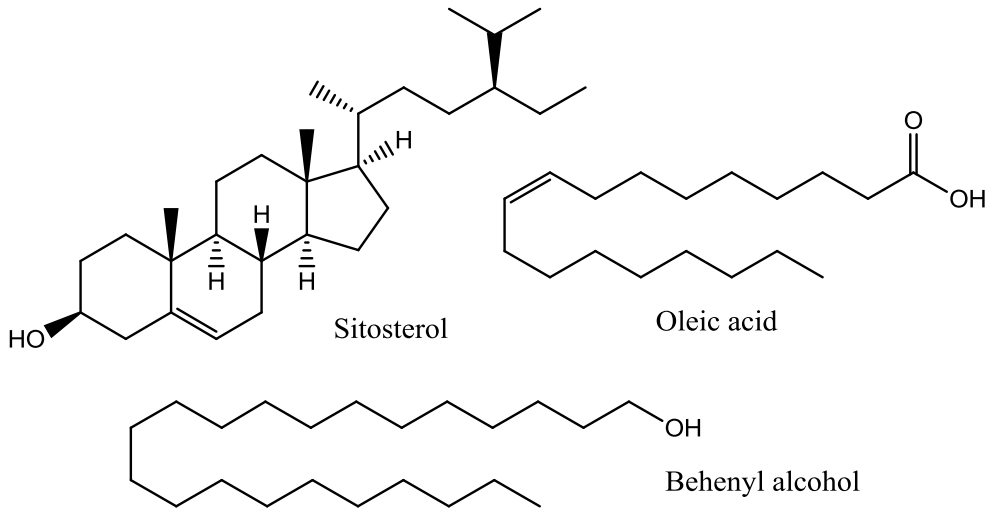


Figure 12. Chemical structures of the sterol sitosterol, fatty acid oleic acid, and the fatty alcohol behenyl alcohol

The phenolic bark constituent included two flavonoids and a phenolic acid. The two flavonoids were identified as taxifolin and catechin (or epicatechin), while the acid was identified as dihydroxybenzoic acid. Both of these compounds are known to occur in Scots pine bark: taxifolin and catechin are the most important bark flavonoids, while the acid is the most prominent simple phenolic in bark (Karonen et al. 2004, Valentín et al.

2010). The chemical structures of these compounds are shown in Figure 13. In addition to the aforementioned extractives, the bark extract also contained a number of compounds that could not be identified.

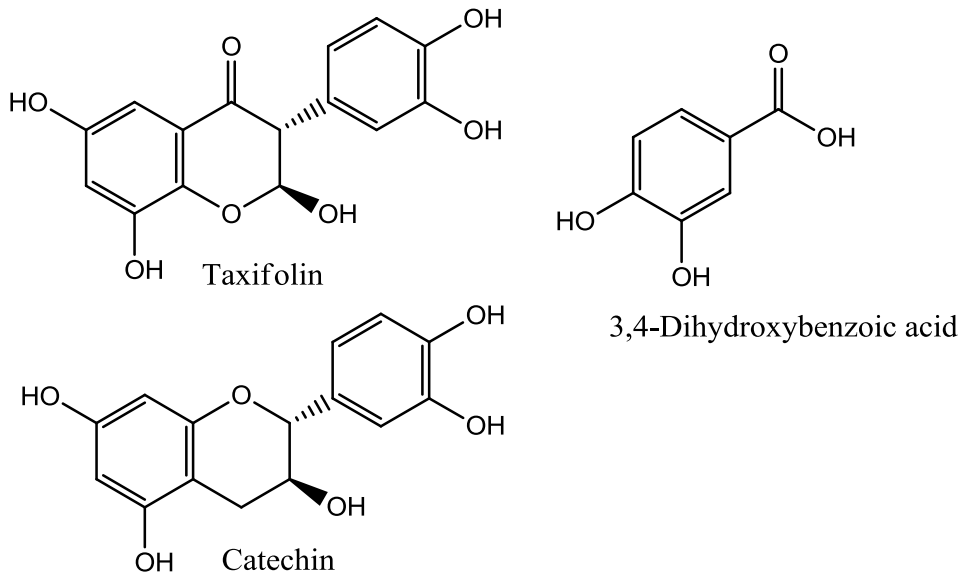


Figure 13. Chemical structures of the bark phenolics

The chromatogram of the knot extract is presented in Figure 14 and shows that the knots contained fewer compounds than the bark. Much like in the bark extract, the dominant class of compounds in the knot extract was the resin acids. The same six resin acids (Figure 11) were present, although their relative proportions were different. Abietic acid was found to be the dominant resin acid, followed by dehydroabietic and isopimaric acids. Abietic acid has been found dominant in previous works as well (Willför et al. 2003a, Hovelstad et al. 2006), but the relative abundances of other resin acids are not as clearly established. Willför et al. (2003a) found palustric and neoabietic acids to be second in abundance, while Hovelstad et al. (2006) found neoabietic acid and the combination of dehydroabietic and levopimaric acids to follow abietic acid.

The knot extract also contained large amounts of pinosylvins. The pinosylvins appeared as a shouldered peak in the chromatogram (retention time 8.55 min), and based on the

presence of the correct ions in the mass spectral data, the main peak was concluded to correspond to pinosylvin monomethyl ether (PSM) and the shoulder to pinosylvin (PS). No pinosylvin dimethyl ether (PSD) was identified. Pinosylvins are known to occur in large amounts in Scots pine knots (Willför et al. 2003a, Hovelstad et al. 2006, Pietarinen et al. 2006), and the identification of pinosylvins was therefore an expected finding. Much like in previous works (Willför et al. 2003a, Hovelstad et al. 2006), the abundance of PSM was found to be higher than that of PS. The amount of pinosylvins in the knot sample was second only to abietic acid.

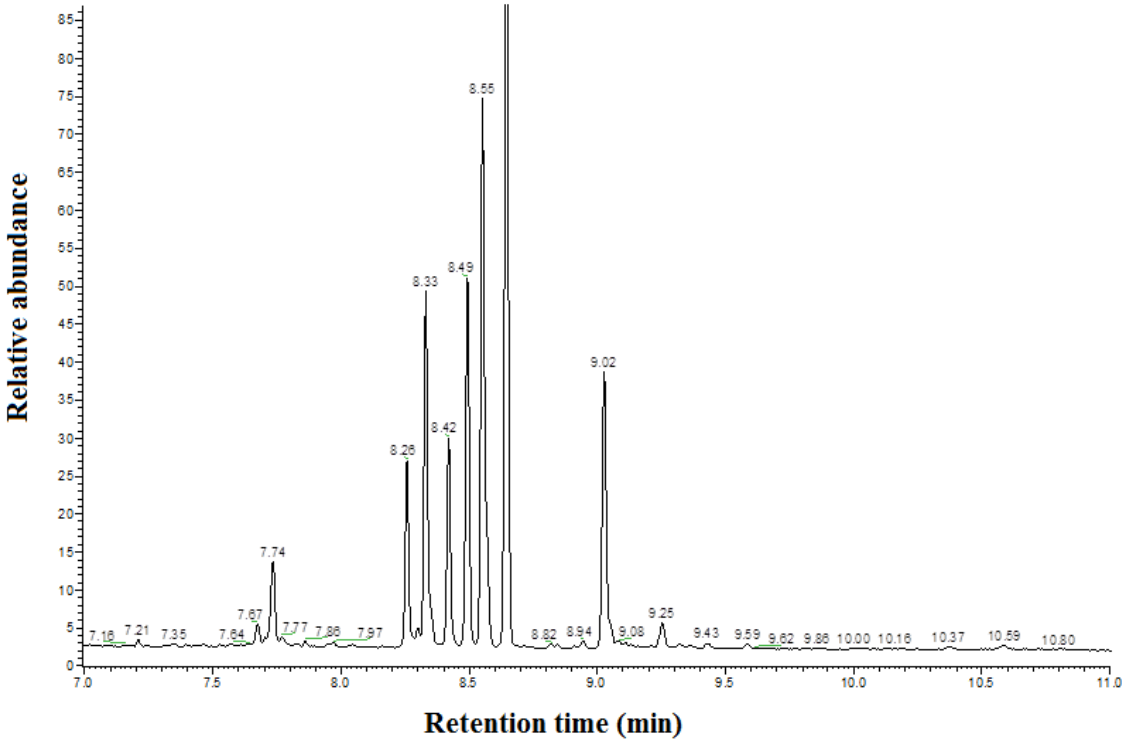


Figure 14. Gas chromatogram of knot extract (retention times 7-11 min)

The third class of compounds identifiable in the knot extract was the fatty acids. Fatty acids have been found to occur in Scots pine knots (Willför et al. 2003a), but their exact composition has not been reported. The fatty acids identified here included only two compounds, oleic (C18:1) and linoleic (C18:2) acids. The abundance of these compounds was found to be significantly lower than that of the resin acids or

pinosylvins. The extract also contained very small amounts of a number of unidentified compounds. Lignans could not be found.

The results of GC-MS are summarised in Table 3.

Table 3. Summary of compounds identified in the bark and knot extracts (+++ compound present in large amounts (relative abundance 100-60%), ++ compound present in moderate amounts (r.a. 60-30%), compound present in small amounts (r.a. 30-0%), - compound not present)

Compound	Bark	Knots
Resin acids		
Pimaric acid	++	+
Isopimaric acid	+	++
Palustric acid	++	++
Dehydroabietic acid	+++	++
Abietic acid	++	+++
Neoabietic acid	+	+
Oxidised resin acids		
Hydroxydehydroabietic acid	+	-
Unidentified	+	-
Flavonoids		
Taxifolin	+	-
Catechin	+	-
Pinosylvins		
PS	-	+++
PSM	-	+++
Simple phenolics		
Dihydroxybenzoic acid	+	-
Fatty acids		
Oleic acid (C18:1)	+	+
Linoleic acid (C18:2)	+	+
Stearic acid (C18)	+	-
Behenic acid (C22)	+	-
Fatty alcohols		
Behenyl alcohol (C22)	+	-
Monosaccharides		
Unidentified	+++	-
Sterols		
Sitosterol	+	-

Analysis of Scots pine bark and knot extracts by GC-MS and FTIR-PAS and UV Raman spectroscopies has provided a clear outline for the chemical composition of

these extracts. GC-MS has shown that the bark extract consists primarily of resin acids and monosaccharides, while the knot extract consists primarily of resin acids and stilbenes. IR and Raman spectroscopies support these findings. Both spectroscopies were able to show the presence of resin acids in the extracts, and in the case of the knot extract, Raman spectroscopy was also able to specifically identify the conjugated resin acids. Raman spectroscopy showed that both extracts contained aromatic compounds: the aromatic signal of the bark extract probably originated from dehydroabietic acid, while in the knot extract the stilbenes were the major contributor. The presence of fatty acids was indicated by Raman spectroscopy, while IR spectroscopy showed that the extracts contain fatty acid esters. Fatty acids could be identified by GC-MS but their esters could not.

While a good understanding of the chemical composition of the extracts could be obtained by the chosen methods, it should be remembered that the analysis is not exhaustive. Many of the compounds visible in GC-MS could not be identified, and the extracts are also likely to contain compounds that are not visible in GC-MS at all. Such compounds include fats, waxes, tannins, proanthocyanidins, and other polyphenolics. Fatty acids esters (fats and waxes) were identified by IR spectroscopy, but due to the nature of the method, their composition or abundance could not be studied.

5.3 Antifungal activity of extracts

The results of the agar plate tests are summarised in Table 4, which shows the antifungal activities (AFA) recorded for the extracts. The results are principally as expected: higher concentrations were more effective towards fungi than lower concentrations, and knot extracts were more effective than bark extracts. As suspected, the bark extracts proved to be inefficient antifungal agents, at least at the concentrations used in this experiment. The extract dramatically increased the growth of fungi at a concentration of 0.1 mg/g, and had no statistically significant effect on fungal growth at a concentration of 1 mg/g. The knot extract, in turn, had no statistically significant effect on growth at the lower concentration, but at the higher concentration the extract inhibited growth notably, particularly in the case of *T. versicolor*. The differences in fungal growth between replicate plates were sometimes large, which accounts for the

statistical insignificance of many AFA values. Agar plates with different amounts of fungal growth are shown in Figure 15.

Table 4. AFA (%) values of extracts for different fungi

Tissue	Concentration	*C. puteana*	*R. placenta*	*T. versicolor*
Bark	**0.1 mg/g**	-95.1[y]	-473.8[y]	-154.4[y]
	1 mg/g	20.0	17.5	5.6
Knots	**0.1 mg/g**	-10.1	-34.8	-4.1
	1 mg/g	53.3[y]	61.4[y]	79.5[y]

[y] statistically significant difference ($p<0.05$) to reference

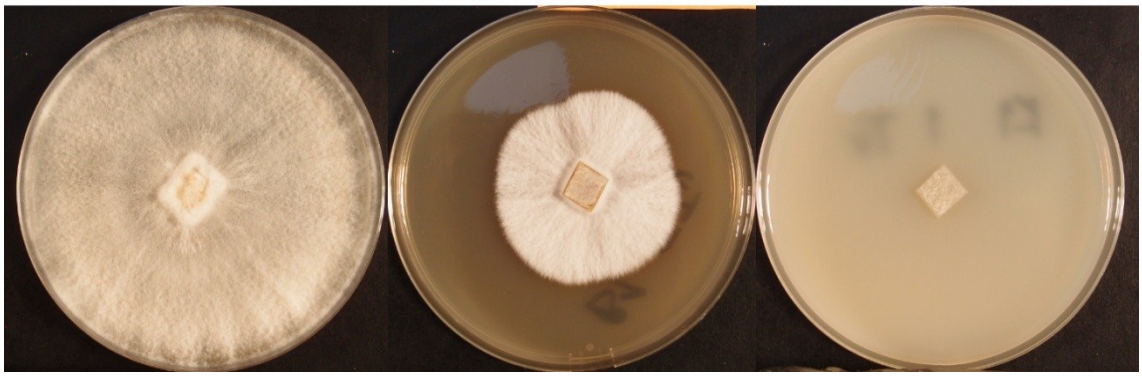


Figure 15. Agar plates with different amounts of fungal growth (left: full growth, middle: intermediate growth, right: no growth)

The effects the two extracts had on rot fungi are a consequence of their chemical composition. The knot extract consisted primarily of resin acids and pinosylvins, with small amounts of fatty acids and traces of other compounds. Pinosylvins are known to be highly toxic to fungi (Celimene et al. 1999, Seppänen et al. 2004), and even resin acids are thought to work in defence against microbial invasion, although the antifungal activity of isolated resin acids has been found lacking (Eberhardt et al. 1994). The knot extract therefore contains an abundance of compounds that are capable of restricting the growth of fungi.

Unlike the knot extract, the bark extract consisted primarily of resin acids and monosaccharide sugars, with small amounts of fatty acids and other compounds. As stated above, the resin acids do possess some antifungal character, but their activity against rot fungi has been shown to be weak. Sugars and fatty acids, on the other hand, are merely nutritional compounds. The bark extract is likely to contain tannins and proanthocyanidins in addition to the compounds identified by GC-MS, but these compounds do not possess significant antifungal activity either (e.g. Scalbert et al. 1998). The bark extract therefore contains no compounds that are capable of significantly inhibiting the growth of fungi. However, moderate fungal inhibitions, like in the work of Alfredsen et al. (2008), could have been seen at a higher concentration.

Chemical composition may also explain why the bark extract had such a strong growth-promoting effect at a low concentration. The bark extract contained sugars, and at a low concentration of antifungal compounds these sugars may have acted as an additional food source, resulting in increased growth. The growth promoting effect of low doses of extractives has been encountered before: similar effects have been recorded for other European bark extracts and the stilbenes pinosylvin and resveratrol (Seppänen et al. 2004, Alfredsen et al. 2008), among many others. The presence of nutritional compounds may account for the growth enhancing effects of extracts but cannot explain why otherwise antifungal compounds would have the same effect. Also, it should be mentioned that while growth promoting effects were seen in the works of Seppänen et al. (2004) and Alfredsen et al. (2008), none of the extracts or chemicals studied increased the growth of fungi by as much as the bark extract of this experiment.

Like many prior investigations, the results of this experiment demonstrate how the efficacy of extractives can vary from one fungus to another. The most significant differences were seen in the inhibition efficiency of the 1 mg/g knot extract and in the growth promoting effects of the 0.1 mg/g bark extract. The 1 mg/g knot extract proved to be more effective towards *T. versicolor* than the two brown rot fungi, while the 0.1 mg/g bark extract was found to have a much stronger effect on *R. placenta* than the other fungi. As a white rot fungus *T. versicolor* is less likely to infect Scots pine than the brown rots, meaning that it may have accumulated less tolerance to the antifungal compounds of pines. Seppänen et al. (2004) found that fungi that are less likely to affect

a particular wood species are more susceptible to its extractives. Another possible explanation is the origin of the strains: the *T. versicolor* used in this experiment is of foreign origin, meaning that it most likely has less resistance towards the extractives of Finnish species. In the case of the bark extract, the reasons for the strong growth of *R. placenta* are not known, but *R. placenta* may simply have been exceptionally well equipped to take advantage of the nutritious compounds present in the bark extract.

The results of the agar plate tests show that while the antifungal activity of Scots pine bark is insufficient, the Scots pine knot extract may have some potential as a wood preservative chemical. However, the results of this experiment should not be considered conclusive. The concentrations used here were relatively low, and higher concentrations are likely to have yielded higher AFA values. A 100 mg/g concentration was originally considered but was abandoned due to difficulties in re-dissolving the bark extract. It should also be remembered that the extractives solutions were autoclaved in this experiment. Extractives are known to undergo chemical changes and degradation at higher temperatures, which means that the autoclaving process may have affected the antifungal properties of the extractives. Last but not least, conclusions on the preservative potential of compounds should not be drawn based on wood-free experiments. Pinosylvins in particular are known to lose toxicity when brought into contact with wood, meaning that the antifungal activity of the knot extract may be much lower in wood (Hart and Shrimpton 1979, Hart 1989 p. 871).

6 CONCLUSIONS

The purpose of this study was to assess the preservative potential of Scots pine bark and knot extractives. Many wood extractives are natural antifungal compounds, and it may therefore be possible to use these extractives as preservative chemicals to improve the decay resistance of wood products. Scots pine was selected as a source of extractives due to its wide-spread occurrence in Finland; bark and knots were chosen because neither of these tissues is used in any high-value commercial applications and because both are known to contain high amounts of extractives.

To assess the preservative potential of extractives from Scots pine bark and knots, the yields of extractives from these tissues were recorded and the ability of the extracts to limit the growth of decay fungi was measured. Tests involving the impregnation of wood should be used to test the preservative potential of chemicals, but in this work a simple wood-free agar plate method was chosen due to time constraints. The chemical composition of extracts was also determined, and the composition of the extracts was used to explain the effects the extracts had on the growth of fungi.

The results of this experiment showed that Scots pine bark and knots are good sources of extractives in terms of yield. Acetone extraction of these tissues yielded 16.8% bark extractives and 24.6% (w/w) knot extractives. Chemical analysis of the extracts by GC-MS and FTIR-PAS and UV Raman spectroscopies showed that the bark extract consisted primarily of monosaccharides and resin acids. The identities of the saccharides could not be elucidated, but they were determined to be hexopyranose sugars, possibly glucose. Six resin acids were identified, with dehydroabietic acid being by far the most abundant. The bark extract also contained of fatty acids, fatty acid esters, fatty alcohols, sterols, oxidised resin acids, small amounts of phenolics, and a number of compounds that could not be identified.

The knot extract, on the other hand, consisted primarily of resin acids and pinosylvins. The same six resin acids were identified as in the bark extract, although their relative abundances were different. In knots abietic acid was the dominant compound, followed by dehydroabietic and isopimaric acids. Pinosylvins were also present in large amounts: pinosylvin and pinosylvin monomethyl ether could be identified in the extract, but no

pinosylvin dimethyl ether was found. The knot extract also contained small amounts of fatty acids and traces of other compounds.

The agar plate tests showed that the knot extract may have some potential as an antifungal agent, while the bark extract does not. The bark extract was found to have no antifungal activity: at a higher concentration it had no statistically significant effect on growth, while at a lower concentration it dramatically increased it. The findings were concluded to be due to the chemical composition of the extract. The extract contained no compounds that possess notable antifungal activity, and at a low concentration the sugars present in the extract may have served to promote fungal growth. The knot extract, on the other hand, could significantly limit the growth of fungi at a higher concentration. The antifungal activity of the extract was concluded to be due to the presence of large amounts of pinosylvins. Pinosylvins are highly antifungal compounds, especially when tested on agar.

While the performed experiments may give some indication as to the preservative potential of Scots pine bark and knot extractives, the results presented here should not be considered definitive. The concentrations tested in this work were low, and the compounds were not tested in contact with wood. However, despite these and other limitations, a few conclusions may still be drawn. First, the bark extract can be concluded to be an insufficient antifungal agent. The extract could not inhibit the growth of fungi, and while higher concentrations may have yielded slightly better results, the extract is unlikely to ever show high antifungal activity, because it simply lacks the highly antifungal compounds. Out of the compounds identified in this work, the resin acids are the only chemicals that have been associated with antifungal activity, and even their activity has been shown to be extremely poor when impregnated into wood. The extract may also contain tannins and proanthocyanidins, but their antifungal activity is not high either.

Unlike the bark extract, the knot extractives can be taken to have some potential as wood preservative chemicals. The knot extract contains known antifungal compounds and was found capable of limiting the growth of fungi at a moderate concentration. Furthermore, extractives could be obtained from knots at high yields, making knots an effective source of chemicals. Pinosylvins could be extracted with acetone, a relatively

inexpensive and nontoxic solvent, and may also be extractable by water. Methods have been developed for the separation of knots from wood chips, and because knots have no industrial applications, their use as a source of chemicals is theoretically feasible.

However, the use of knot extractives in wood preservation is not without problems. The main problem related specifically to Scots pine knots is that their antifungal potential is largely dependent on pinosylvins, which are known to lose toxicity in contact with wood. The antifungal activity of knot extracts may therefore be significantly lower when the extracts are used to impregnate wood. Knot extractives are also subject to the problems associated with the use of wood extractives in general. Namely, extractives are leachable by water, degradable by fungi, and vary in activity from one fungus to another. The extent of the leaching and degradation problems is not fully known, but the variations in activity are well documented and were also seen in this experiment. Further study is needed to determine whether extractives can be used in wood impregnation.

7 FURTHER RESEARCH

The antifungal properties of many wood extractives have been determined, but the question of whether extractives can function as effective preservative chemicals still remains unanswered. Further research is therefore being planned: the research started here will be expanded to cover wood extractives in general and will address the issues associated with the use of extractives in preservatives. Issues to be addressed include toxicity and its variations, leachability, and degradability.

Leachability will, naturally, be addressed in impregnation experiments. The aim is to study different types of extractives to determine the severity of the leaching problem and to see whether leachability varies from one extractives compound to another. Both the amount of extractives removed and the effect of leaching on durability will be assessed. Furthermore, the ability of extractives to penetrate the wood cell wall will be studied, since cell wall penetration is required for the potential elimination of leaching. Different types of extractives will again be studied to determine which, if any, can

penetrate the cell wall and to what extent. Finally, once the nature and severity of the leaching problem is known, solutions to the problem will be attempted.

The ability of rot fungi to degrade impregnated extractives will also be studied. The extent of degradation will be investigated, as will the mechanisms of degradation and the chemicals produced by fungi. As the enzymes of fungi are highly specific, it is expected that degradation of extractives will vary from one fungus to another, and also from one extractives compound to another. A number of different types of extractives and fungi will therefore be studied. The possibilities of formulating extractives mixtures that have improved resistance to degradation should be assessed.

Research into the antifungal properties of wood extractives will also be continued. The research started here will be continued by impregnation tests with Scots pine extractives and also with other extractives. Variations in activity from one fungus to another will be addressed, and attempts will be made to identify compounds and compound mixtures that show minimal variation. Finally, methods and modifications that could improve the antifungal activity of extractives will be sought. By combining all the aspects of preservative functions discussed above, an understanding of the preservative potential of wood extractives will hopefully be obtained.

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