Evgen Multia

Potential and utilization of water extracts from spruce bark

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Major in Biomass Refining

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Thesis supervisor:

Prof. Tapani Vuorinen

Thesis advisors:

M.Sc. (Tech.) Jinze Dou

M.Sc. (Tech.) Kirsi Tuominen

AALTO UNIVERSITY
SCHOOL OF CHEMICAL ENGINEERING

ABSTRACT OF THE
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The aim of this thesis was to investigate potential ways to utilize water extracts from spruce bark. Spruce bark has been found to contain three main stilbene glucosides (astringin, isorhapontin, polydatin), which have potential in the treatment of skin aging and cosmetics applications. Since stilbene glucosides are water soluble, they are dissolved into the debarking process waters of the pulp and paper industry. These waters are not fully utilized at the moment, and being considered as a waste stream, which adds to the costs of the mill by increasing COD levels of the waste water. This waste stream could potentially be transformed to a source of stilbene glucosides, to provide additional revenue to the mill.

In the experimental part, temperature effect on hot water extraction yields of industrial spruce bark was studied. Mechanical pressing of heated spruce bark was performed with a laboratory scale mechanical press to study the effect on the COD levels. Finally, bark press waters obtained from the mill were characterized, with partial purification of stilbene glucosides by ultrafiltration with 2 and 5 kDa filters. The yields were calculated by gravimetric and spectroscopic analysis. Two dimensional (2D) solution-state ^{1}H–^{13}C correlation NMR spectroscopy provided the structural verification of the stilbene glucosides and other compounds present in the samples.

Extraction yield of industrial spruce bark was increasing 1.4 times every 20 °C, on average, reaching yield of 5.0% at 80 °C. The mechanical pressing was able to press out 26±5% of the total mass of the sample. COD level was 75,100 mg/L for 80 °C sample. This was two times higher compared to bark press sample of taken from a paper mill, which had COD level of 37,500 mg/L. Ultrafiltration could remove 90% of polyphenols and permeated 14% of the stilbenes of the feed. With 2D-HSQC NMR, all three major stilbene glucosides were identified from the filtrate and the feed.

Keywords: Astringin; bark press waters; COD; debarking; hot water extraction; HSQC; isorhapontin; polydatin; skin aging; stilbene glucosides; ultrafiltration; UV-vis

Preface

I would like to thank Professor Tapani Vuorinen and Sappi Kirkniemi mill for the opportunity to research this interesting topic. I greatly appreciate the patience and support of Professor Vuorinen. I had a pleasure to have as my supervisor doctoral student Jinze Dou, who helped me with all of the aspects related to the thesis. His invaluable input to this thesis and time invested is greatly appreciated. I learned much during our discussions and his expertise in NMR made the NMR experiments appear much easier that they actually were. My second supervisor was Kirsi Tuominen from Sappi Kirkniemi mill, who made the collaboration with the mill pleasant and exciting. I would like to thank Kirsi for her contribution and discussions that greatly helped with the completion of this thesis.

I would also like to thank rest of my Aalto University colleagues, friends and family for the support and encouragement.

Espoo, 16.04.2018

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Symbols and abbreviations

Symbols

δ	Chemical shift (ppm)
AU	Absorbance units
b	Path length in the cuvette (cm)
^{13}C	Carbon-13
c_{Fe}	Concentration of iron(II) solution (mol/L)
Da	Daltons
DF	Dilution factor
^{1}H	Hydrogen
^{3}H	Tritium
Hz	Hertz
J	J-coupling (Hz)
kWh	Kilowatt-hour (equal to 3.6 MJ)
MJ	Megajoule
MWh	Megawatt-hour
V_3	Consumption of iron(II) solution by the zero sample (mL)
V_4	Consumption of iron(II) solution by the sample (mL)
V_5	Sample volume (mL)

Abbreviations

CPD	Cyclobutane pyrmidine dimers
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
GC-FID	Gas chromatography–flame ionization detector
HMW	High molecular weight compounds
HSQC	Heteronuclear single quantum coherencespectroscopy
NHEK	Normal human epidermal keratinocytes
NMR	Nuclear magnetic resonance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TOC	Total organic carbon
UVA	Ultraviolet A (315–400 nm)
UVB	Ultraviolet B (280–315 nm)
UVC	Ultraviolet C (100–280 nm)
UV-vis	Ultraviolet–visible spectroscopy

1 Introduction

Forest industry has already taken major steps into producing biochemicals from the biomass. The biochemicals are potential source of numerous product innovations and can act as alternatives to oil-based chemicals. [1] It is not surprising that Finland published Bioeconomy Strategy already in 2014 [2], that forecasts the bioeconomy to be the next source of economic growth. The vision is to refine each ingredient in the wood into an end use with potential economic added value. [1]

Spruce bark has this potential. At the moment, it is used industrially mainly for energy purposes at the mill. [3] The mill can extract more energy from the bark by dewatering it first with mechanical bark press. [4] However, debarking waters and bark press waters are considered wastewaters, and are highly toxic with high concentration of dissolved compounds. The spruce bark contains water-soluble compounds, which are extracted during the debarking and dewatering. Due to the toxicity, the mill needs to treat the wastewaters in a costly process. To reduce the costs, some of the valuable compounds found in the wastewaters could be extracted, serving also as an additional source of revenue for the mill. [5]

The spruce bark is a rich source of sugars, tannins and stilbenoids, all of which are dissolved during the debarking process into the wastewaters. [5, 6, 7] This thesis has its focus in the potential utilization of spruce bark stilbenoids, due to their positive health effects [7, 8, 9], with the emphasis on the treatment of skin aging [10, 11]. Stability of the stilbenoids in extract mixture and as isolated compounds is also elaborated in the literature part [7].

In the experimental part of the thesis, effect of temperature and extraction time on extraction yields of water soluble compounds of the industrial spruce bark will be studied. This is done to determine the best possible conditions to maximize the yield of stilbene glucosides. The studies will be done on a small scale with a microwave reactor by adding additional water to dried spruce bark. Total yields will be determined by gravimetric analysis, and stilbene glucoside yields with UV-vis spectorscopy using a stilbene glucoside standard. Laboratory scale mechanical press will be used to test the pressing efficiency on the chemical oxygend demand (COD) levels of heated spruce bark without any additional water. The aim is to see, if the water already present in the spruce bark is sufficient to extract water soluble compounds, and thus increase the COD levels at elevated temperatures. Finally, ultrafiltration of bark press waters from a paper mill will be studied for a possibility to isolate stilbene glucosides from high molecular weight compounds present in these waters. The aim is to find industrially viable option for stilbene glucosides extraction. Structural verification of stilbene glucosides in the filtrates and the bark press waters will be analyzed with two-dimensional nuclear magnetic resonance (NMR).

Literature review

2 Spruce bark in pulp and paper industry

In 2016, Finnish forest industry consumed 67.4 Mm^3 of wood, which was 7% more than the average of five-years. [12] Total of 23.47 Mm^3 of spruce roundwood was used by wood products industry (14.02 Mm^3), and pulp and paper industry (9.45 Mm^3). Latter was distributed between mechanical pulp production (4.94 Mm^3) and chemical pulp production (4.51 Mm^3). [13] In this thesis, mainly the spruce bark used by the pulp and paper industry will be covered. Since 9-15% of the volume of spruce roundwood is bark [14], the Finnish pulp and paper industry produced 0.85-1.42 Mm^3 of bark in 2016. This would amount to 320,000-540,000 tonnes of spruce bark, using an average dry density of 380 kg/m^3 [15].

Bark composition of Norway spruce is 26.6% cellulose, 9.2% hemicelluloses, 11.8% lignin, and 32.1% of extractives [16]. The pulp and paper mills debark mainly with drum debarker [15], which uses 0.6-2 m^3 of water/m^3 of roundwood [5]. The spruce bark contains 12-20.9% of water-soluble compounds [5, 6], and the temperature of the water plays a major role in the increase of the extraction yield. Already 12% of water-soluble compounds are released at 30 °C, 60 min [5], while 20.9% extraction yield can be achieved at 90 °C, 120 min [6]. Debarking conditions at the pulp and paper mills being closer to 30 °C than 90 °C, it would be safer to use extraction yield of 12% when estimating the amount water-soluble compounds. Calculating based on this estimate, approximately 38,000-65,000 tonnes of water-soluble compounds have been dissolved into the debarking process waters of the Finnish pulp and paper industry in 2016. This could be almost doubled to 67,000-113,000 tonnes, with the increase of the temperature of the debarking water to 90 °C. The estimate is not taking into account possible losses of the extractives during the storage of the logs. [5, 6]

2.1 Spruce bark dewatering

At the moment, most of the bark harvested is fully utilized [16], mostly by burning the bark for energy purposes at the mills [3], with some energy production at power plants [15]. Coniferous bark has a net calorific value 5.0-9.0 MJ/kg at typical moisture content (50-65% moisture), while dry bark has net calorific value of 18.5-20.0 MJ/kg [17, 18]. Thus, to increase the net calorific value of the bark, the mills use bark presses for dewatering the bark by mechanical pressing. The mills mainly burn the bark in their own boilers, but another option would be to utilize the excess bark in external power boilers, which would require dewatering and additional drying.

[4] It is possible to reduce spruce bark moisture content to 51-60% with dewatering [19]. Operation of the bark press consumes about 5 kWh/tonne of removed water [20]. Thus, at electricity price of 0.059 euros/kWh for industrial consumers in Finland [21], the operating of the bark press costs about 0.295 euros/tonne of removed water. This water also contains water soluble extractives, that could be utilized. [5]

Figure 1 illustrates the debarking process with dewatering for an integrated pulp and paper mill. The mill has two debarking drums for softwood, and uses a ratio of 80% spruce and 20% pine. After the debarking, the bark is sent to a hammer mill to reduce the size. Then the bark goes through the bark press that reduces the moisture content from 80% to 61% before being burned at the bark boiler. Debarking water is sent to the water purification plant. [4]

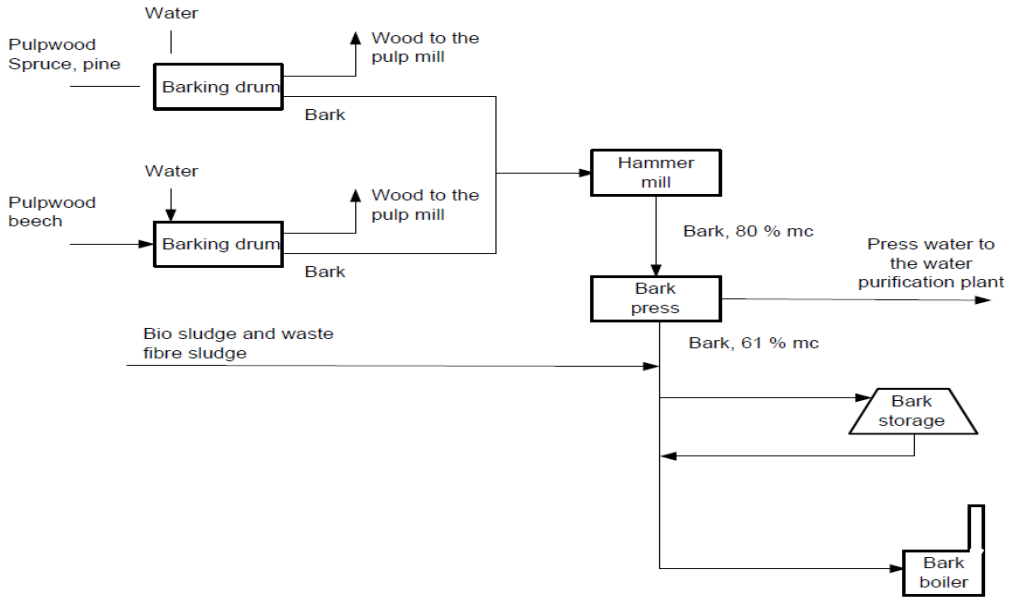

Figure 1: Bark handling at integrated pulp and paper mill (Stora Enso Nymölla). [4]

There is also a notable seasonal variation in the bark moisture content (Figure 2A) in Finland, with decrease in moisture content in the summer time. Pressing pressure and heated press head of the bark press also have an effect on the dewatering process (Figure 2B). Pressing time of 30 seconds was used in these experiments. Overall, the dewatering increased rapidly with the pressure increase up to 10 bar, with only slight increase from 10-20 bar. Dewatering from 72 to 65.1% moisture content can be achieved with 20 bar and no heating of the press head. Heating the press head to 100 and 150 °C resulted in dewatering to a moisture content of 61.8%. Dewatering can also be enhanced by increasing the pressing time. [4]

The efficiency of dewatering can be increased with decreasing the particle size of the bark and increasing the temperature, either by adding heated water in the process that will increase the temperature of the bark, or using a heated press

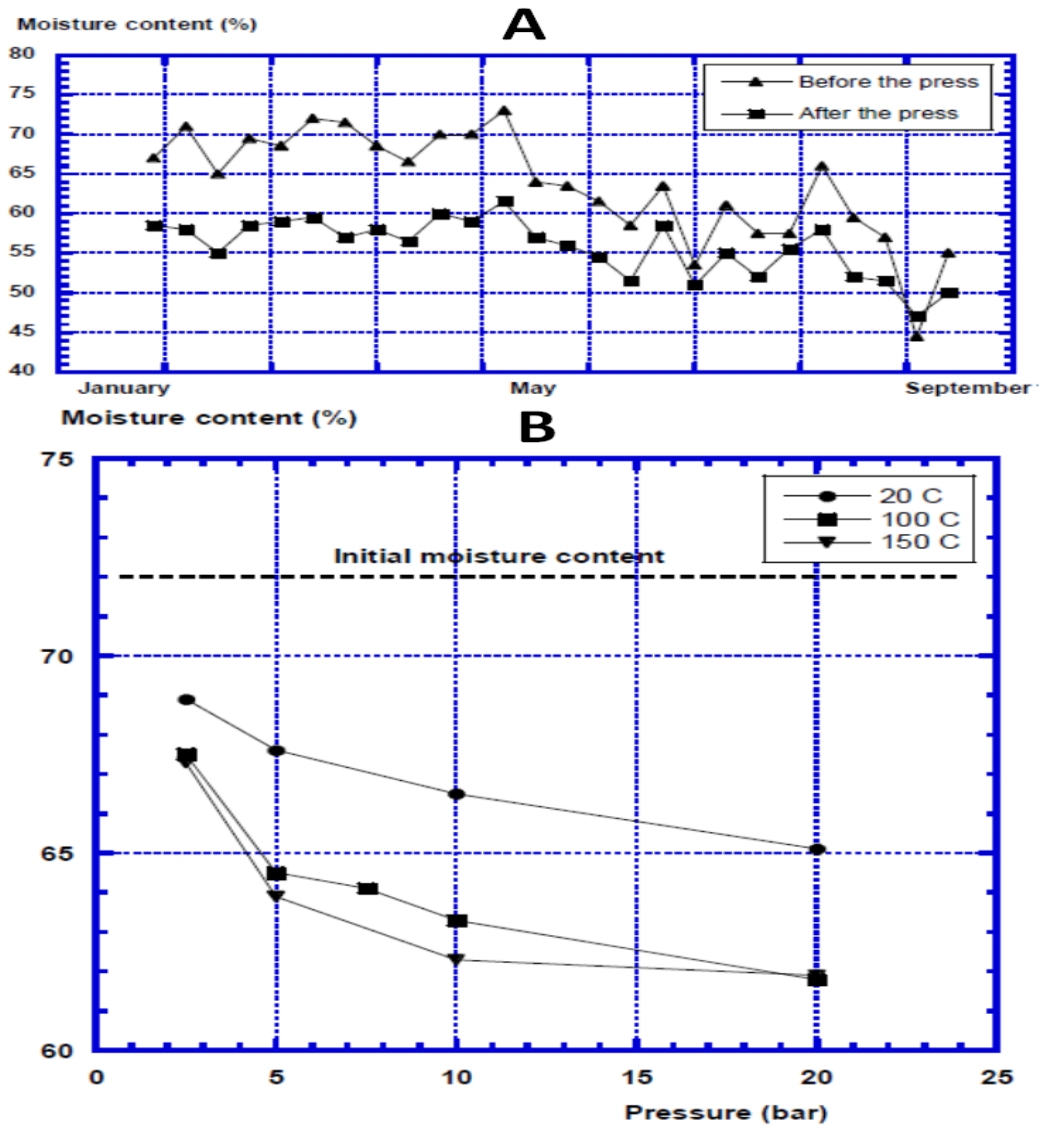

Figure 2: Seasonal variations of moisture content in bark pressing (A). Effect of pressing pressure and press head temperatures on bark moisture content (B). Modified from [4].

head. [4] Increased dewatering after the heating has also been noticed with other biomass materials. [22] Further reducing the moisture content of the bark from 60% (5.6 MJ/kg) to 40% (9.6 MJ/kg) can be used to increase the energy content with additional drying step. [4] The spruce bark can be alternatively sold after dewatering at a price of 15.8 euros/MWh (wood fuel price for industrial solid by-products for the industry) [23], if it is not burned at the mill in the bark boiler to produce energy. This would make the spruce bark at 60% moisture content worth 0.025 euros/kg.

2.2 Spruce bark debarking water and bark press water compositions

Debarking wastewaters are highly toxic with high concentration of dissolved compounds. Most prevalent compounds being tannins, classified as hydrolyzable tannins and condensed tannins. [5] Debarking water and bark press water compositions from Metsä-Serla's Lielahti mill taken in March were analyzed (Table 1). The compositions as percentage of total organic carbon (TOC) are presented in Figure 3.

Table 1: Composition of debarking waters. Modified from [5].

	Debarking water Calc. TOC [mg/L]	**Bark press water** Calc. TOC [mg/L]
Monosaccharides		
Fructose	100	400
Glucose	85	870
Succrose	170	990
Unidentified oligosaccharides	90	540
Stilbene glucosides		
Isorhapontin and astringin	280	2,640
Catechin	5	45
Stilbenoids	5	20
Resin acids	8	40
Polyphenols	240	2,470
TOC		
Total identified	980	8,020
Total	1,930	16,100

From the analysis we can see that the debarking water (1,930 mg/L) was more diluted compared to the bark press water (16,100 mg/L). Extraction of water soluble compounds starts already at the debarking and compounds that are not dissolved easily in the debarking water are pressed from the bark during the bark pressing. This results in increased TOC concentration due to a smaller amounts of water used in the bark pressing, since there is mostly moisture from the bark. Both waters were approximately 50% unidentified, including the polysaccharides. The sugars (mono-, di- and oligosaccharides) accounted for 24% of total TOC, stilbene glucosides 14%, and polyphenols 12% for the debarking water. For the bark press water, the sugars accounted for 17%, stilbene glucosides 17%, and polyphenols 15%, of total TOC. [5]

These results confirm that the debarking waters contain interesting compounds like polyphenols and stilbenoids in high concentrations. Being considered as wastewaters, there is no utilization of these streams, but they can potentially serve as a good source of stilbenoids and polyphenols. Also, considering that wastewater treatment

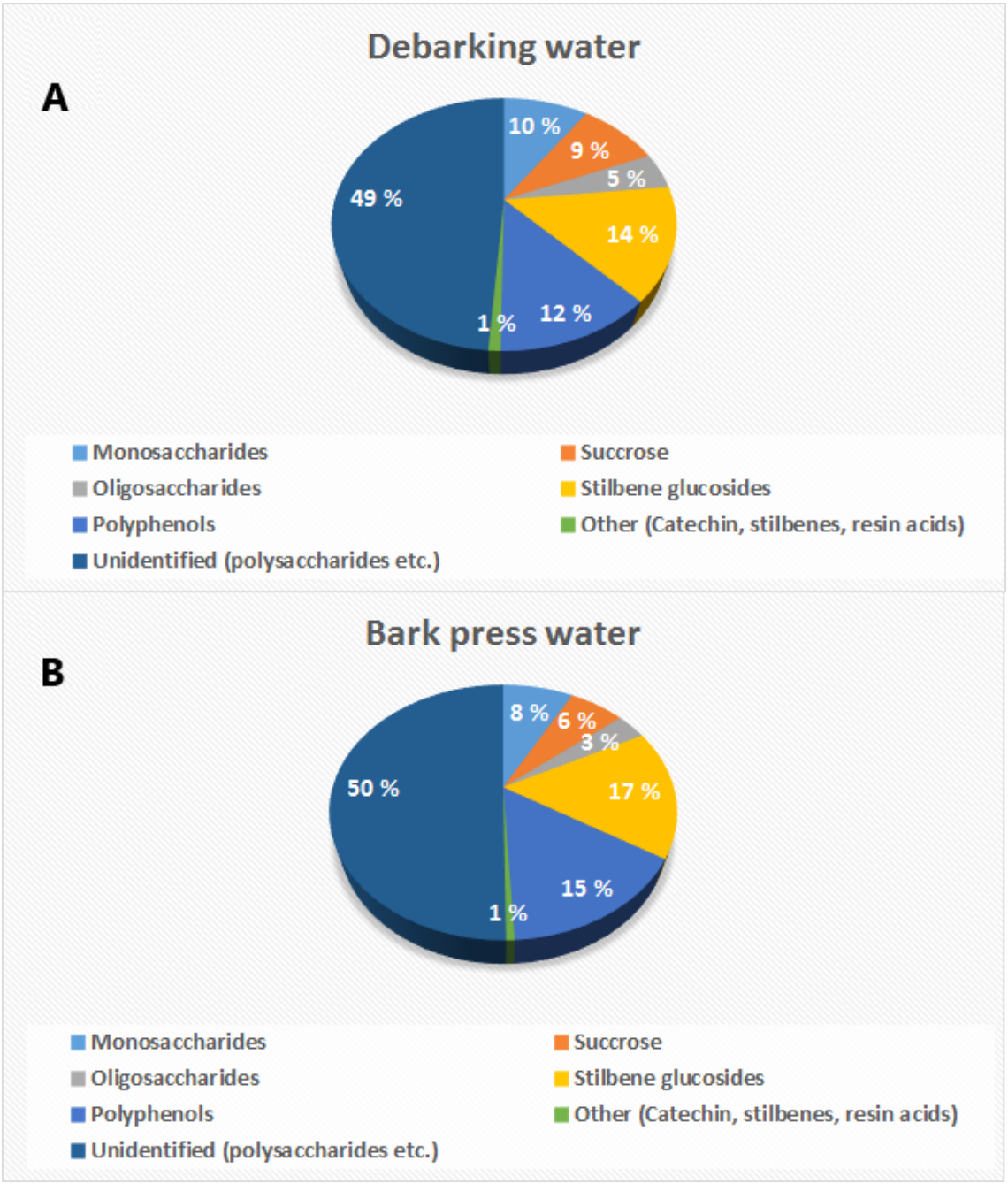

Figure 3: Debarking water (A) and bark press water compositions (B) taken from Metsä-Serla's Lielahti Mill in March. Data from [5].

is a costly process, extraction of these compounds from the wastewaters would results in a lower chemical oxygen demand (COD) of the waste streams and reduction of waste treatment costs. The extracted compounds could also serve as a new source of revenue for the mill. However, there is a need to take into account variations due to the seasons and the debarking conditions on the compositions of the debarking waters. [5]

2.3 Increasing the extraction yields of water soluble compounds from spruce bark

The temperature effect on extraction yields has been evaluated using industrial spruce bark from a dry debarking process. [6] In the dry debarking, the water is only used for de-icing of the logs. [24] Figure 4A shows the positive correlation of temperature on overall extraction yield, while Figure 4B shows the same effect on the extraction yield of free monosaccharides and bound sugars. Solids content in Figure 4 were measured as % of total mass (w/w, bark/water) on dry basis. In wet debarking process, extraction yields can be possibly increased in similar manner to increasing the concentration of water soluble extractives already present in the debarking effluent, prior to the bark pressing. [6]

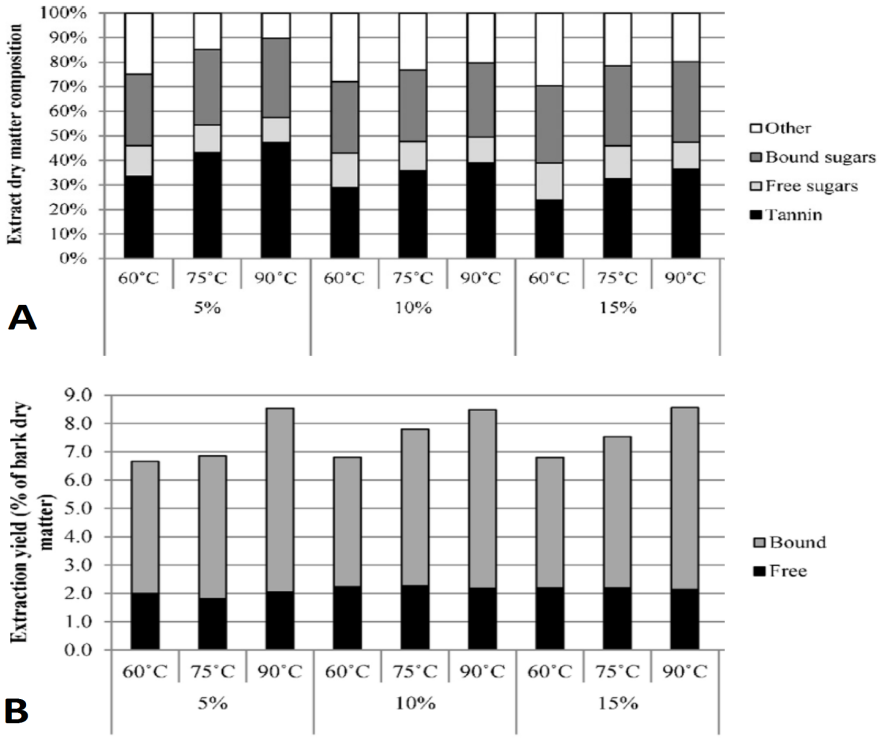

Figure 4: Compositions of water extraction yields (A) and sugar yields (B) at different temperatures and solids %. Modified from [6].

Free sugars (mono-, oligo- and polysaccharides) were found to remain constant (1.8-2.3% of dry bark) with the increase of the temperature, while strong positive correlation was observed for bound sugars (stilbenes and other glycosidic compounds) as seen in Figure 4B. A strong positive effect of temperature was also noticed for tannin yields (Figure 4A). Extracts had a number average molar mass of 1.85 kDa and weight average molar mass of 3.40 kDa. It was also concluded that there is no possibility to selectively increase either tannin or sugar extraction yields by changing the temperature and the solids content. [6]

3 Tannin-type polyphenols in spruce bark

Polyphenols and their structurally most complex group, tannins, have main biological functions in defense against herbivores, pathogens, and UVB radiation. [25, 26] They have also been found to have positive effects on human health [27]. Tannins are mainly water soluble plant polyphenols, having the ability to precipitate alkaloids, gelatin and proteins. They can be classified as hydrolysable tannins and condensed tannins. [15, 28] Typically tannins have molecular weight of 500-3,000 Da [29], but with the increase of degree of polymerization, tannins of 28,000 Da have been observed [30]. Basic structures of hydrolysable and condensed tannins are presented in Figure 5.

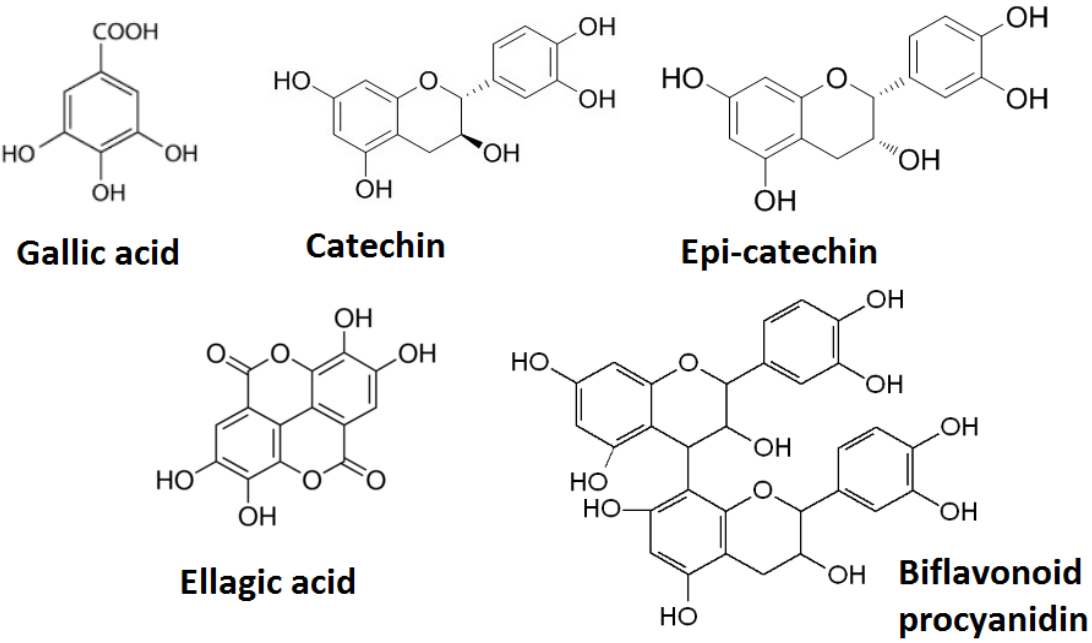

Figure 5: Basic structures of tannins. Drawn by ChemDraw.

Sugar core bound to gallic or ellagic acid is the structural foundation of hydrolysable tannins. These ester bonds are easily hydrolysable, hence the name hydrolysable tannins. [15] Softwood species like spruce produce mainly condensed tannins. [31] The structure of condensed tannins is based on three ring flavonoid units bound to each other via carbon bond [15], one example (biflavonoid procyanidin) is shown in Figure 5. Molecular weight and color intensity of the tannins increase when going from the inner bark to the outer bark, while the water solubility decreases. [15, 32]

3.1 Spruce bark tannins and their utilization

Tannins could be potentially utilized in many different applications, especially in quality leather hide production and oenological applications. Previous uses of the spruce bark have been mainly for leather tanning, which exists even to this date but in smaller scale. [15] Global production of tannins has been approximately 200,000 tonnes per year. [33] Tannin content in spruce bark ranges from 4-15%. [15, 32] Concentration of spruce tannin could be accomplished with evaporation, in similar manner to quebracho tannin [34], which can be concentrated from 10% to 55% by evaporation, and eventually spray dried. Previous efforts in commercial tannin production from softwood (pine bark) suffered from low extraction yields and low quality of the extract. [15]

Tannins can be used as animal feed, dispersants or coagulants, and insulating foams. [15, 35] They can be applied as wood adhesives, with either phenol-formaldehyde resins, or even formaldehyde free tannin adhesives being possible. [33, 36] This has been demonstarted by using spruce bark extract to replace quebracho extract for particleboard (20% replaced) and medium density fibreboard production (60% replaced) without affecting the properties. [37] Technologically, tannin based formaldehyde-free adhesives could be applied on industrial scale, but spruce bark tannin has not yet shown technical success in this application. [15, 33] After the technical success, the spruce bark tannin should also be economically competitive with phenol (1,500 euros/tonne). [15] For example, a paper mill producing 50,000 tonnes of spruce bark per year could produce approximately 5,250 tonnes of tannin, which would amount to 2.6% of the global market. Competitors at the global market produce around 25,000 tonnes/a (Indunor [38]) of quebracho tannin, 15,000 tonnes/a of chestnut tannins (Ledoga [39]), and 15,000 tonnes/a of synthetic tannins (Silvachimica [40]). Kemppainen [15] has proposed a multiproduct bark biorefinery concept found in Figure 6.

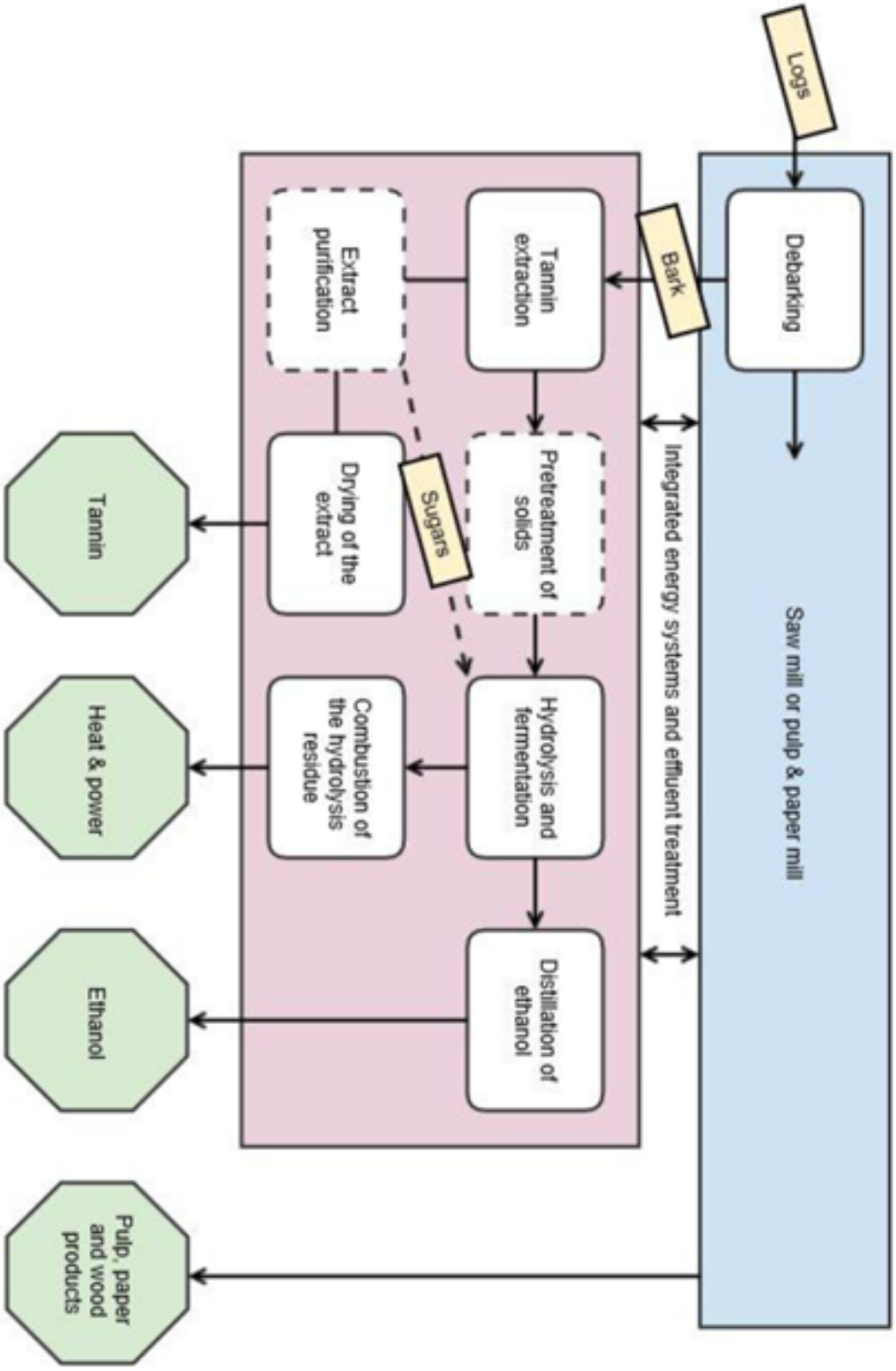

Figure 6: Bark biorefinery concept for tannin and ethanol production. [15]

4 Stilbenoids from spruce bark

Stilbenoids or stilbene derivatives have a basic structure of 1,2-diphenylethene and can be found as *trans*- and *cis*-isomers, with *cis*-isomer being less stable due to steric hindrance. [7, 41] Their main function is to act as antimicrobial and antioxidative substances with stilbenoid synthesis increasing when plant undergoes biotic and abiotic stresses. [7, 42] Spuce bark contains 5-10% of stilbenoids [15, 5, 28], which are mainly present as *trans*-stilbene glucosides, with small amounts of *cis*-isomers being present [28]. Industrial bark contains 1-2% of stilbenes which are eventually dissolved in the debarking process waters. [28] Most abundant stilbene glucosides being isorhapontin, astringin, and polydatin or piceid (10% of the total amount of stilbenes), with smaller amounts of aglucones due to their poor water solubility. [15, 28] Since stilbenoids (Figure 7) have a reactive OH groups, they could potentially be used as a starting material for chemical synthesis. [7] In medicinal, nutraceutical and cosmetics applications stilbenoids have already been utilized. [41]

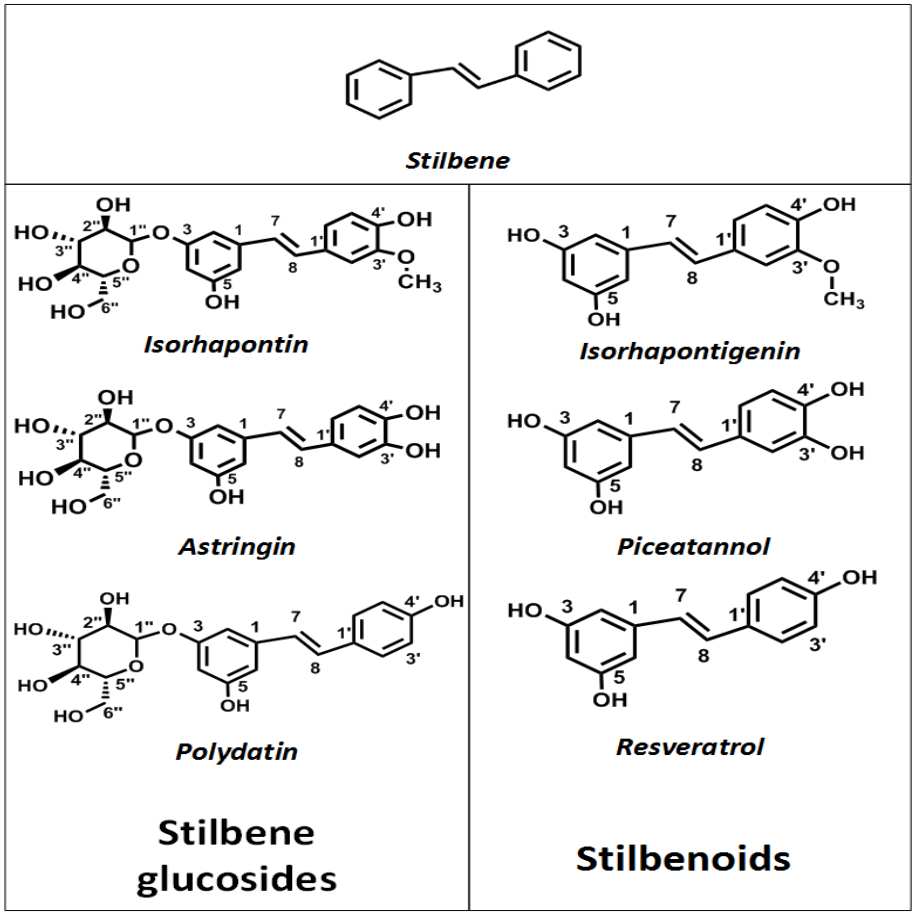

Figure 7: Stuctures of stilbenes and stilbene glucosides found in spruce bark. Drawn by ChemDraw.

Suggested biosynthesis pathway of astringin is shown in Figure 8. Stilbene synthase mediates the biosynthesis pathway [42], and depending on the starting stilbenoid, different reactions are needed. Resveratrol undergoes 3'-hydroxylation and glucosylation to add the missing OH-group and glucose. For piceatannol only glucosylation is needed, since it is the astringin aglucone. Isorhapontin forms when piceatannol undergoes glucosylation and 3'-O-methylation. When starting from resveratrol following reactions are needed: 3'-hydroxylation, glucosylation and 3'-O-methylation. [7] Stilbene glucosides are used to produce aglucones by the tree when the tree undergoes a fungal attack. [44] Aglucones can be produced by the hydrolysis of the glucosides. [83] Some complex structures are also produced when tannins and stilbenoids react. [14]

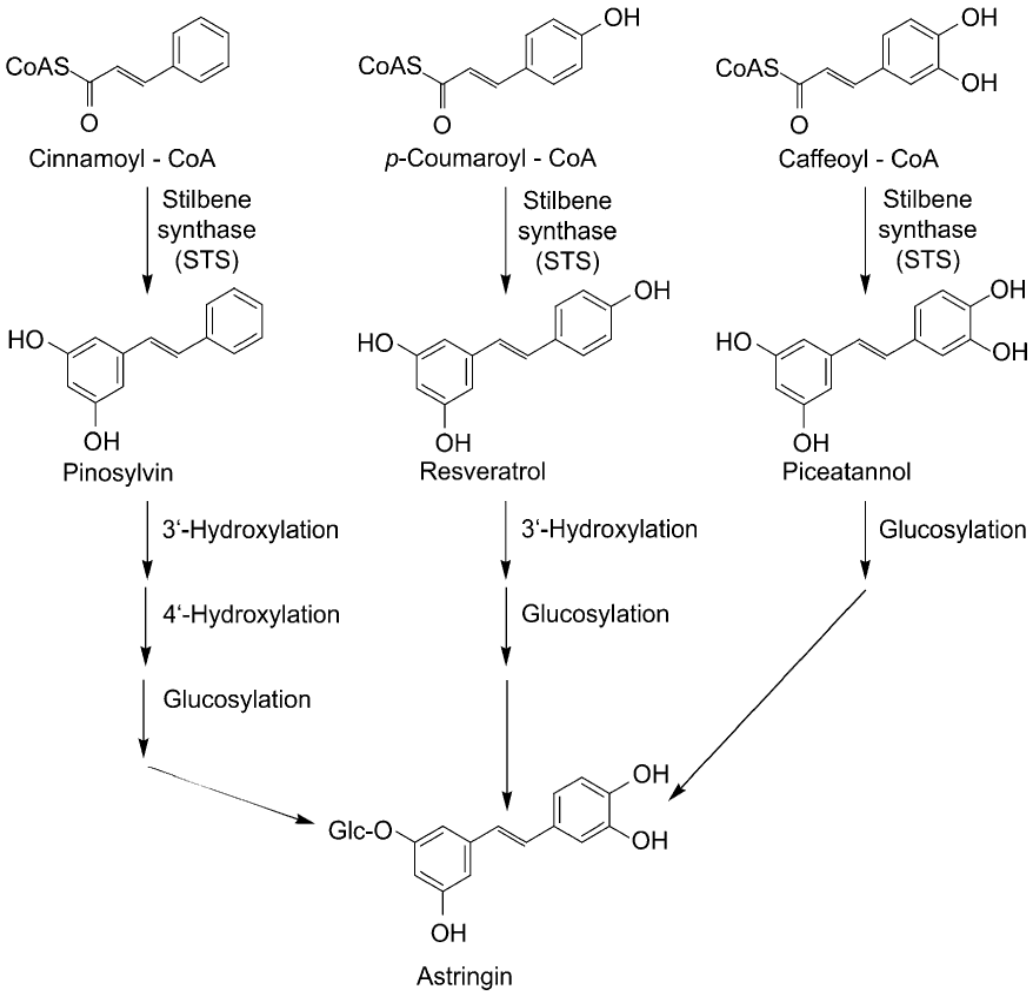

Figure 8: Astringin biosynthesis pathway in Norway spruce. Modified from [43].

4.1 Piceatannol, isorhapontigenin and resveratrol

Trans-resveretrol is the most studied stilbenoid with increasing interest, shown in Figure 9. It has found to have a wide range of benefits for human health (Figure 10) and it is found in small quantities in various foods like berries, nuts, grapes. [8] The interest for resveratrol has been due to its presence in black grapes and thus also in red wines. [28] Red wines usually have concentrations of resveratrol in the range of 30-45 μg/L. [46] Originally resveratrol was extracted from Japanese knotweed (*Polygonum cuspidatum*), and used in Japanese and Chinese traditional medicines to treat skin inflammations, fungal, liver, and cardiovascular diseases. [47] Reseveratrol has also found to have anticancer, anti-aethrogenic, anti-inflammatory, anti-microbial, anti-oxidative and estrogenic activity. [9] Variety of good reviews on health benefits of resveratrol can be found in the literature [8, 48, 49].

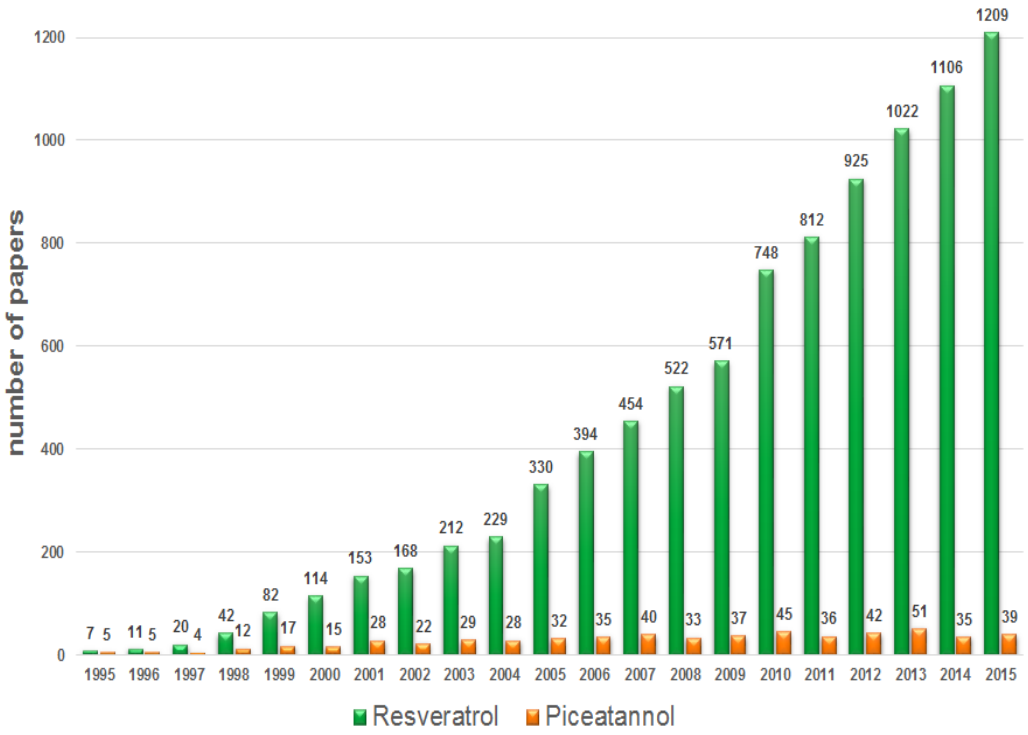

Figure 9: Publications indexed in PubMed referring to "resveratrol" and "piceatannol" from 1995 to 2015.

Piceatannol and isorhapontigenin are less extensively researched analogues of resveratrol with similar health effects. Fresh spruce bark contains probably the highest concentrations of these stilbenes in nature. [28] In addition, resveratrol is found in quantities of 10 mg/g in the spruce bark, that is hundred times more compared to the skin of black grapes (50-160 μg/g). [9, 28] Other type of Japanese knotweed (*Fallopia japonica*) can contain up to 25 mg/g of resveratrol [50], but it needs to be taken into account that resveratrol is present in lower concentrations in the spruce

bark (1% of all stilbenes) compared to piceatannol and isorhapontigenin [28]. Even though piceatannol is less studied, it also has wide variety of biological activity, and can be found in grapes, passion fruit, white tea, and Japanese knotweed [9]. Concentration of piceatannol in red wines ranges 7-14 μg/L. [46] Isorhapontigenin is the least studied stilbenoid found in the spruce bark, but it also has many health benefits. [7]

4.2 Beneficial health effects of stilbenes

Resveratrol and its presence in red wines has been associated partly with "French paradox", or low rate of cardiovascular disease in France, while there is high intake of saturated fat. [8, 51] It has been found to have positive effect in treating type 2 diabetes, obesity, cardiovascular disease, cancer and skin disorders (Figure 10). [8]

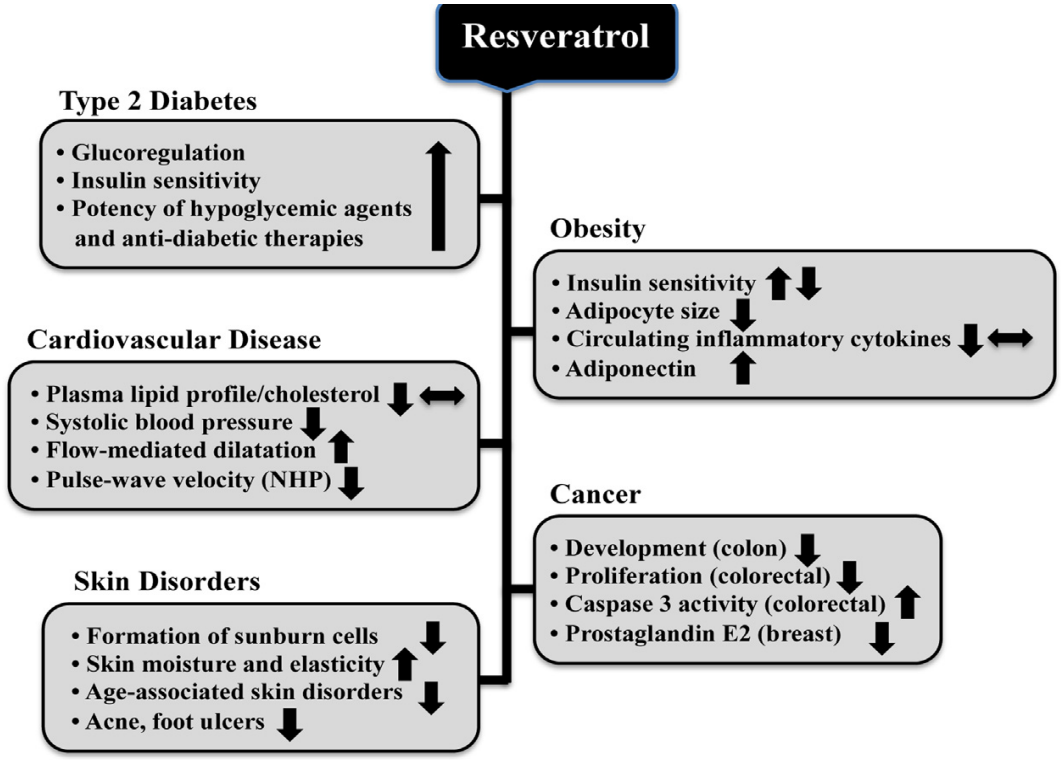

Figure 10: Resveratrol effects on human health (downwards arrow reduces and upwards arrow increases). [8]

More detailed account on health benefits of other stilbenes found in spruce bark (piceatannol [9], isorhapontigenin, astringin, isorhapontin, and etc.) can be found in the literature [7].

4.3 Production of stilbenoids for commercial applications

Production of stilbenoids for commercial application has been proposed by Latva-Mäenpää (Figure 11). Stilbenoids found in Norway spruce have real potential as commercial biochemicals with potential applications as food supplements, pharmaceuticals and cosmetics. [7] Commercial cosmetics product for UV-damage prevention (Rose Myrtle Extract BG80) by Maruzen Pharmaceuticals Co has already been developed. [52] It contains rose myrtle extract with piceatannol as an active stilbenoid. [53]

At the moment the market price for bulk resveratrol is approximately 300 euros/kg when buying in quantities of 100 kg. [54] According to a Frost and Sullivan report, resveratrol market was valued at 40 million euros in 2012. [55] United States accounted for 90% of the use of resveratrol, with the main market being food supplements, followed by Europe, and with growing regions in Asia Pacific. [55] Newer report from Front Research, Resveratrol Global Market and Forecast Research, reports that the plant extracts market would be worth 9.4 billion euros by 2020. [56] Global resveratrol market was expected to grow 4.5% annually from 2016-2021 and United States having 35% of global demand in 2016. [57] Gen Consulting company's report, Global Resveratrol Market Outlook 2016-2021, identified major players in the global resveratrol market collected in Table 2. [58]

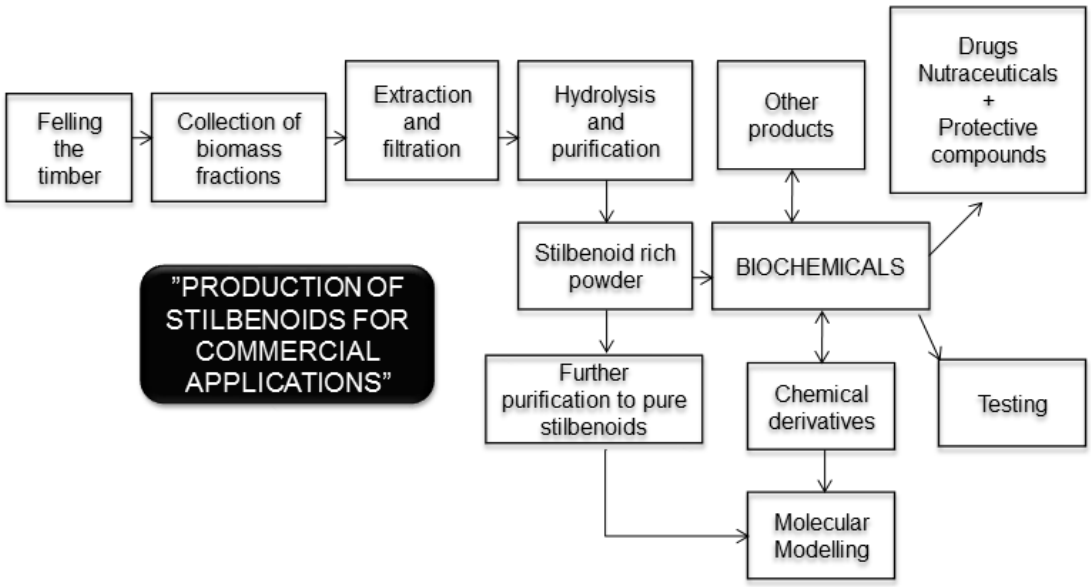

Figure 11: Production of stilbenoids for commercial applications. [7]

Table 2: Major players in the global resveratrol market in 2016. [58]

Company	**Country**
areDSM	Netherlands
Naturex	France
Evolva	Switzerland
Sabinsa	USA
InterHealth	USA
Maypro	USA
Chengdu Yazhong	China
JF-NATURAL	China
Jiangxi Hengxiang	China
Great Forest Biomedical	China
Xi'an Gaoyuan Bio-Chem	China
Kerui Nanhai	China
Shanghai DND	China
Jiaxing Taixin Pharmaceutical	China
Ci Yuan Bitechnology	China
Xi'an Sinuote	China

4.4 Stability of stilbenoids

Stability of stilbenoids to photo-oxidation needs to be taken into account before extracting these compounds from spruce bark, to ensure that bark extracts would be useful for further applications. While stilbenoids are found in nature mainly as *trans*-isomers, exposure to UV radiation may isomerize stilbenoids to *cis*-isomers (Figure 12). [7] It has been studied that *trans*-resveratrol and its glucoside remain stable as solids under (Good Manufacturing Practice pharmaceutical protocols) 60% humidity and 25 °C, as well as at 75% humidity and 40 °C. [60] When these compounds are in solution there is degradation to the *cis*-isomers. [61]

Stability is especially important to take into consideration for the possibilities of utilization of debarking waters. Stilbenoids in a crude extract of spruce root bark were found to be stable after exposure to fluorescent light for at least one week, with slow increase in degradation after that period. This was suspected to be due to variety of compounds present in the crude extract that reduce the light exposure of the stilbenoids. Similar behavior could be expected with spruce bark debarking waters. Solid stilbenoid glucosides (*trans*-astringin, *trans*-isorhapontin and *trans*-piceid) were also stable under fluorescent light, ambient conditions, light protected conditions and under the contact with atmospheric air. [7]

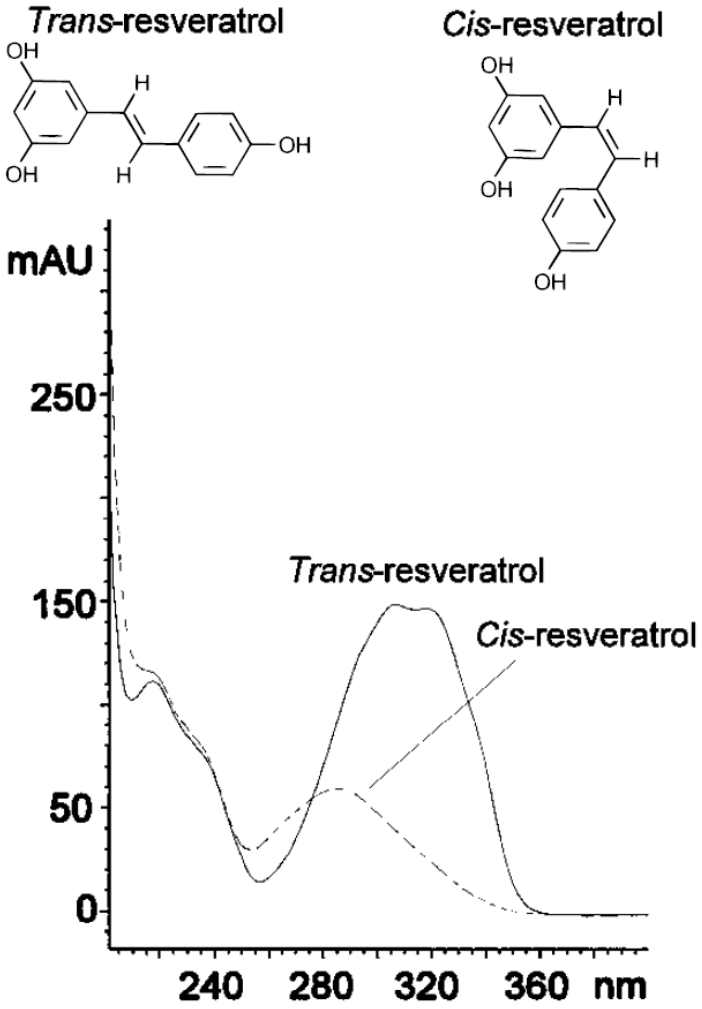

Figure 12: UV spectra of *cis*- and *trans*-reveratrol and their structures. Modified from [59].

Isolated stilbenoids from spruce root bark were evaluated for stability towards fluorescent light and UV light (Figure 13). It was found that *trans*-isorhapontingenin and *trans*-isorhapontin were the most stable stilbenoids after 2 weeks of exposure to fluorescent light, while *trans*-astringin and *trans*-piceid being least stable (Figure 13A). Methoxy substituent in the structure of both *trans*- isorhapontingenin and *trans*-isorhapontin, possibly increased the stability and provides steric hindrance, reducing the formation of *cis*-isomers. When exposed to UV light (366 nm), the isomerisation to form *cis*-isomers was more rapid (Figure 13B). Most of the *trans*-isomers were isomerized within 10-30 minutes after the exposure. Most stable compounds being *trans*-astringin and *trans*-isorhapontin. [7]

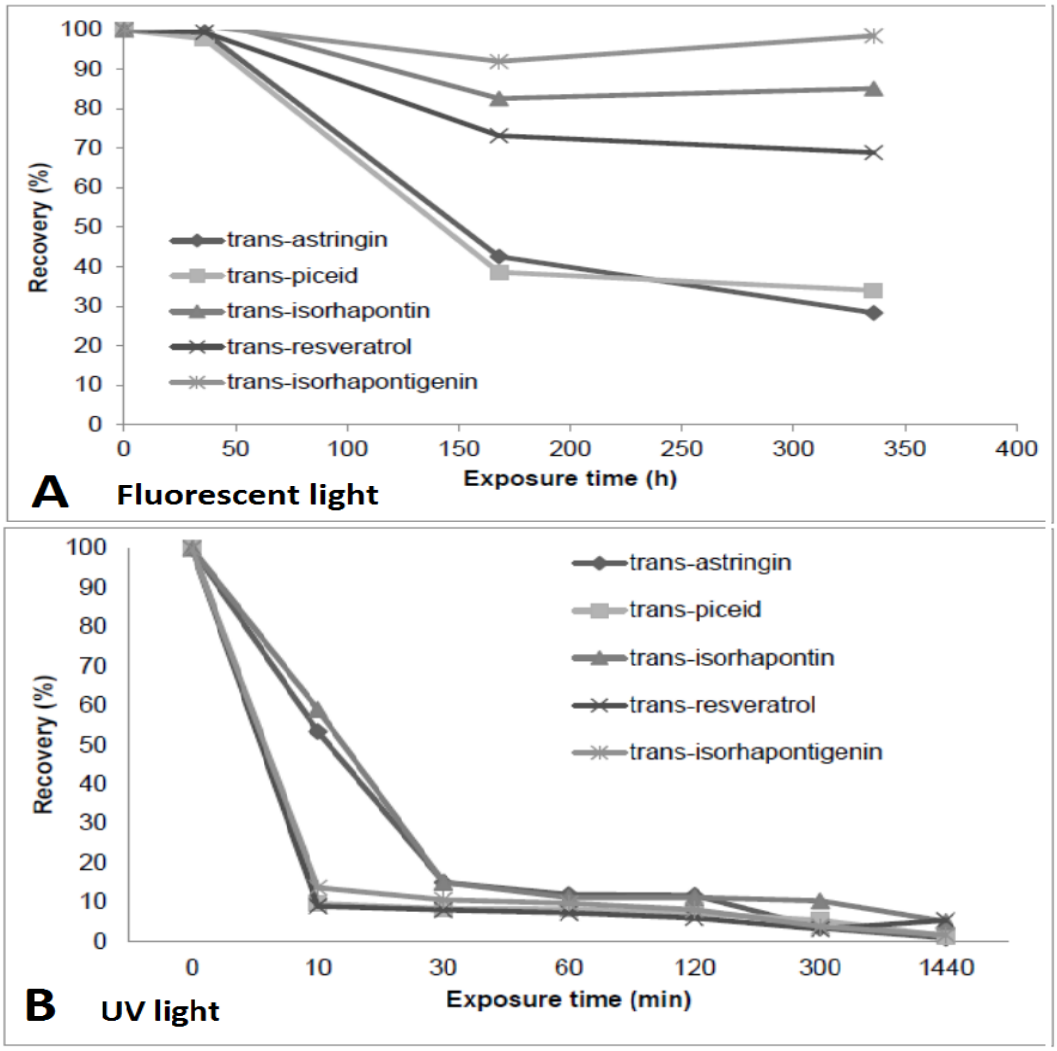

Figure 13: Stability of *trans*-stilbenoids to fluorescent light (A) and UV light (B). Modified from [7].

These results need to be taken into account when applying stilbenoids for cosmetics applications, especially topically, since there would be exposure to UV radiation. For *trans*-resveratrol, other issues being limited permeability on the skin, and its low water solubility. [62] Encapsulation approach has been developed to overcome these issues, as well as to enhance the properties, aestetic attributes and performance of cosmetic products. [63] These include vesicular delivery systems: liposomes, niosomes, ultrasomes, photosomes, transferosomes, ethosomes, aquasomes, silicon vesicles, microemulsions, liquid crystals, microspheres, microsponges, nanoemulsions, nanospheres, nanotopes, nanocrystals, fullerens, cyclodextrins, and cubosomes. [64, 65] Methylation of polyphenols has also found to have dramatic effect on stability, since it blocks conjugation reactions. [66, 67]

5 Skin aging and stilbenes

Skin aging (natural or intrinsic and extrinsic or photoaging) has been associated with increased production of reactive oxygen species (ROS). [10] This results in disturbance of mitochondrial function and acute stress response to solar radiation. [68] Skin aging has been also associated with a depletion of naturally occurring antioxidants that act as defence mechanism against free radical damage. [69] ROS accelerates skin aging by damaging cell membranes, proteins and DNA. ROS upregulates the factors (activator protein 1 and nuclear factor-kB) that accelerate skin aging. [10] Activator protein 1 is responsible in production of metalloproteinases [10], that induces premature skin aging [70], since they are enzymes responsible of breaking down collagen [10]. Wrinkling is believed to be caused by the loss of dermal collagen. [10] Collagen lose of 1% per year has been observed in post-menopausal women that results in fragile, thin and wrinkled skin. [71] Skin aging is being treated in these cases with estrogen replacement therapy. [10]

It has been demonstated that there is a down regulation of activator protein 1 and nuclear factor-kB by resveratrol, which preserves dermal collagen and reduces skin inflammation. [10, 72, 73] In addition, since stilbene structure is similar to synthetic estrogen (diethylstilbesterol), it is a phytoestrogen with possibility to be used in estrogen replacement therapy. [10] Topical application of resveratrol analogues can protect the skin from UV radiation induced damage and reduce the formation of sunburn cells. [67, 74] Resveratrol-procyanidin blend has also been found to improve skin moisture and elasticity. [75] Anti-acneic treatment of acne vulgaris with resveratrol containing gel has been reported, that showed 53.8% reduction in clinical leseions on the resveratrol treated sides compared to 6.1% on vesicle treated sides [76]. Other application of resveratrol could be as a skin lightener. [10] Resveratrol specific sites in human skin have also been found. [77]

5.1 UV radiation and the skin

The skin provides a large surface area (1.5-2.0 m^2) for the exposure to UV radiation. [78] UV exposure might initiate skin disorders like skin cancer, hypopigmentation, heperpigmentation, wrinkling, scaling and skin dryness. [78, 79, 80, 81] While there is a genetic factor in development of skin diseases, the UV radiation exposure plays the most important role. [78]

UV radiation can be divided into UVC (200–290 nm), UVB (290–320 nm), and UVA (320-400 nm). Since UVC radiation is the most energetic, it usually stops at the ozone layer and does not reach the surface of the earth, but on the skin it could penetrate 60-80 μm and damage DNA. UVB radiation (5% of total solar UV

radiation) on the other hand already penetrates 160-180 μm and is responsible for most of the non-melanoma and melanoma skin cancers. [78] UVB induces oxidative stress, DNA damage, skin aging [79, 80, 81], and has various effects on the immune system [82]. While skin has its own protection against UVB radiation, long exposure of UVB radiation leads to depletion of skins own defence system which eventually leads to skin disorders. [78] UVA radiation (90-95% of total solar UV radiation) penetrates the skin deepest, into the dermis of the skin up to 1000 μm. [78] As is the case with UVB radiation, excessive exposure to UVA radiation leads to the skin cancers. [83] UVA radiation induces ROS formation that damages proteins, lipids and DNA. [84] It is also responsible for photoaging, like skin sagging, due to oxidative stress on the skin. [85] Figure 14 shows the penetration of UV radiation into the human skin.

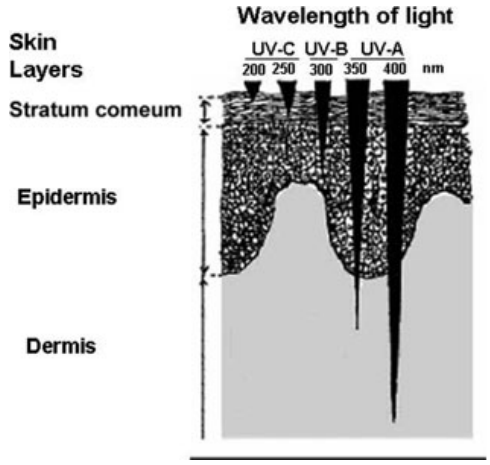

Figure 14: Penetration of UV-light into human skin. [86]

5.2 Skin protection with phenolic compounds

Phenolic compounds like catechins and stilbenes found in the spruce bark have a potential in treating and preventing UV radiation induced skin disorders. They exhibit low toxicity due to their natural origin and could be applied in treating causes and effects related to skin: aging, diseases, and damage (wounds and burns). Health effects can be achieved either by pure compounds (resveratrol, piceatannol and other stilbenes) or combination of compounds in an extract. Treating of skin conditions like skin cancers, psoriasis, rosacea, acne vulgaris, skin allergies, atopic dermatitis, dermatophytosis, wounds, incised wounds, chronic wounds, and burns with phenolic compounds has been reviewed in the literature. [11]

Oxidative stress is based on many factors and mechanisms (Figure 15), with ROS being toxic and mutagenic. [11] Phenolic compounds can act as antioxidants to inhibit formation of ROS [87], trapp ROS, reduce the chelated metal ions to promote

extinction of singlet oxygen [88], interrupt the free radical reactions in lipid peroxidations [89] as well as protect other compounds that have antioxidant activities [11, 90]. Skin has its own ways to defend against oxidative stress with antioxidant enzymes and molecules like vitamins, ubiquinone and glutathione [91], but this is often not sufficient against ROS [11]. By adding natural antioxidants to the diet or applying them externally, it is possible to increase the antioxidant activity against ROS. [11] External antioxidants that can be utilized are vitamins (C and E), lipoic acid, coenzyme Q, melatonin, resveratrol, curcumin and other polyphenols [92], since they are safe and have better biological activity compared to synthetic antioxidants [93].

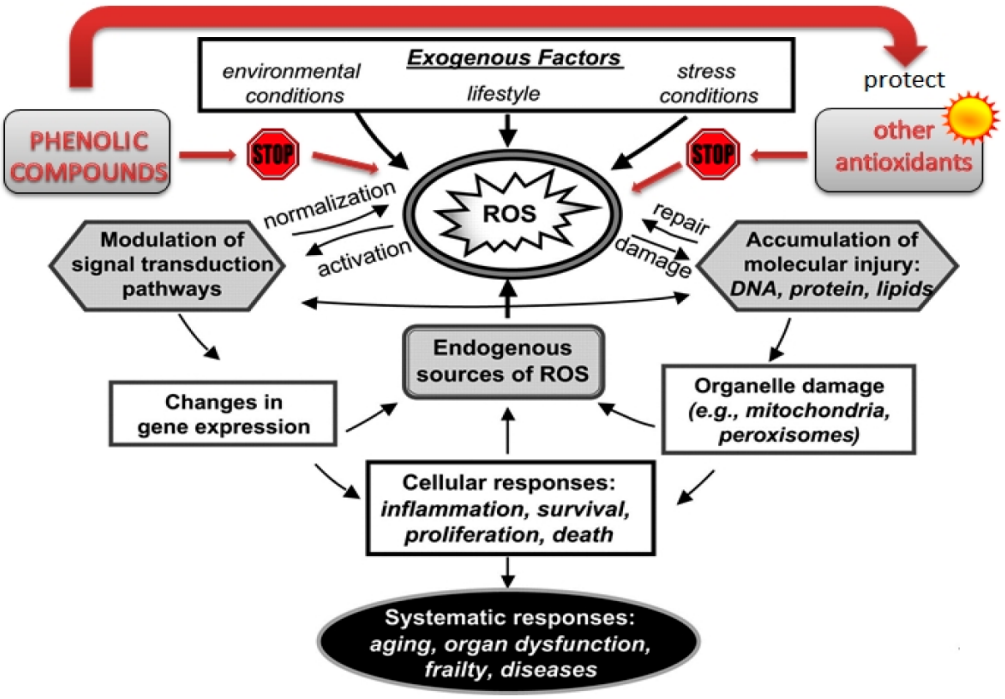

Figure 15: Factors involved in ROS formation with antioxidants and phenolic compounds acting as inhibitors. [11]

Inflammation is body's defence mechanism against irritation, damage or allergies that produces free radicals. Reactive oxygen and nitrogen (RNS) species activate also activator protein 1 and nuclear factor-kB that lead to inflammation which appears as redness or swelling. [11] Polyphenols could prevent the inflammation by inhibition of lipid peroxidation. [94]

Antibiotics are used to treat dermal infections or diseases [11], that sometimes leads to negative effects on skin's own microflora and develops resistant bacterial strains [95]. With over 90% of bacteria (*staphylococci*, *pneumococci* and *enterococci*) isolated from wounds being resistant to antibiotics. [11] Polyphenols could act as a non-toxic solution to this problem with their antibacterial, antiviral and antifungal properties. [11, 96]

5.3 Preventing skin aging with phenolic compounds

Skin aging can be noticed from visible signs, which are caused by the loss of elasticity of the skin: wrinkles, dry and flaccid skin, and inflammation or hyperpigmentation (age spots). Oxidative stress reactions disturb skin cells and skin condition can be affected by many factors: alcohol, environmental factors, genetics, nutrition and smoking. [11]

To slow down the skin aging, people use nutrition, sports and cosmetics. Phenolic compounds, dietary or topical, could be used to slow down the skin aging. There is also a preference for natural compounds among consumers, which makes phenolic compounds appealing for cosmetics applications. Figure 16 combines the effects of different phenolic compounds to slow down the skin aging. These are effective skin cell renewal, inhibition of melanin synthesis, and stimulation of collagen and elastin. Stilbenes can inhibit the melanin production in epidermis, but also help with protection and synthesis of collagen and elastin in dermis. [11] Figure 17 illustrates the distribution of resveratrol when applied to human skin sections.

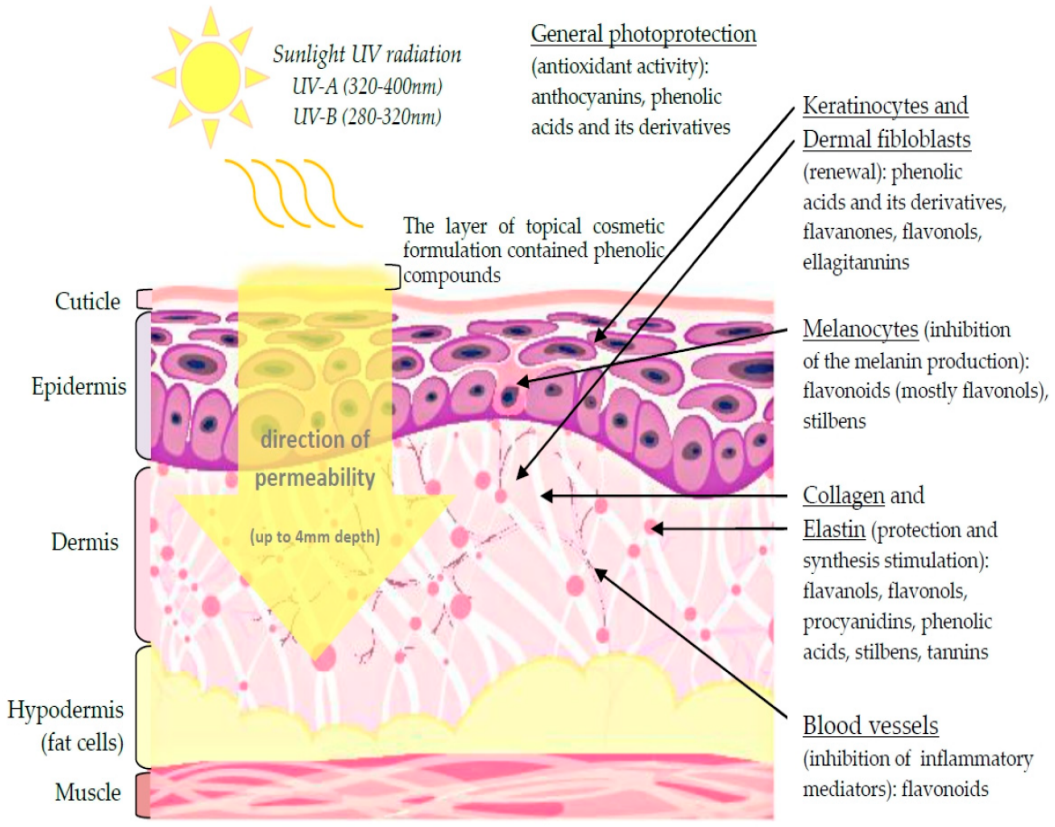

Figure 16: Effects of phenolic compounds on treating skin aging at different layers of the skin structure. [11]

Figure 17A shows that most of [^{3}H]-resveratrol was found in epidermis, where intensity of the signal is dependable of [^{3}H]-resveratrol concentration. Similar distribution (black dots) was observed when human skin was exposed to liquid emulsion (Figure 17B), where [^{3}H]-resveratrol binding sites were in granular keranocytes (90% of cells in the epidermis). [97]

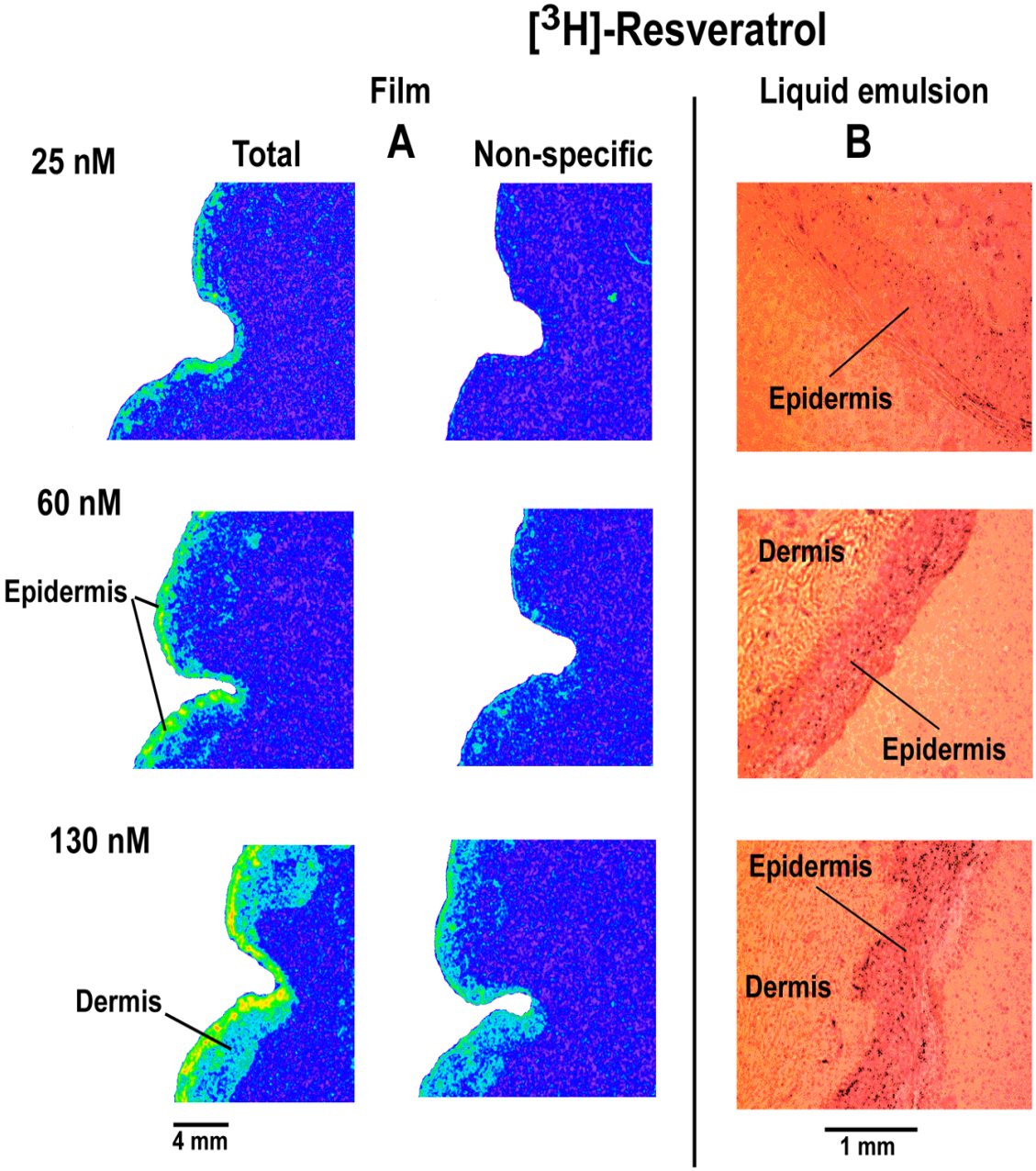

Figure 17: [^{3}H]-resveratrol binding sites on human skin. (A) Skin sections incubated for 8 months with [^{3}H]-resveratrol and stained with hematoxylin/eosin. (B) Photomicrograph of skin sections incubated with [^{3}H]-resveratrol and exposed to liquid emulsion for 8 months. [97]

With the aging, also activity of matrix metalloproteinases may become excessive. This is not wanted effect, since while matrix metalloproteinases are regulated by protein inhibitors, there is a homeostasis, and collagen and elastin synthesis is in control. With the imbalance in homeostasis, skin tissue looses its composition and formes wrinkles. Wrinkle formation can be minimized with the inhibition of matrix metalloproteinases (Figure 18), which in turn restores damaged collagen fibers. [11] Plant polyphenols act as inhibitors for matrix metalloproteinases (collagenases and elastases). [98] Phenolic compounds like catechin, epicatechin, gallic acid, resveratrol have found to have inhibitory action against matrix metalloproteinases, with gallic acid having the strongest activity. [11]

Human skin has specialized cells called melanocytes that produce melanin in their organelles called melanosomes. Melanin is thus skin's own pigments that determines the skin pigmentation and protects the skin from UV radiation. Melanin is transferred to keratinocytes through dendrites and is derived from tyrosine by oxidative reactions that involve tyrosinase. Skin uses melanin for photoprotection absorbing 50-70% of UV radiation. UV exposure results in excessive production of melanin (hyperpigmentation), which may lead to skin disorders, but is also considered a cosmetic problem. [11] The cosmetic problem has increased the research to find depigmentation agents. [99, 100]

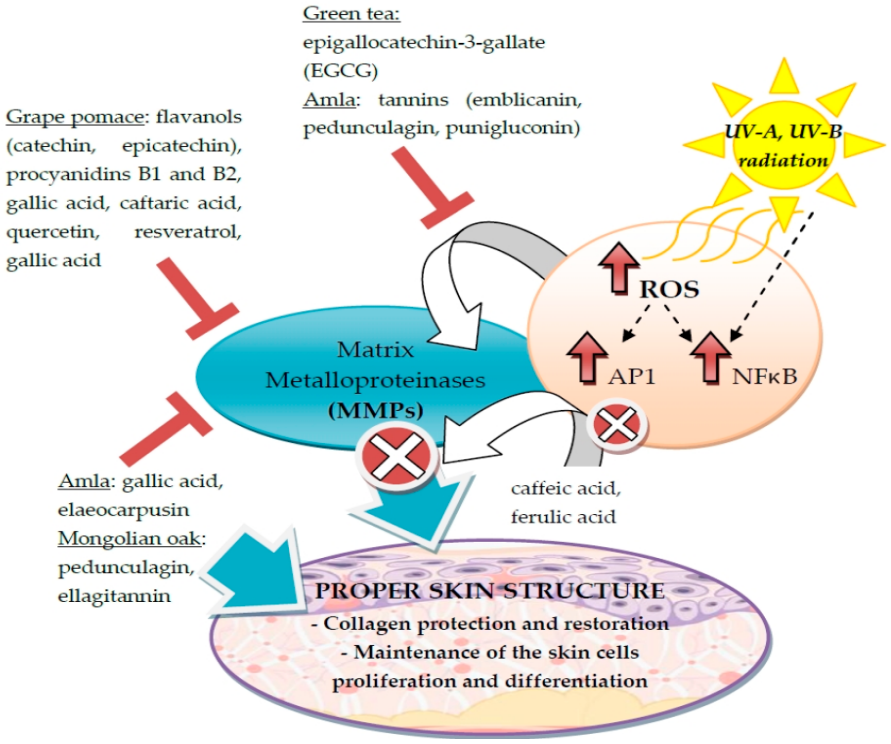

Figure 18: Regulation of matrix metalloproteinases by phenolic compounds. [11]

Figure 19 shows the melanogenesis and inhibition of tyrosinase by resveratrol, since it has similar structure to tyrosine. Resveratrol and oxyresveratrol have been found to be effective tyrosinase inhibitors. [101, 102] Another interesting tyrosinase suppressor is an extract from the leaf of *Morus alba*. It has been used in traditional medicine and its inhibitory effect on melanogenesis is due to combination of 20 different phenolic compounds found in the extract: 8 benzofurans, 10 flavonoids, stilbene and chalcone. [11, 103]

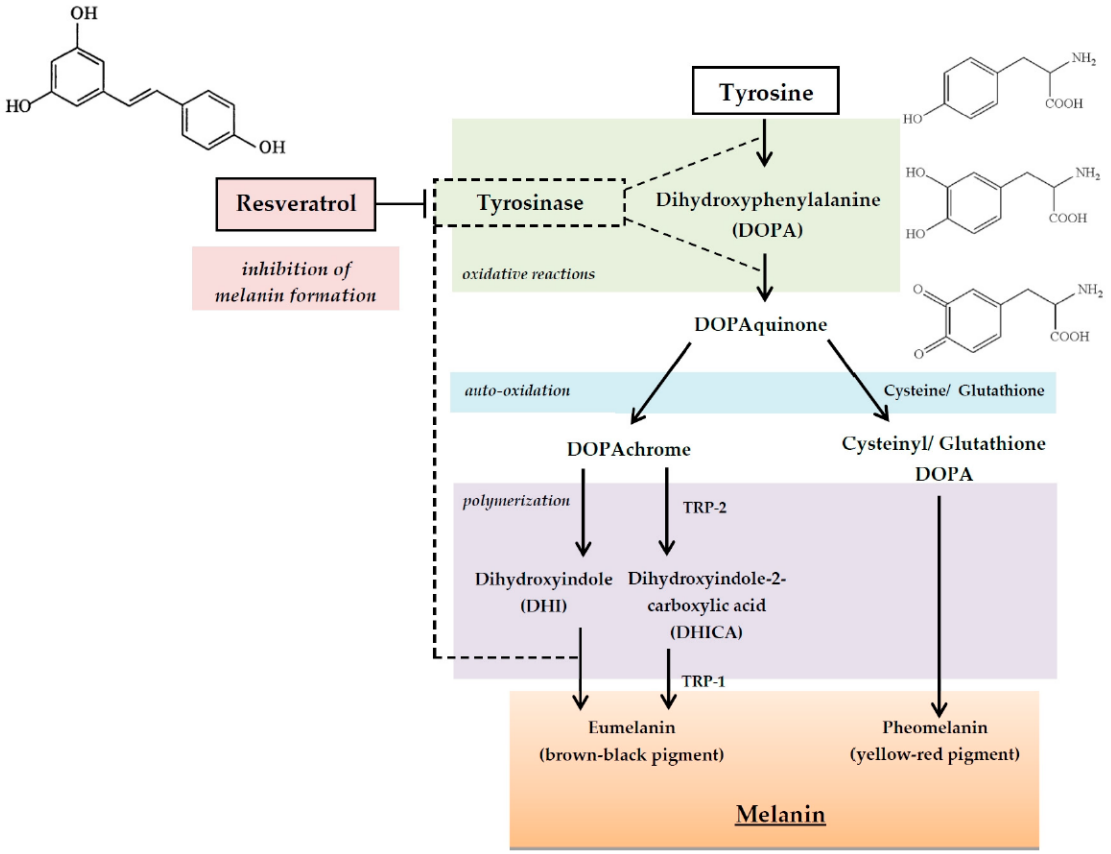

Figure 19: Inhibition of melanogenesis by resveratrol. [11]

5.4 Passion fruit seed extract and piceatannol

Passion fruit (*Passiflora edulis*) seed extract has been found to contain high concentrations of piceatannol also found in the spruce bark extracts. Seed extract was found to have positive effect on skin cells by promoting synthesis of collagen and inhibition of melanogenesis. This is suspected to be due to piceatannol, resveratrol and scirpusin B content of the extract. [104]

To test the effect of soluble collagen synthesis and inhibition of melanin synthesis different concentrations of passion fruit seed extracts were applied on in melanin-

producing human melanoma cells and human dermal fibroblast cells. Results are shown in Figure 20. The soluble collagen synthesis increased significantly when applying different concentrations of passion fruit seed extracts. [104]

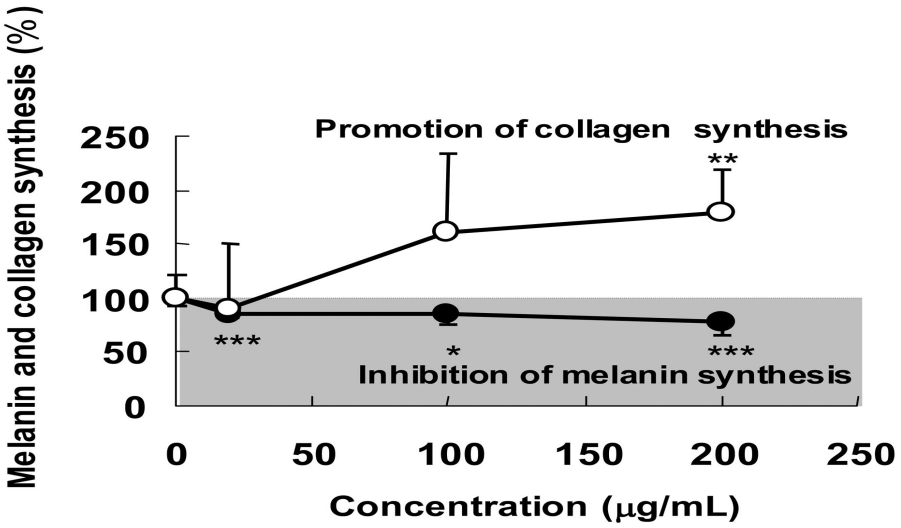

Figure 20: Melanin and collagen synthesis after application of different concentrations of the passion fruit seed extracts. [104]

Contribution of piceatannol was evaluated by preparing extracts and commercial piceatannol to the same concentrations regarding the piceatannol concentration. From the Figure 21, it can be seen that extracts yielded stronger effect on the inhibition of melanin, with commercial piceatannol also giving a strong effect. No significant difference on the promotion of collagen synthesis was noticed. [104]

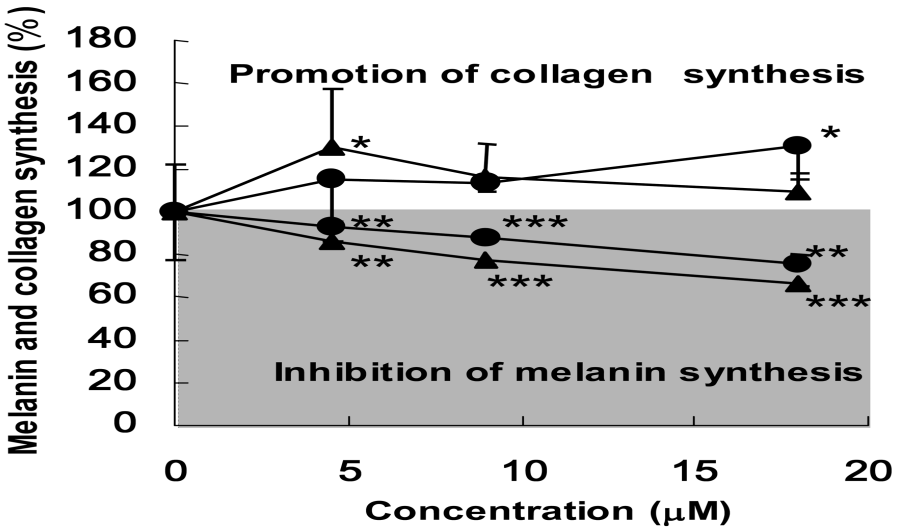

Figure 21: Melanin and collagen synthesis: comparison of the passion fruit seed extracts (Δ) with piceatannol (o). [104]

In addition, a comparison between piceatannol and resveratrol was made (Figure

22), since both are present in passion fruit seed extracts. Significant increase in collagen synthesis can be noticed with 5 μM of piceatannol, while 10 μM is needed for resveratrol. [104] Inhibition of melanin synthesis was also stronger for piceatannol [104], which has been also noticed previously due to higher antioxidant activity of piceatannol [105].

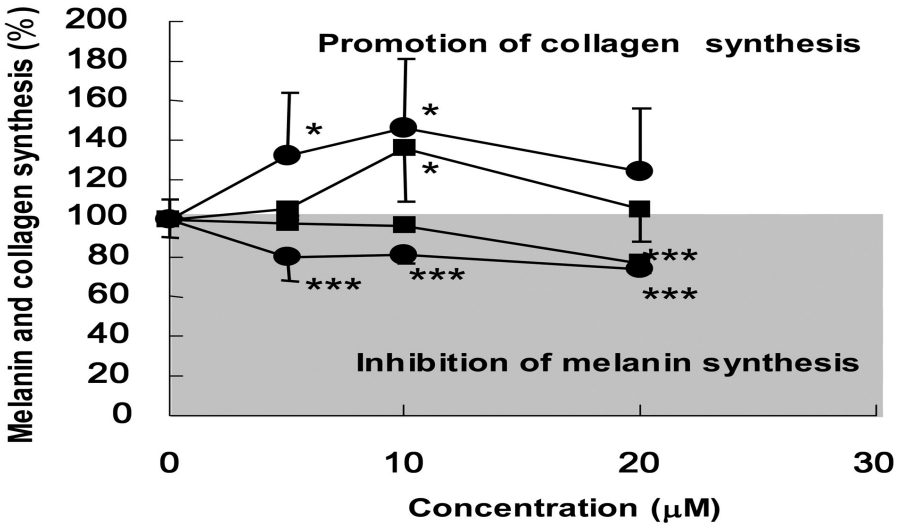

Figure 22: Melanin and collagen synthesis: comparison of resveratrol (■) with piceatannol (o). [104]

The results indicated that the passion fruit seed extract can be applied in the diet or topically to reduce melanogenesis, wrinkles and skin damage mostly due to piceatannol acitivity. [104]

5.5 Rose myrtle extract and piceatannol

Extracts from fruit of rose myrtle (*Rhodomyrtus tomentosa*) were tested as inhibitor of cell deaths of UVB induced cytotoxisity on cultured normal human epidermal keratinocytes (NHEK). [106] UVB radiation damages DNA by increasing the formation of *cis-syn* cyclobutane pyrmidine dimers (CPD) and other pyrimidone photoproducts. [107] Rose myrtle extract was found to contain piceatannol and piceatannol-4'-O-β-D-glucopyranoside, and protective effect of piceatannol on UVB irradiated NHEK was found to be due to an enhancement in DNA repair. UVB radiation (50 mJ/cm^2) exposed NHEK cell viability was increased when treated with rose myrtle extract and piceatannol, while piceatannol-4'-O-β-D-glucopyranoside was not found to have any significant effect (Figure 23). Viability was tested with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h after the treatment. Rose myrtle extract and piceatannol were also found to promote removal of CPD photoproducts (Figure 24). Rose myrtle extract and piceatannol

incubated NHEK were irradiated with UVB (80 mJ/cm^2) and CPD was determined by DNA-enzyme-linked immunoassay. [106]

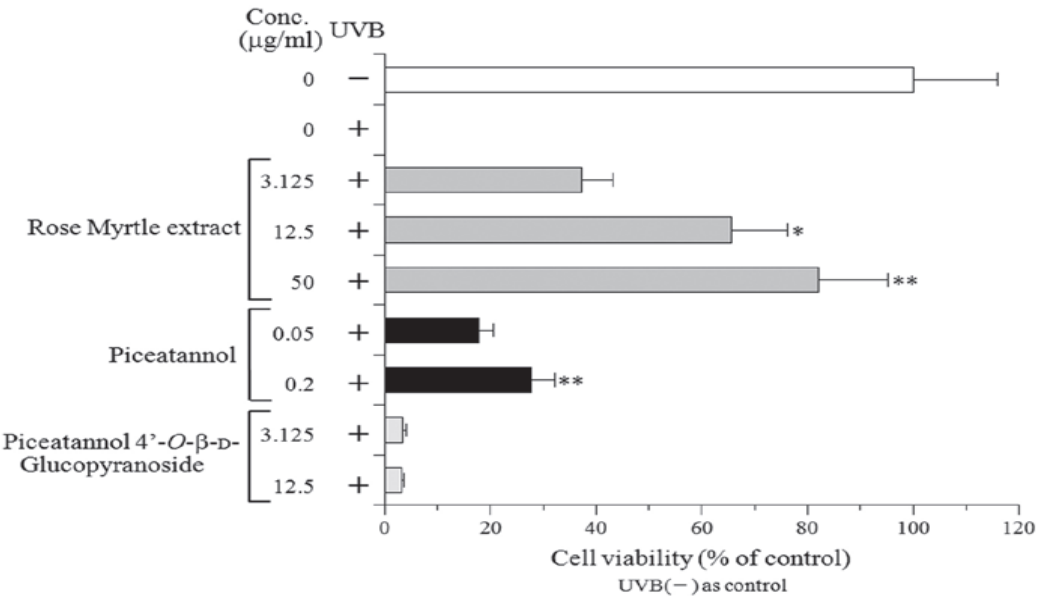

Figure 23: Cell viability after exposure of NHEK to UVB radiation. [106]

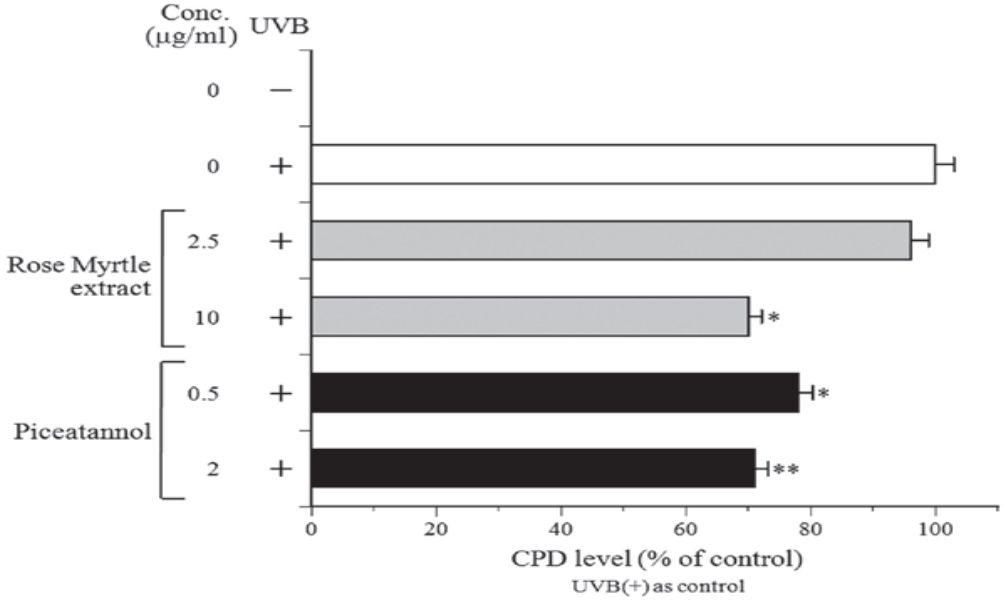

Figure 24: Effect of rose myrtle extract and piceatannol on CPD production in NHEK induced UVB radiation. [106]

The results suggested that rose myrtle extract and piceatannol may be used for the photoprotection of the skin, due to enhancement of DNA repair and suppression of inflammation. [106]

5.6 Cosmetics market

Based on Allied Market Research report (2016) on cosmetics market, the global cosmetics market is expected to reach 386 billion euros by 2022. An annual growth of 4.3% is expected during the period 2016-2022. The growth is due to the rise in disposable incomes and growth of global economies. In addition, changing climatic conditions and lifestyle preferences increase the demand for skin and sun care products, increasing the market growth for cosmetics (Figure 25). [108]

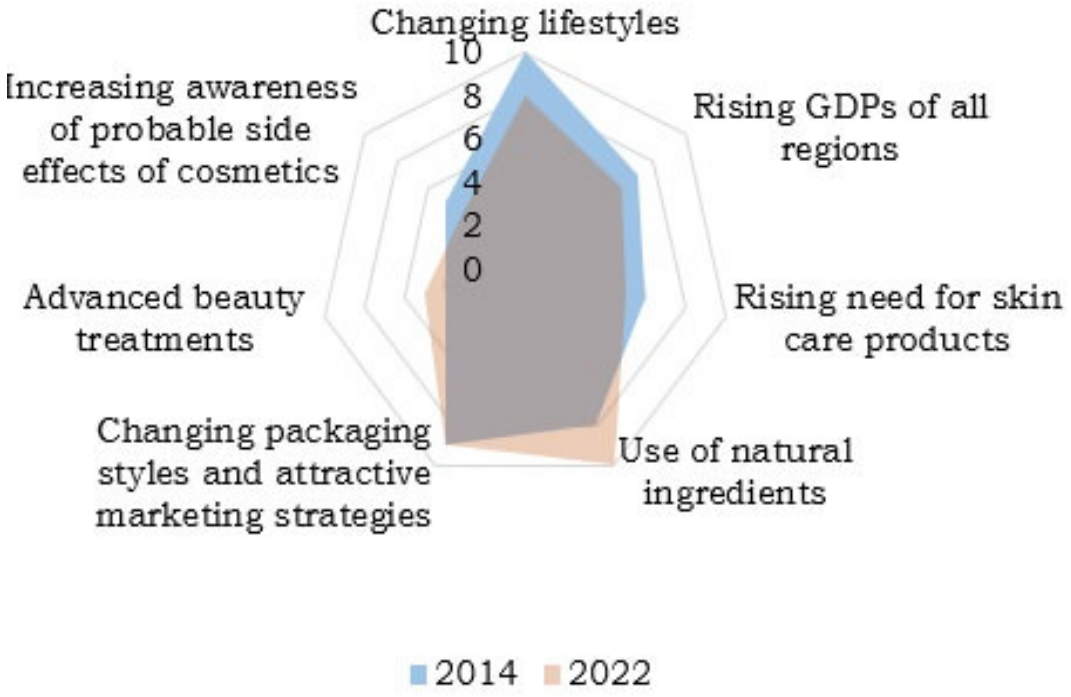

Figure 25: Factors impacting global cosmetics market. [108]

The growth of cosmetics market is fueled by improvement in the lifestyles with increasing amount of disposable income and better awareness of customers regarding the usage of cosmetics. Men have also started consuming more cosmetics (fragrances and deodorants) and have contributed to the growth of cosmetics market. [108]

Increase in the usage of natural and organic ingredients in cosmetics has been observed among manufacturers. Consumers have increased their demand for natural based products, since herbal cosmetics are less prone to side effects. [108] These trends can potentially create possibilities for utilization of the spruce bark extracts in cosmetics market. Polyphenols found in spruce bark can act as active ingredients for cosmetics products. [109] For example, topical resveratrol formulation for commercial applications has been suggested that utilizes dendrimer nanotechnology. [110] In addition, 1% resveratrol product has been developed for Calidora Skin Clinics by FAMAR (Athens, Greece). [111]

Experimental

6 Materials and methods

Experimental section is divided into parts (Figure 26) where temperature effect on the hot water extraction yields of spruce bark was studied in addition to ultrafiltration of spruce bark press water from Sappi Kirkniemi mill. In the hot water extraction with microwave reactor and mechanical pressing of bark water, industrial spruce bark from the wet debarking process was used. The bark was collected from Sappi Kirkniemi mill in February 2017 from the conveyor belt of the mill. Bark press waters used for ultrafiltration experiments were collected from Sappi Kirkniemi mill in March and May 2017. Chemicals used in this thesis were from Sigma-Aldrich, unless noted otherwise.

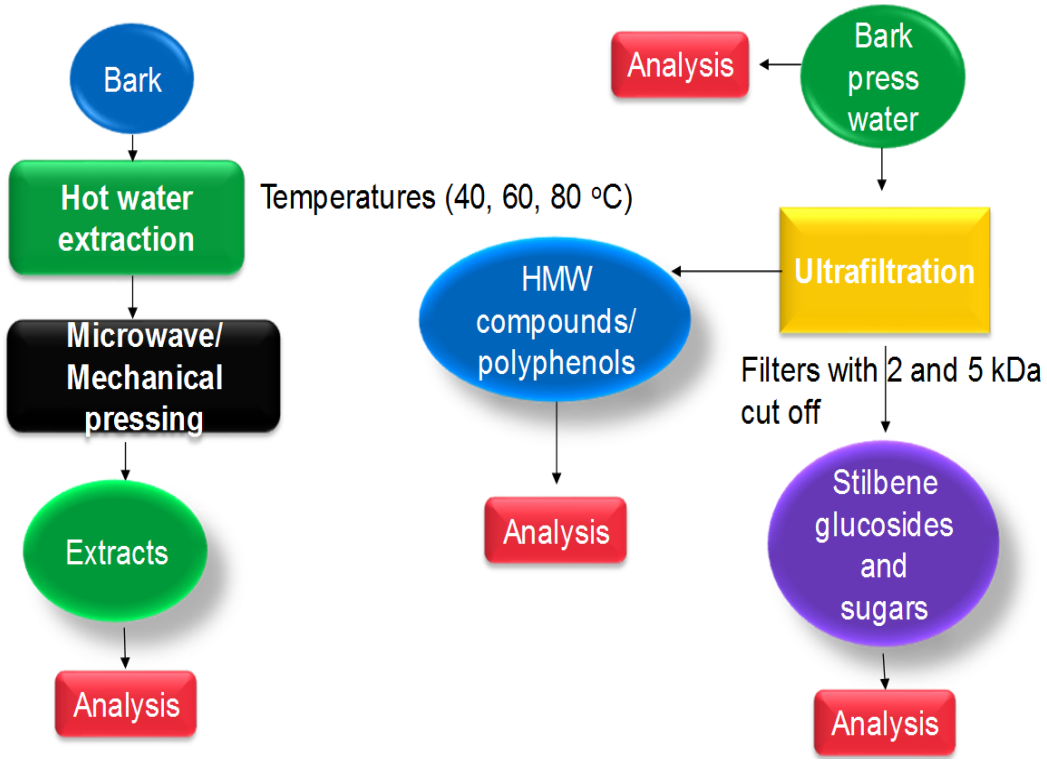

Figure 26: Experimental set-up of the thesis. HMW: high molecular weight compounds.

6.1 Hot water extraction with microwave reactor

The effect of temperature on extraction yields of polyphenols and stilbenes was tested with a Monowave 300 single-mode microwave reactor (Anton Paar GmbH) and 25 mL borosilicate glass reaction vessel. 1.5 g of oven dried bark cut to small pieces was dissolved in 15 mL of Milli-Q water. The solution was mixed with magnetic stirrer (600 rpm) during the extraction. Microwave reactor was operated with power of 850 W. Hot water extraction was done at 40, 60 and 80 °C with different reaction times (10, 20, 40 and 60 min). The extracted solution was filtered through a crucible (pore size 10-16 μm) and dried in the oven (Memmert) at 60 °C overnight. Total extraction yield was determined by averaging three repetitions. Figure 27 shows experimental set-up using microwave reactor.

Figure 27: Experimental set-up of hot water extraction with microwave reactor.

6.2 Mechanical pressing and steam oven extractions

In mechanical pressing 300 g of bark was used. Moisture content of the bark was determined gravimetrically (Precisa 205 ASCS) by placing bark samples in the 105 °C oven (VWR venti-line) overnight method (SCAN-C 39:97). Extractions were done in Air-o-Steam Touchline steam oven (Electorlux) at different temperatures (40, 60 and 80 °C) and extraction times (20, 60 and 120 min) without additional water. The samples were placed in sealed polyethylene bags that had holes cut into the corners (Figure 28A) and moisture content inside the oven was set to 90% for the extraction.

Mechanical pressing was conducted after steam oven extractions with Christensen mechanical press (Figure A1) by applying 20 tonnes over the area of 304.8 cm^2 for 10 minutes resulting in a pressure of 64.4 bar. Tailor made vessel for holding the bark was used (Figure 29). The vessel was disassembled to collect the extracted solution (Figure A2). Extracts were then centrifuged (Eppendorf centrifuge 5804R) at 8,000 rpm for 30 minutes (Figure A3).

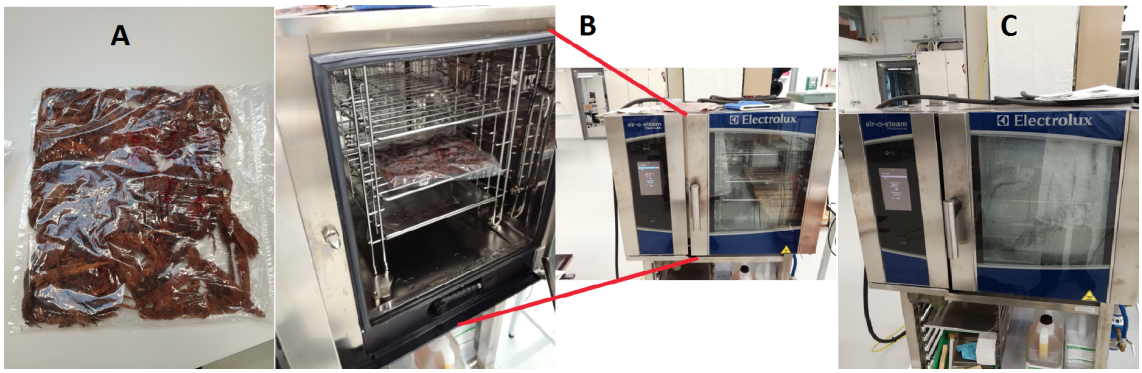

Figure 28: Experimental set-up of steam oven extractions.(A) Bark was sealed in polyethylene bag,(B) sample was placed in the middle of the oven, and oven was set to a wanted extraction time and temperature. (C) Extraction was performed at the moisture content of 90%.

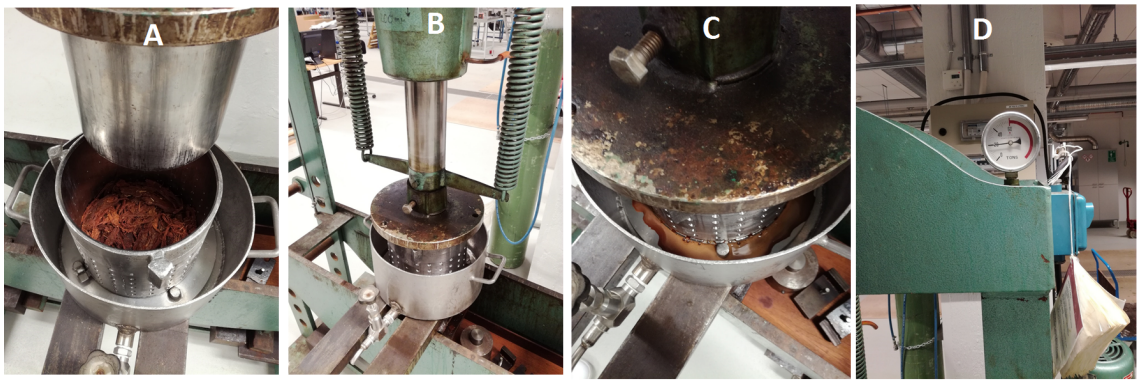

Figure 29: Mechanical pressing procedure: (A) bark is placed in tailor made pressing vessel; (B, C and D) the press is lowered until wanted pressing pressure reached.

6.3 Chemical oxygen demand test

Chemical oxygen demand (COD) test was applied for the mechanically pressed samples according to the standard method (SFS 5504) by averaging three repetitions, and the COD levels were determined using the following equation:

$$COD = 8,000 \cdot c_{Fe} \cdot (V_3 - V_4)/V_5, \tag{1}$$

where V_3 is the consumption of iron(II) solution by the zero sample (mL), V_4 is the consumption of iron(II) solution by the sample (mL), V_5 is the sample volume (mL) and c_{Fe} is the concentration of iron(II) solution (mol/L).

6.4 Ultrafiltration

Ultrafiltration of bark press samples from Sappi Kirkniemi mill were done using Alfalaval Labstak M20-0.72 ultrafiltartion unit (Figure 30) using 2 kDa (Alfalaval GR 90PP) and 5 kDa (Alfalaval GR 95PP) filters (Figure 30C). 700 mL of bark press sample (March and May) were diluted with water to a final volume of 4 L (Figure 30B) and placed in the filtration tank. Samples were filtrated for two hours.

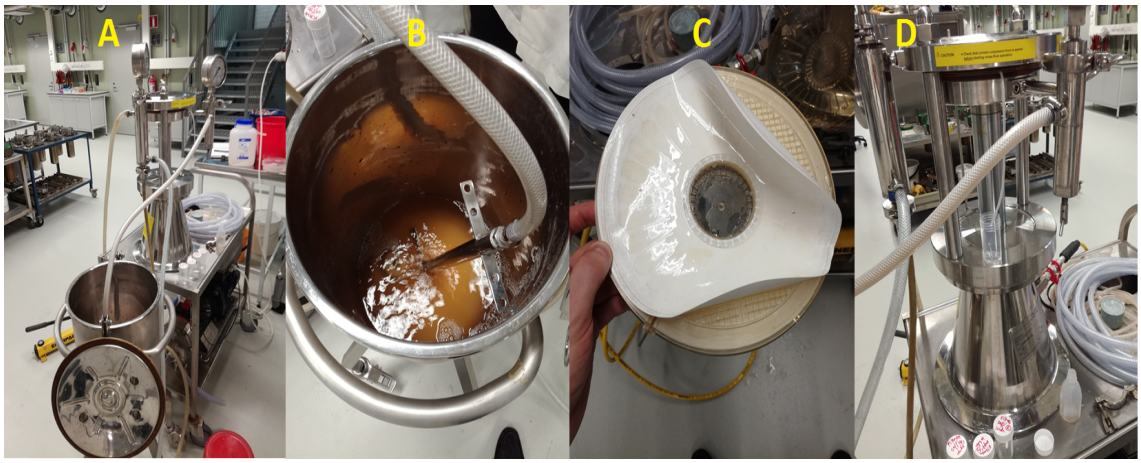

Figure 30: Ultrafiltration set-up: (A) ultrafiltration unit; (B) sample; (C) filter and (D) collection of filtrate.

6.5 UV-vis spectroscopy

A UV-vis spectrophotometer (Shimadzu UV-2550) was used to measure polyphenol and stilbene concentrations from the samples. Samples were diluted to achieve absorbance range under 1 AU. Polyphenols were determined according to protocol [112] based on phenolic adsorption at 280 nm. Following equation was applied to calculate the concentration:

$$phenol_{total} = [A280 \cdot DF \cdot (1cm/b)] - 4, \tag{2}$$

where DF is the dilution factor, b is the path length in the cuvette, and 4 is used as a correction factor for nonphenolic absorbance [112]. The results are reported in absorbance units (AU).

Due to a lack of standard compounds for all of the stilbenes, trans-piceid (polydatin) was used as an external standard for the quantification of all of the stilbene glucosides found in the samples [113, 114]. Calibration curve was prepared by measuring three times UV-vis spectra of polydatin (Figure 31) at different concentra-

tions (1, 4, 6, 8, 10, and 12 μg/mL). Resulting calibration curve (Figure A4) was prepared using averaged absorbance values at 320 nm and the linear equation was y=0.07124x+0.0132.

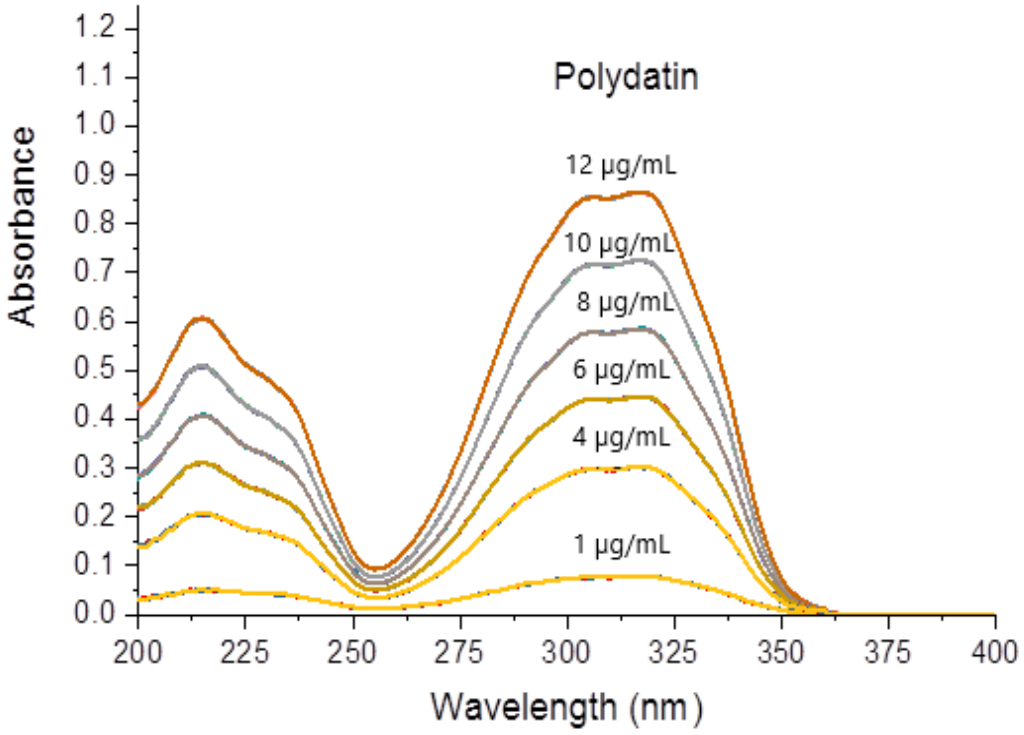

Figure 31: UV-vis spectra of polydatin at different concentrations.

6.6 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR experiments were utilized to obtain information on the composition of the extracts were performed in solution-state in dimethyl sulfoxide (DMSO)-d_6/pyridine-d_5 (4:1) at 27 °C on a Bruker Avance III spectrometer (Figure A5A). NMR spectrometer was operating at 400.13 MHz (^{1}H) and 100.61 MHz (^{13}C). The data was processed using TopSpin 3.0 software. The ^{1}H chemical shifts were referenced to DMSO at 2.49 ppm and the ^{13}C chemical shifts at 39.5 ppm. Heteronuclear single quantum coherence spectroscopy (HSQC) was performed for all of the samples to obtain ^{1}H–^{13}C HSQC spectras. Spectral widths of 12 ppm for ^{1}H and 219 ppm for ^{13}C were used. DMSO-d_6/pyridine-d_5 was obtained from Merck and resveratrol was from Evolva Sa. Samples were prepared by dissolving 50 mg of sample in DMSO-d_6/pyridine-d_5 in NMR tubes (Figure A5B).

Information that can be extracted from HSQC spectra is shown in Figure 32. The HSQC spectra of hot water extracts is divided between aliphatic region, sidechain and saccharide region, polysaccharide anomeric region, and aromatic region. [115]

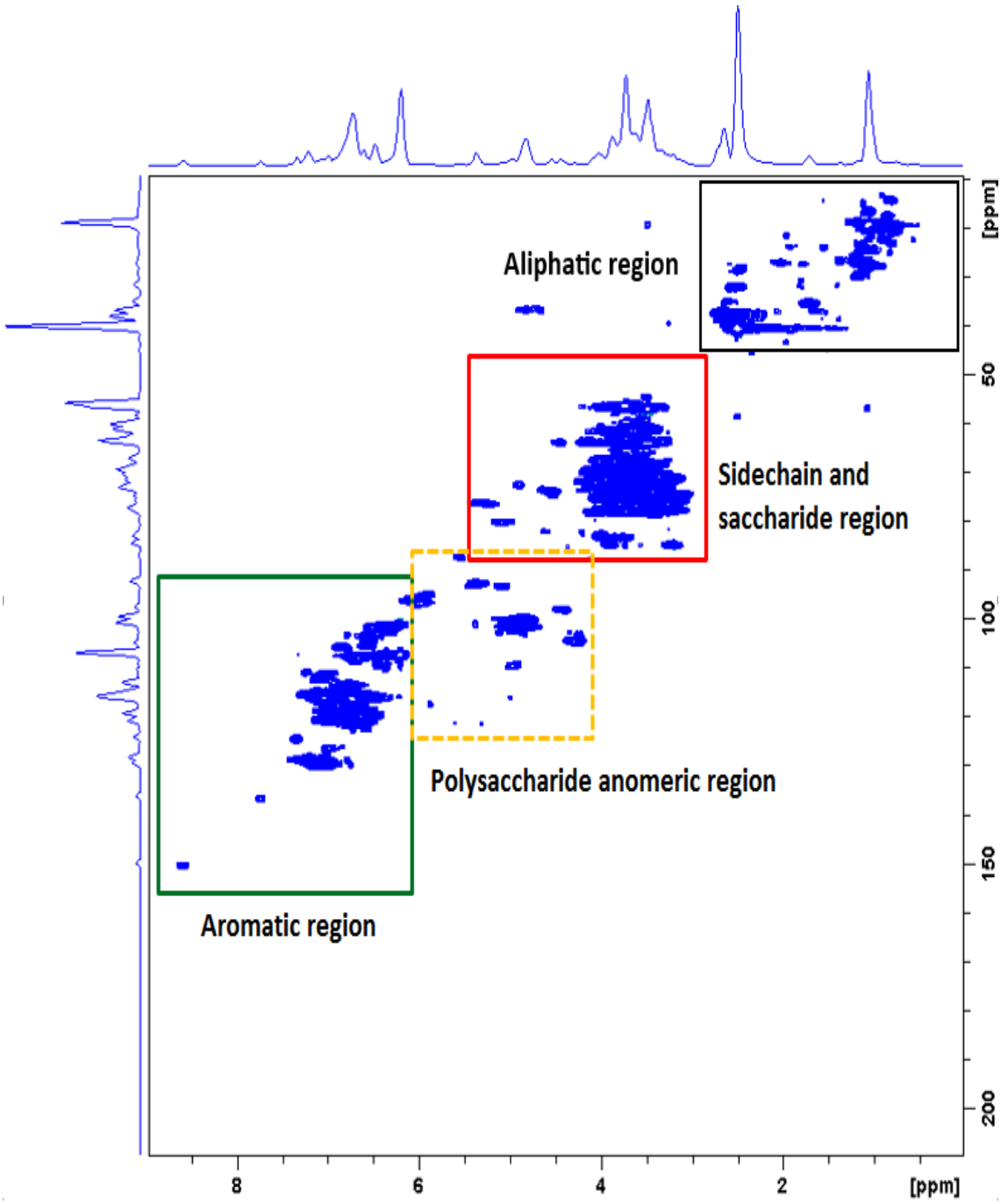

Figure 32: HSQC spectra and observed regions: aliphatic region in black, sidechain and saccharide region in red, polysaccharide anomeric region in yellow and aromatic region in green.

7 Results and discussion

7.1 Hot water extraction with microwave reactor

Triplicate hot water extractions with microwave reactor were done at three temperatures (40, 60 and 80 °C) and four different extraction times (10, 20, 40, and 60 min). The extracts were visibly more intense in brown color with extraction temperature increase (from 40 to 80 °C) already at 10 min extraction time (Figure A7), indicating that increasing concentrations of polyphenols were extracting from the spruce bark. Total extraction yields were determined gravimetrically (Figure 33). The extracts were also analyzed with UV-vis (Figure A6) to obtain quantitative information on total hot water extraction yields of polyphenols and stilbenes (Figure 34 and 35). Averaged UV-vis spectra show that increasing absorbance correlates with temperature. Higher yields of extractives were obtained by increasing the temperature and saturation of the yields for each temperature were already observed at 20 min (Figure 33). The extraction yield was increasing on average 1.4 times every 20 °C. At 40 °C the extraction yields ranged from 1.93-2.88%, 3.00-4.04% at 60 °C, and 4.04-4.99% at 80 °C, respectively. Relatively low water extraction yields compared to the literature (12-20.9%) [5, 6] could be the result of spruce bark taken from the mill being already extracted during the storage period at the mill site. More experiments would be needed to confirm this assumption.

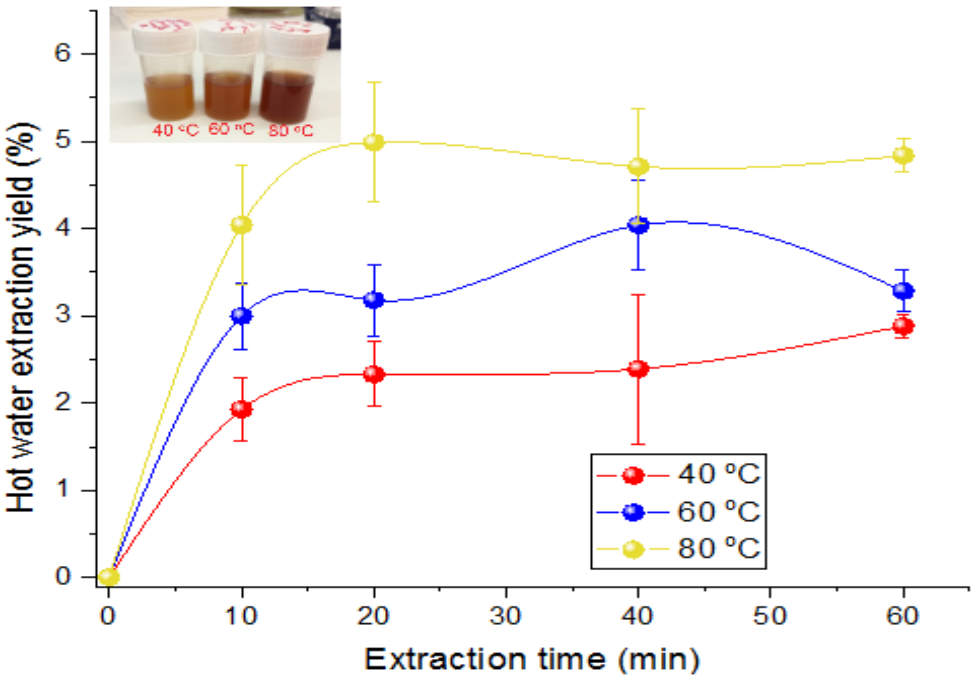

Figure 33: Total hot water extraction yields at different temperatures and extraction times of hot water extracts done with microwave reactor.

The extraction yields of polyphenols (Figure 34) and stilbenes (Figure 35) followed the trend of total extraction yields. Polyphenols extraction yields were affected by the temperature the most. From the UV-vis spectra, it was quantified that at 40 °C extraction yields ranged from 1.30-2.20 AU, 5.85-9.94 AU at 60 °C, and 11.17-16.50 AU at 80 °C, respectively. The extraction yield of polyphenols was increasing 4.4 times from 40 °C to 60 °C and 1.9 times from 60 °C to 80 °C, which was also noticeable from the brown color change (Figure A7).

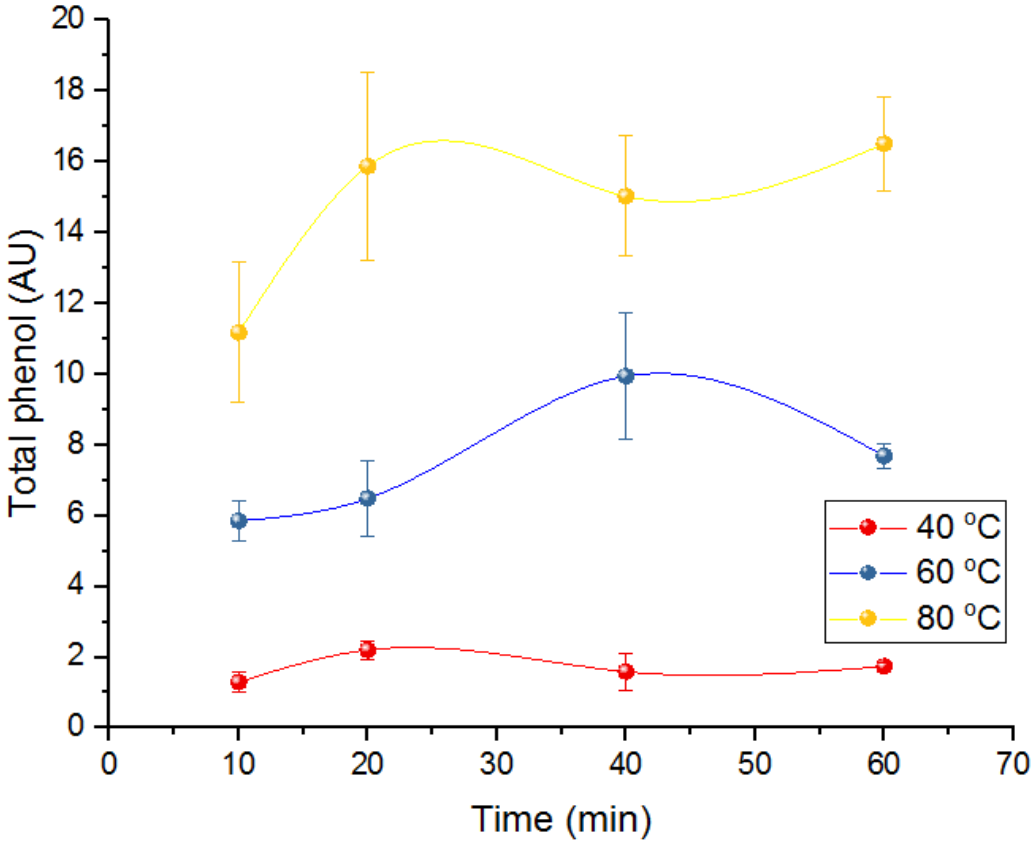

Figure 34: Extraction yields of polyphenols (280 nm) at different temperatures and extraction times of hot water extracts done with microwave reactor.

The extraction yields of stilbenes (Figure 35) were calculated from the calibration curve obtained measuring the polydatin standard solutions at 320 nm (Figure A4), and thus the concentrations are presented as polydatin μg/mL. At 40 °C extraction yields ranged from 28.32-34.52 polydatin μg/mL, 57.96-91.23 polydatin μg/mL at 60 °C, and 93.52-139.18 polydatin μg/mL at 80 °C, respectively. The extraction yield of stilbenes was increasing 2.3 times from 40 °C to 60 °C and 1.7 times from 60 °C to 80 °C. These results are also affected by a possible extraction of the stilbenes during the storage and the debarking process. More experiments are needed to determine if the stilbenes are still present after the storage, and if the stilbenes are extracted during the debarking process into the debarking waters. This would lead to a possibility to isolate the stilbenes from the debarking waters directly.

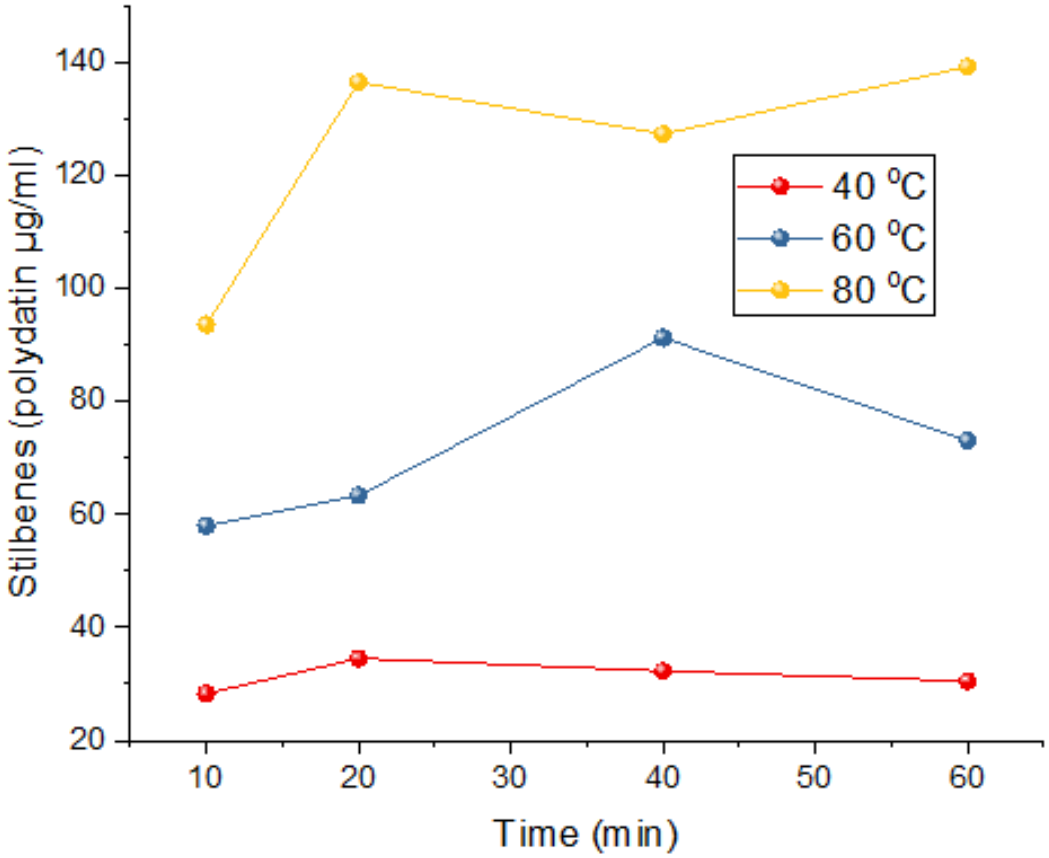

Figure 35: Extraction yields of stilbenes (320 nm) at different temperatures and extraction times of hot water extracts done with microwave reactor.

These results confirmed that the extraction yield is close to maximum already at the extraction time of 20 min. Since the increase of the extraction temperature increases the polyhpenols concentration more effectively compared to stilbenes, such extraction temperature needs to be selected that gives sufficient concentrations of stilbenes and minimizes the concentration of the polyphenols. To maximize the extraction yields of both polyphenols and stilbenes high temperatures should be preferred. Other means of separation (e.g., ultrafiltration, chromatography) of the stilbenes could be employed in this case. The ultrafiltration is the most appealing one, since it is less expensive than chromatography. By selecting the appropriate molecular weight cut off, it could reduce the high molecular weight compound (polyphenols) and pass through the low molecular weight compounds (stilbenes).

7.2 Mechanical pressing of spruce bark

Mechanical pressing after the steam oven extractions of the spruce bark were conducted to investigate if the moisture present in the bark is sufficient to increase the extraction yields without any additional water. This would lead to a process that would only need heating of the spruce bark. Average moisture content of the spruce bark used in the experiments was 68.1±0.9 % (n=3), which is in agreement with the literature [4]. Samples of 300 g were selected for these experiments so that approximately 2/3 of the sample was water, which would be used for extraction. The pressure (64.4 bar) and the pressing time (10 min) were both higher compared to the literature [4]. The conditions were selected to maximize the extraction yield. Selected pressing conditions pressed out 26±5 % of the total mass of the sample (Figure 36).

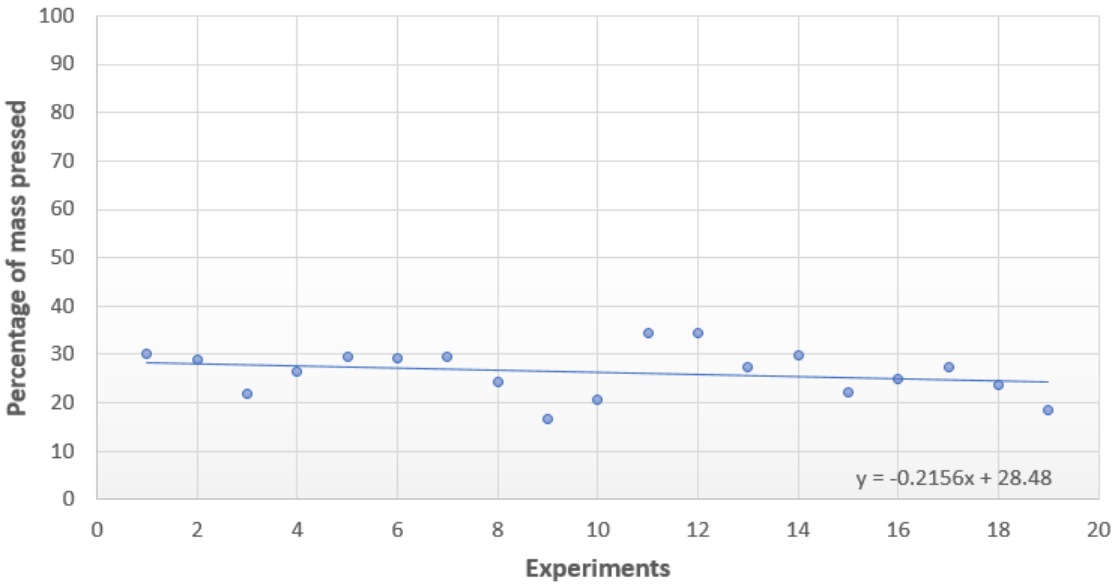

Figure 36: Pressing efficiency of mechanical pressing.

Chemical oxygen demand (COD) test was used to quantify the amount of organic compounds that were pressed out after the steam oven extractions. The results show the effect of the extraction temperature and time on the concentration of the organic compounds extracted and pressed out from the bark during the mechanical pressing (Figure 37). March bark press sample (COD 37,500±1,100 mg/L) was the sample that was pressed at the paper mill with bark press found at the mill. No heating sample (COD 48,700±500 mg/L) was a sample that was pressed out as a control experiment without any steam oven extraction. The COD levels ranged from 39,300-48,400 mg/L at 40 °C, 41,300-73,300 mg/L at 60 °C, and 55,100-75,100 mg/L at 80 °C, respectively. No apparent effect on the COD levels were noted at 40 °C compared to the control. Long extraction times and higher extraction temperatures were also causing some issues with the mechanical pressing, since softened spruce

bark easily squeezed out of the pressing vessel and was not pressed uniformly.

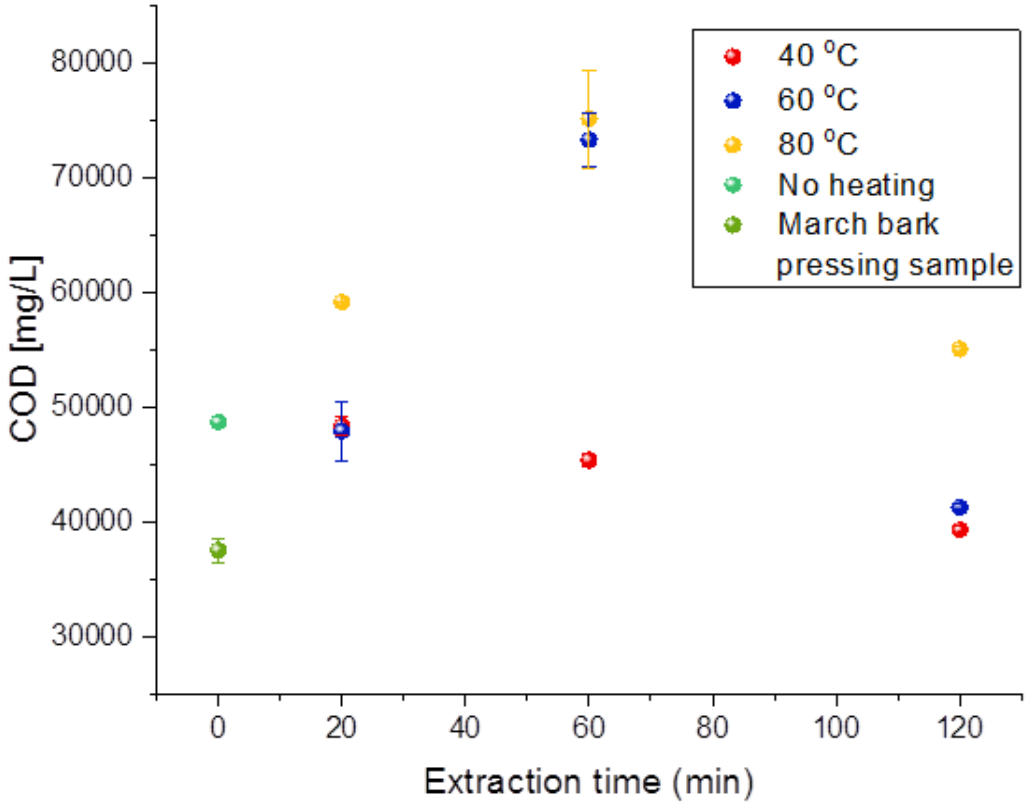

Figure 37: COD test results from the mechanical pressing after the steam oven extraction at different extraction times and temperatures. March bark pressing sample pressed at the paper mill.

At 80 °C there was clear elevation in the COD levels compared to the control experiment, which indicates that extraction yields of the extracted compounds increase due to the increase of extraction temperature. Highest COD levels achieved at 80 °C and 60 min extraction time were 1.5 times higher compared to the control experiment. Results also show that more extreme pressing conditions, which were used in the experiments, can press more organic compounds (1.3 times) out of the bark compared to the pressing conditions used at the paper mill. At 80 °C extraction, it is possible to increase the COD levels 1.5-2 times compared to the conditions used at the mill. For the applications where the bark press water would be further utilized, it would be important to optimize the pressing conditions. As shown in previous experiments, the stilbenes were already extracted from industrial spruce bark before the bark ended up at the bark press, and thus the increase of the COD levels related to the pressing conditions and extraction temperature were most likely due to the increase of concentration of the polyphenols and not the stilbenes. However, the experiments showed that there is no need for additional extraction water in addition to the moisture found already in the spruce bark to increase the concentration of the organic compounds.

7.3 Ultrafiltration of bark press waters

Ultrafiltration experiments were conducted with the bark press water sample that were taken directly from the mill and diluted (700 mL to 4L) to ensure that the filter would not get blocked. UV-vis spectra (Figure 38) of the sample taken in March differed from the sample taken in May, which is most likely due to different storage conditions used for the May bark. The spectra, even though being for the spruce bark extracts, resembles Figure 12, where spectrum of March sample resemble that of *cis*-resveratrol and the spectrum of May sample resemble that of *trans*-resveratrol. Spectra and previous experiments lead to conclusion, that the freshness of the logs before they are debarked and the bark is pressed is important for the preservation of stilbenes (290-320 nm in the UV-vis spectra) in the bark. The UV-vis spectra of the May sample, which was pressed out from more fresh bark compared to the March sample, contained peaks at the region where stilbenes are found (290-320 nm), while the March sample did not. This leads to a conclusions that there were no *trans*-stilbenes present in the March sample, and that the stilbenes were possibly converted to *cis*-form. Chromatography should be used to confirm this. Some preliminary gas chromatography with flame ionization detector (GC-FID) experiments showed that there were almost no stilbenes found in the March sample using commercially available stilbenes as a reference.

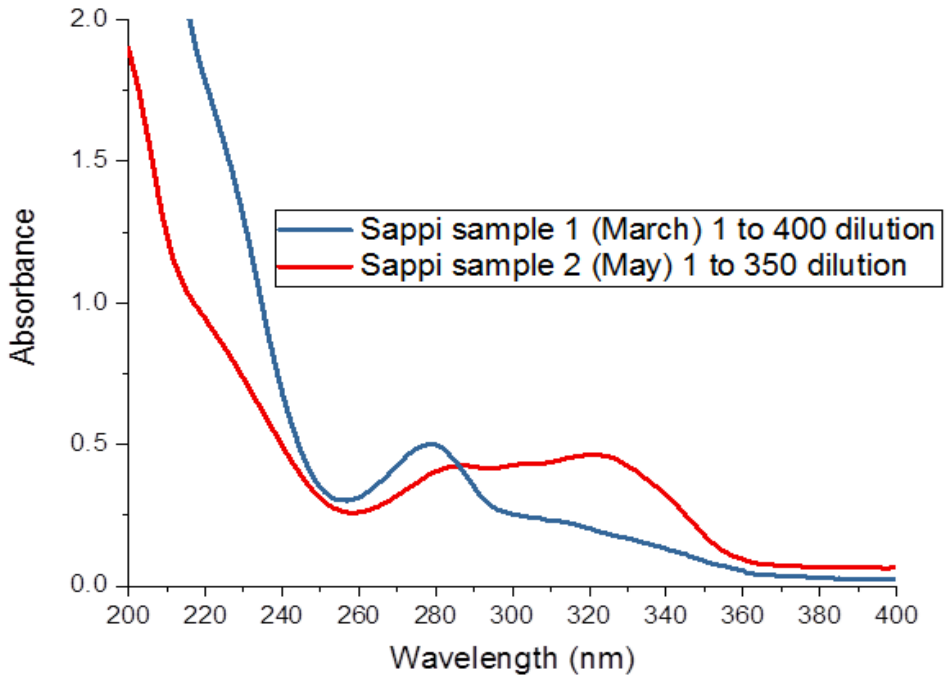

Figure 38: UV-vis spectra of bark press water samples pressed at the paper mill.

Since the May sample absorbed at UV-regions where stilbenes are found, it was selected for the ultrafiltration experiments. Both 2 kDa and 5 kDa filters were used for May sample (Table 3).The filters with these cut off levels were selected to filter high molecular weight compounds (polyphenols, polysaccharides) and to allow for stilbene glucosides to pass through. Stilbene glucosides that can be found in the spruce bark have molecular weight around 500 g/mol or 0.5 kDa. Bark press waters also contain sugars and other low molecular weight compounds that would be passing through the filter along with the stilbenes. 5 kDa filtrate contained 8.8% of stilbenes out of the total mass of the filtrate, leaving 91.2% of the mass for other low molecular weight compounds. Process diagram of the ultrafiltration with 5 kDa filter is depicted in Figure 39. From the diagram it can be seen that the polyphenols were concentrated in the process, resulting in a low polyphenol concentration in the filtrate (13 AU) and higher concentration (184 AU) compared to original feed concentration (137 AU). To further isolate the stilbene glucosides from the filtrate, additional purification steps should be applied. This could be possibly done with additional step, by polymer resin (XAD-7HP) filled column, to remove the soluble sugars, which will pass through the resin [7].

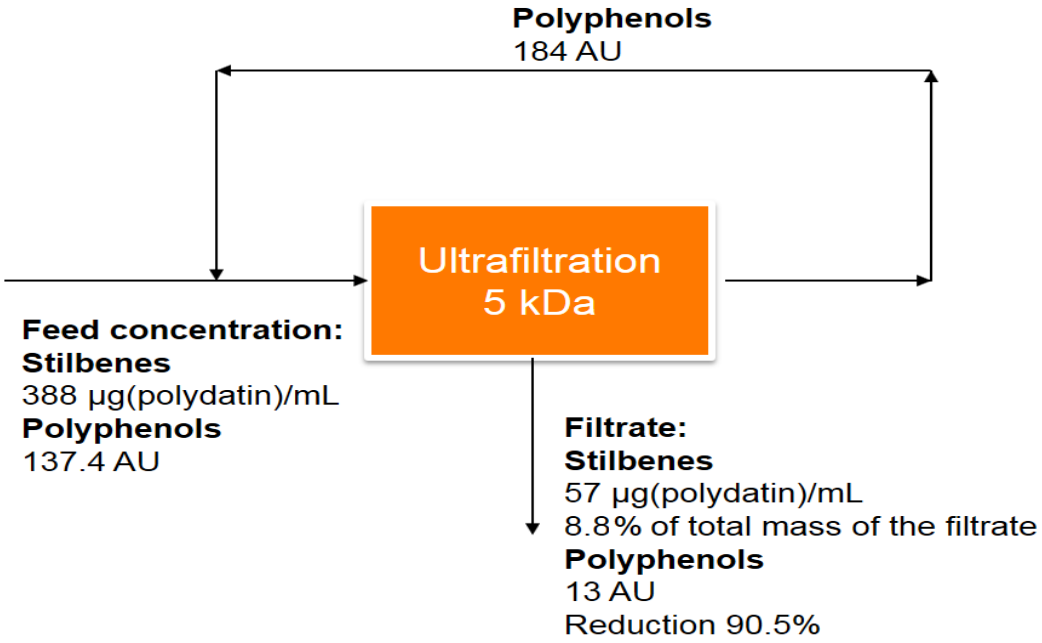

Figure 39: Process diagram of ultrafiltration of May bark press sample with 5 kDa filter.

Both 2 kDa and 5 kDa filters were effective in reducing the polyphenols by approximately 90% from feed concentration. The filters also successfully isolated approximately 14% of the stilbenes in the feed. From Table 3, it can be seen that 5 kDa filter had slightly better reduction of polyphenols (90.5%) and had slightly higher concentration of stilbenes (14.8%) compared to 2 kDa filter.

Table 3: Ultrafiltration of May sample with 2 kDa and 5 kDa filters.

Feed (700 mL May sample in 4 L)	**2 kDa filtrate**	**5 kDa filtrate**
Polyphenols		
Feed 137.4 AU	15 AU	13 AU
Reduction from feed concentration (%)	89.3	90.5
Stilbenes		
Feed 388 μg(polydatin)/mL	53.5 μg/mL	57.4 μg/mL
% of feed concentration	13.9	14.8

Efficiency of different filters on different feed concentrations should be thoroughly tested before further conclusions can be made. Since the feed in the mill will be undiluted, the undiluted samples should be tested in laboratory conditions. These tests were omitted in this thesis due to possibility of blockage in the lab scale ultrafiltration unit. However, the results show that the ultrafiltration is a viable way of purifying stilbenes from the bark press waters. Figure 40 shows that UV-vis spectra of the filtrates is similar to that of *trans*-resveratrol (Figure 12) and other stilbenes (Figure 31). In addition, the filtrates lost the brownish color that was present in the feed, which indicates that most of the polyphenols did not pass the filter, supported by the UV-vis spectra.

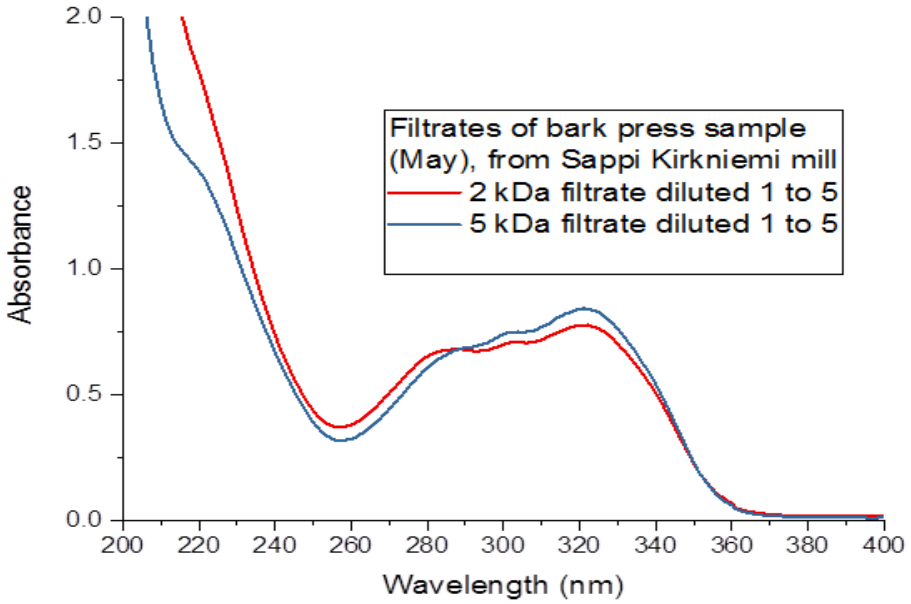

Figure 40: UV-vis spectra of ultrafiltrates (2 and 5 kDa filters) of May bark press sample.

7.4 NMR spectroscopy of stilbenes, bark press waters and ultrafiltrates

Bark press waters obtained from the mill, mechanically pressed hot water extract, ultrafiltrates, and commercially available stilbenes were subjected to 2D ^{1}H–^{13}C HSQC NMR for structural verification of the stilbene glucosides present in the samples.

Sidechain and saccharide region (60-105 nm) confirms the presence of the sugars in the samples and aromatic region (110-150 ppm) confirms the aromatic compounds. Stilbene glucosides (Figure A8 and A9) contain both sugar region as well as aromatic region, while resveratrol has chemical shifts only in aromatic region (Figure A10). Astringin standard was not available commercially, and the chemical shifts were obtained from the literature. The chemical shifts of isorhapontin and polydatin also agreed well with the literature. [113] Isorhapontin with chemical formula and corresponding chemical 2D ^{1}H–^{13}C HSQC shifts are illustrated in the Figure A8. Polydatin is illustrated in a similar fashion in Figure A9. ^{1}H and ^{13}C NMR chemical shifts of isorhapontin, polydatin, and astringin are collected in the Tables 4 and 5.

Table 4: ^{1}H NMR chemical shifts for isorhapontin, polydatin, and astringin in DMSO-d_6/pyridine-d_5. Multiplicity and coupling constants in parenthesis.

	Isorhapontin	**Polydatin**	**Astringin [113]**
Proton	δ (ppm)	δ (ppm)	δ (ppm)
H-2'	7.21 (d, 1.9)	7.39 (d, 8.5)	6.96 (d, 2.1)
H-8	7.01 (d, 16.3)	7.12 (d, 16.3)	6.94 (d, 16.3)
H-6'	7.00 (dd, 8.2, 1.9)	7.39 (d, 8.5)	6.83 (dd, 8.2, 2.0)
H-5'	6.83 (d, 8.1)	6.83 (d, 8.7)	6.71 (d, 8.1)
H-2	6.86 (s)	6.86 (s)	6.70 (s)
H-6	6.70 (s)	6.69 (s)	6.55 (s)
H-4	6.51 (t, 2.1)	6.51 (t, 2.1)	6.33 (t, 2.1)
H-1"	4.96 (d, 7.6)	4.96 (d, 7.6)	4.80 (d, 7.6)
CH_3O	3.81 (s)	n/a	n/a
H-2"	3.41 (m)	3.42 (m)	3.41 (m)
H-4"	3.36 (m)	3.35 (m)	3.35 (m)
H-5"	3.44 (m)	3.46 (m)	3.44 (m)
H-6"a	3.84 (m)	3.86 (m)	3.84 (m)
H-6"b	3.63 (m)	3.64 (m)	3.63 (m)

Coupling constants in Hz
Multiplicity (s singlet; d doublet; t triplet;m multiplet) [113].
n/a not applicable

Table 5: ^{13}C NMR chemical shifts for isorhapontin, polydatin, and astringin in DMSO-d_6/pyridine-d_5.

	Isorhapontin	**Polydatin**	**Astringin [113]**
Carbon	δ (ppm)	δ (ppm)	δ (ppm)
C-1	129.2	-	129.1
C-1'	139.7	-	139.5
C-1"	100.6	100.8	100.9
C-2	104.7	104.8	105.1
C-2'	110.3	-	113.4
C-2"	73.3	73.3	73.4
C-3	159.1	-	159.0
C-3'	148.1	-	145.5
C-3"	76.8	-	76.7
CH_3O	55.5	n/a	n/a
C-4	102.8	102.7	103.0
C-4'	146.9	-	145.8
C-4"	69.6	69.8	69.9
C-5	158.6	-	158.4
C-5'	115.6	115.5	115.9
C-5"	77.2	77.1	77.3
C-6	107.3	107.1	107.2
C-6'	120.2	-	118.9
C-6"	60.6	60.7	60.9
C-7	125.7	-	125.2
C-8	129.1	128.4	129.0

All the ^{13}C chemical shifts were collected from the HSQC and [113].
n/a not applicable
- Undefined values

Signal at δ 3.81 (s) ppm represents methoxy groups (CH_3O) three protons (Figure A8), which is only present in the isorhapontin. The anomeric centre (H-1") of the β-glycoside unit [116] can be found at δ 4.96 (d, J=7.6 Hz) ppm for both isorhapontin (Figure A8) and polydatin (Figure A9). [113] Apart from the methoxy group, the NMR spectrum of isorhapontin is similar to that of polydatin. Characteristic signals of β-glycoside unit can be found for both in the region δ 3.86-3.35 ppm. Both have trisubstituted aromatic ring protons (H-2, H-4 and H-6) in the aromatic ring connected to the sugar. [113]

Mechanical pressing sample (Figure A11), which was from the spruce bark collected in February from the mill, did not contain *trans*-stilbene glucosides according to the 2D ^{1}H–^{13}C HSQC NMR spectrum. Neither did the bark press water sample

(March) (Figure A12). This confirms that the stilbene glucosides in the winter bark from the mill are either isomerized to *cis*-form or extracted already in the storage of the bark. Additional research is needed to find the best possible period of the year for stilbene glucoside extraction of the spruce bark found at the mill. However, all of the three major stilbene glucosides were identified in the bark press water sample (May) (Figure A13) and its filtrates 2 kDa (Figure 41) and 5 kDa (Figure A14).

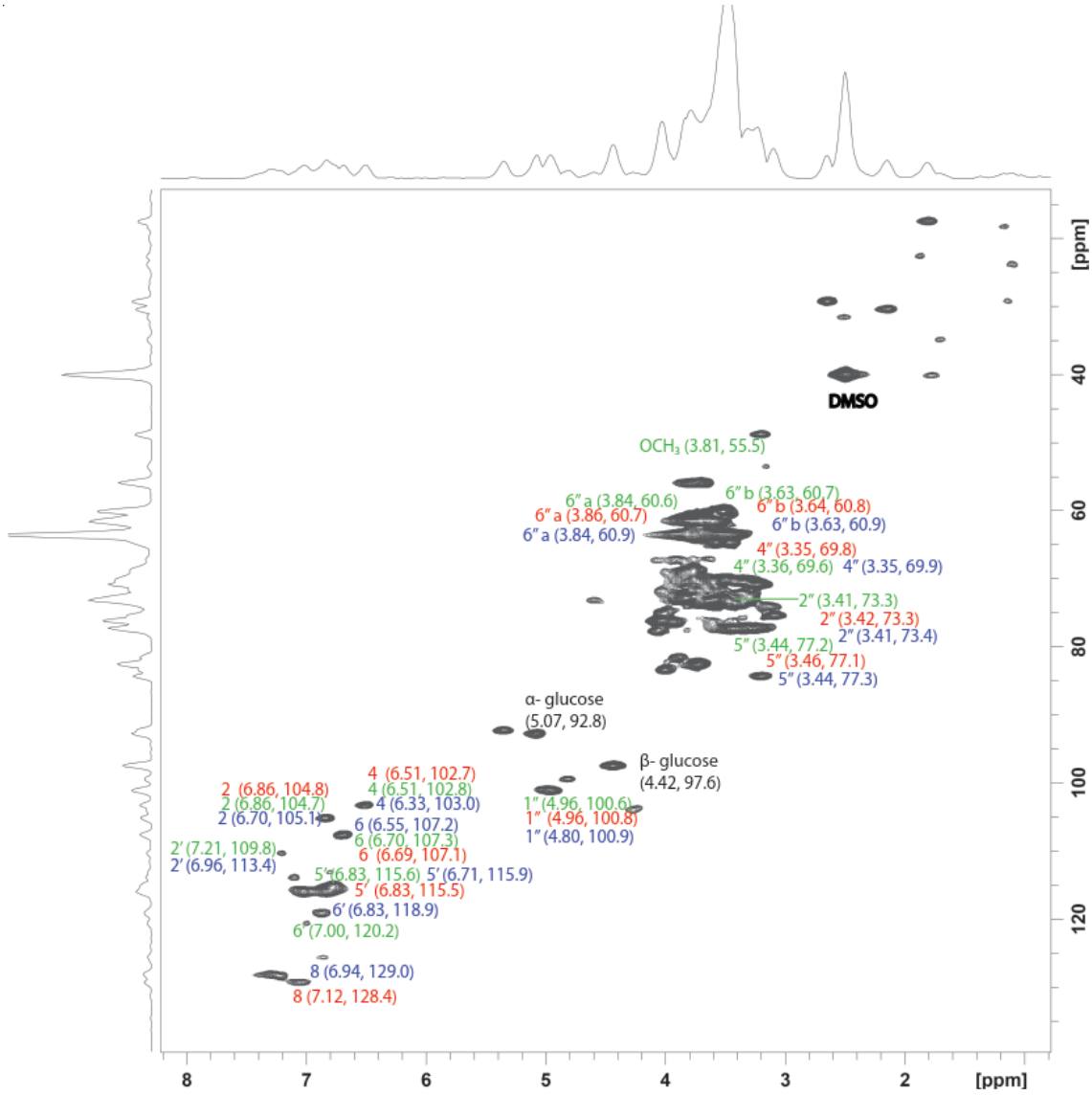

Figure 41: 2D ^{1}H–^{13}C HSQC NMR spectrum of 2 kDa filtrate of bark press water sample (May) in DMSO-d_6/pyridine-d_5: isorhapontin (green); polydatin (red); astringin (blue).

Filtrates contain major overlapping in the sidechain and saccharide region (60-100 ppm), thus the further step would be to remove the sugars from the filtrates using the polymer resin to obtain an enriched isolate of the stilbene glucosides.

8 Conclusion and future research

In the literature part, spruce bark utilization in pulp and paper industry was studied. The spruce bark harvested in Finland, is fully utilized, mostly by burning to produce energy. At typical moisture content (50-65% moisture) spruce bark produces 5.0-9.0 MJ/kg of energy, while dry bark has net calorific value of 18.5-20.0 MJ/kg. Thus, dewatering and bark pressing is utilized at the mill to increase the net calorific value, and burned at bark boiler to produce energy. Apart from using spruce bark for energy purposes at the mill in the bark boiler, it could be sold as a wood fuel at price of 0.025 euros/kg (60% moisture content). Debarking wastewater is highly toxic with high concentration of dissolved compounds (sugars, stilbenoids, and tannins). The spruce bark contains 12-20.9% of water-soluble compounds, depending on the extraction temperature, that are dissolved in the debarking wastewaters. These waters are not utilized at the moment, and are considered a waste stream, which adds to the costs of the mill by increasing COD levels of the wastewater. Aim of the literature part was to find compounds, which would potentially provide additional revenue to the mill.

Tannin-type polyphenols content in spruce bark ranges from 4-15%. Tannins could be potentially utilized in many different applications, especially in quality leather hide production, oenological applications, as animal feed, dispersants or coagulants, and insulating foams. They can be applied as wood adhesives, with either phenol-formaldehyde resins, or even formaldehyde free tannin adhesives. Technologically, tannin based formaldehyde-free adhesives could be applied on industrial scale, but spruce bark tannin has not yet shown technical success in this application. After the technical success, the spruce bark tannin should also be economically competitive with phenol (1,500 euros/tonne). A paper mill producing 50,000 tonnes of spruce bark per year could potentially produce approximately 5,250 tonnes of tannin, which would amount to 2.6% of the global market.

Spuce bark contains 5-10% of stilbenoids, while industrial bark contains 1-2% of stilbenoids, which are eventually dissolved in the debarking process waters. Most abundant being stilbene glucosides: isorhapontin, astringin, and polydatin. Only small amounts of aglucones (piceatannol, resveratrol, and isorhapontigenin) are present due to their poor water solubility. Aglucones can be produced by the hydrolysis of the glucosides. Some complex structures can be also produced when tannins and stilbenoids react. Stability of stilbenoids to photo-oxidation needs to be taken into account before extracting these compounds from spruce bark to ensure that bark extracts would be useful for further applications. Global resveratrol market is expected to grow 4.5% annually from 2016-2021. At the moment the market price for bulk resveratrol being approximately 300 euros/ kg when buying quantities of 100 kg. Higher prices are expected for stilbene glucosides, piceatannol and isorhapontigenin due to their presently lower demand and scarcity.

Stilbenoids have been found to have positive effect in treating type 2 diabetes, obesity, cardiovascular disease, cancer and skin disorders. This thesis reviewed application of stilbenoids for the treatment of skin aging, and their possible application in cosmetics. Resveratrol-procyanidin blend has been found to improve skin moisture and elasticity. Anti-acneic treatment of acne vulgaris with resveratrol containing gel has been also reported, that showed 53.8% reduction in clinical leseions on the resveratrol treated sides compared to 6.1% on vesicle treated sides. Other application of resveratrol could be as a skin lightener. Passion fruit seed extract with positive effect on skin cells, has been found to contain high concentrations of piceatannol, which also found in the spruce bark extracts. Seed extract was found to have positive effect on skin cells by promoting synthesis of collagen and inhibition of melanogenesis. This is suspected to be due to piceatannol, resveratrol and scirpusin B content of the extract. Extracts from fruit of rose myrtle on the other hand, were tested as inhibitor cell deaths of UVB induced cytotoxisity on cultured normal human epidermal keratinocytes (NHEK). The results suggested that rose myrtle extract and piceatannol may be used for the photoprotection of the skin, due to enhancement of DNA repair and suppression of inflammation. Piceatannol and isorhapontigenin are less extensively researched analogues of resveratrol with similar health effects to resveratrol. Fresh spruce bark contains probably the highest concentrations of these stilbenoids in nature. The global cosmetics market is expected to reach 386 billion euros by 2022, with annual growth of 4.3%. The growth is due to the rise in disposable incomes and growth of global economies. In addition, changing climatic conditions and lifestyle preferences increase the demand for skin and sun care products. Increase in the usage of natural and organic ingredients in cosmetics has also been observed among manufacturers. Consumers have increased their demand for natural based products, since herbal cosmetics are less prone to side effects. Thus, industrial isolation stilbenoids for cosmetics applications should be considered based on the literature review.

In the experimental part, temperature effect on the hot water extraction yields of spruce bark was studied in addition to ultrafiltration of spruce bark press water from the paper mill. Extraction yield was increasing 1.4 times every 20 °C, reaching 4.04-4.99% at 80 °C. Relatively low extraction yields compared to the literature (12-20.9%) could be the result of spruce bark taken from the mill being already extracted during the storage at the mill site and the debarking process. More experiments would be needed to confirm this assumption. The extraction yield of polyphenols was increasing 4.4 times from 40 °C to 60 °C and 1.9 times from 60 °C to 80 °C. The extraction yield of stilbenoids was increasing 2.3 times from 40 °C to 60 °C and 1.7 times from 60 °C to 80 °C. These results are also affected by a possible extraction of the stilbenoids during the storage and the debarking process. More experiments are needed to determine if the stilbenoids are still present after the storage, and if they are extracted during the debarking process into the debarking waters. This would lead to a possibility to isolate the stilbenoids from the debarking waters directly and wastewaters could potentially be transformed to a source of stilbene glucosides and polyphenols for cosmetics, to provide additional revenue to the mill.

Mechanical pressing after steam oven extractions of the spruce bark were conducted to investigate if the moisture present in the bark is sufficient to increase the extraction yields without any additional water. Average moisture content of the spruce bark used in the experiments was 68.1±0.9 % (n=3). Selected pressing conditions pressed out 26±5 % of the total mass of the sample. Results also show that more extreme pressing conditions, which were used in the experiments, can press more organic compounds (1.3 times) out of the bark compared to the pressing conditions used at the paper mill. At 80 °C extraction it is possible to increase the COD levels 1.5-2 times compared to the conditions used at the mill. Results indicated that the stilbenoids were already extracted from industrial spruce bark before the bark ended up at the bark press, and thus the increase of the COD levels related to the pressing conditions and extraction temperature were most likely due to the increase of concentration of the polyphenols and not the stilbenoids. However, the experiments showed that there is no need for additional extraction water in addition to the moisture found already in the spruce bark to increase the concentration of the organic compounds.

Ultrafiltration experiments with 2 kDa and 5 kDa filters were conducted with the bark press water sample that were taken directly from the mill. Filters with these cut off levels were selected to filter high molecular weight compounds (polyphenols, polysaccharides) and to allow for stilbene glucosides to pass through. The sample taken in March differed from the sample taken in May, which is most likely due to different storage conditions used for the May bark. Chromatography should be used to confirm this. Some preliminary gas chromatography with flame ionization detector (GC-FID) experiments showed that there were almost no stilbenoids found in the March sample using commercially available stilbenoids as a reference. Both 2 kDa and 5 kDa filters were effective in reduction of the polyphenols by approximately 90% from feed concentration. The filters also successfully isolated approximately 14% of the stilbenoids in the feed. Efficiency of different filters on different feed concentrations should be thoroughly tested before further conclusions can be made. Since the feed in the mill will be undiluted, the undiluted samples should be tested in laboratory conditions. These tests were omitted in this thesis due to possibility of blockage in the lab scale ultrafiltration unit. However, the results show that the ultrafiltration is a viable way of purifying stilbenoids from the bark press waters. Bark press waters obtained from the mill, mechanically pressed hot water extract, ultrafiltrates, and commercially available stilbenoids were subjected to 2D ^{1}H–^{13}C HSQC NMR for structural verification of the stilbene glucosides present in the samples. All of the three major stilbene glucosides were identified in the bark press water sample (May) and its filtrates 2 kDa and 5 kDa, but not in bark press water sample (March) nor mechanically pressed samples. This was most likely due to the spruce logs at the mill being relatively fresh compared to the winter period. Filtrates contain major overlapping in the sidechain and saccharide region (60-100 ppm), thus the further step would be to remove the sugars from the filtrates using the polymer resin by adsorption-desorption to obtain an enriched isolate of the stilbene glucosides.

Isolation strategy for industrial stilbene glucosides and low molecular weight (LMW) polyphenols for cosmetics purpose is presented in Figure 42. In the strategy, the ultrafiltration units will transfer the filtrate to the polymer resin column that will adsorb stilbene glucosides and low molecular weight (LMW) polyphenols until the capacity of the resin is reached. The compounds can be then desorbed and collected for further applications. Parallel resin columns can be utilized in case where continuous adsorption process is preferred. Second column will be used while the first one is desorbed.

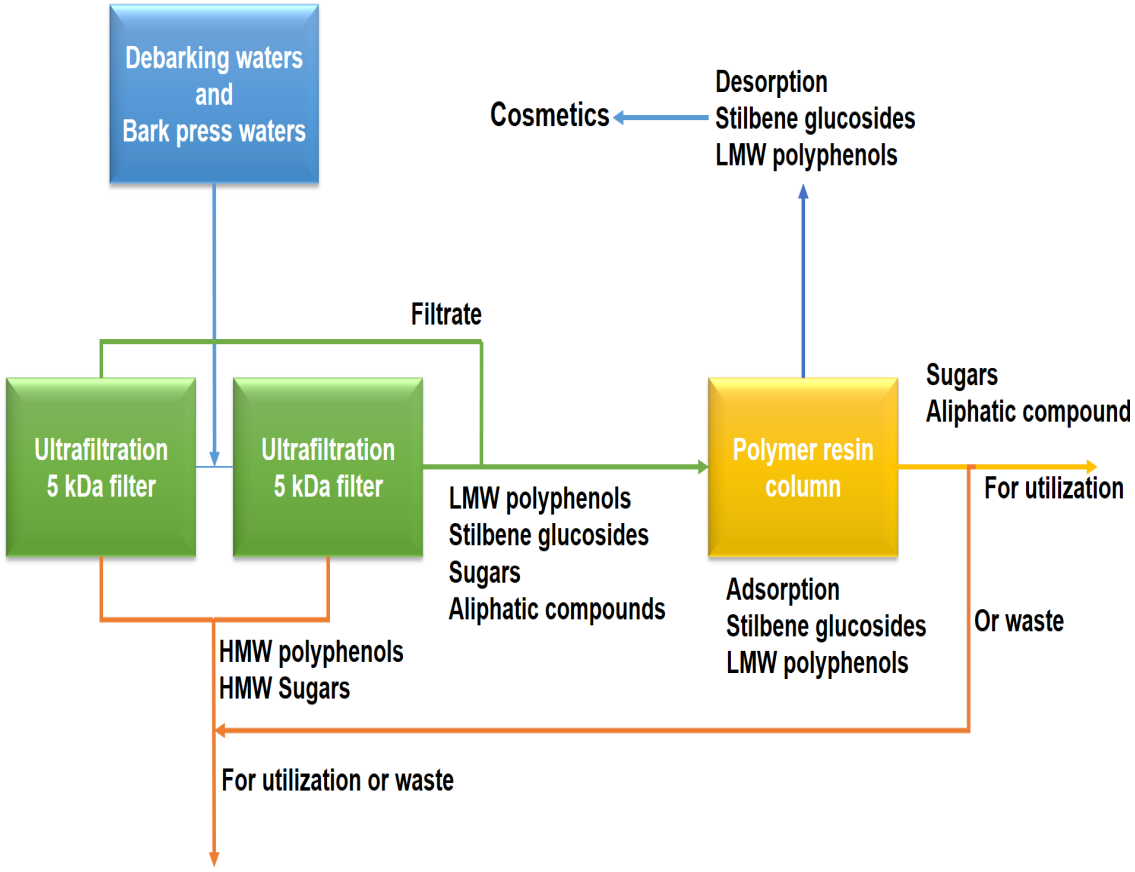

Figure 42: Strategy for industrial isolation of stilbene glucosides and low molecular weight (LMW) polyphenols for cosmetics purpose.

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A Appendix

Figure A1: Mechanical press applied to press spruce bark after steam assisted extraction of water soluble extractives.

Figure A2: Disassembling the pressing vessel: (A) Removing the pressing piston; (B) removing the plate holding the bark; (C and D) collecting the extracts in a container.

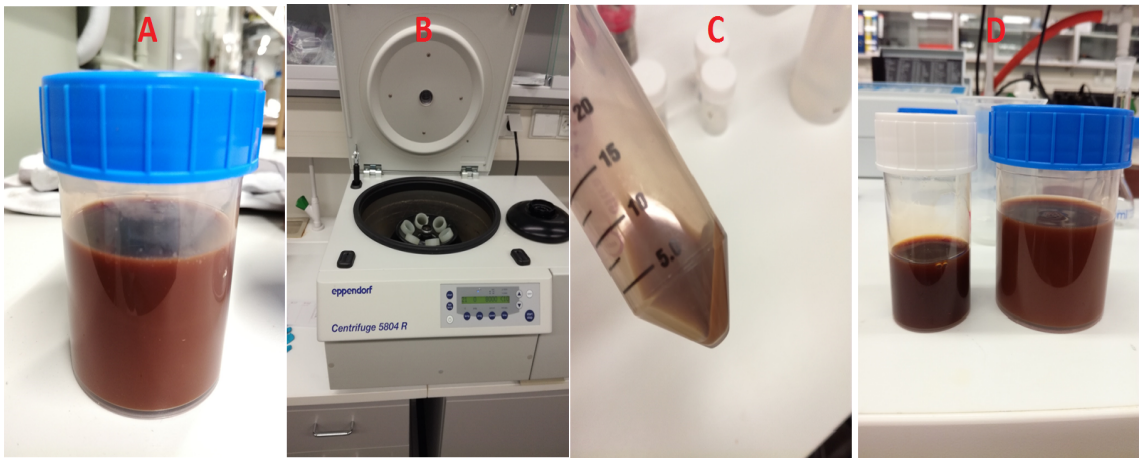

Figure A3: Centrifugation of the mechanically pressed sample: (A) sample; (B) centrifuge; (C) precipitate left after centrifugation; (D) centrifuged sample (white) and original sample (blue).

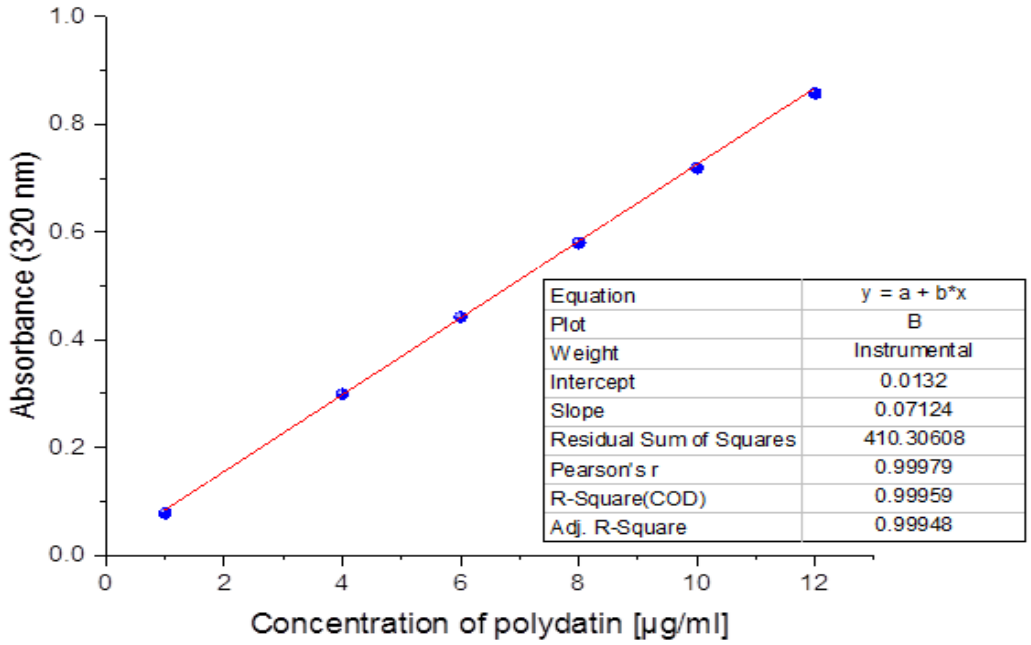

Figure A4: Calibration curve of polydatin used for estimation of stilbene concentrations.

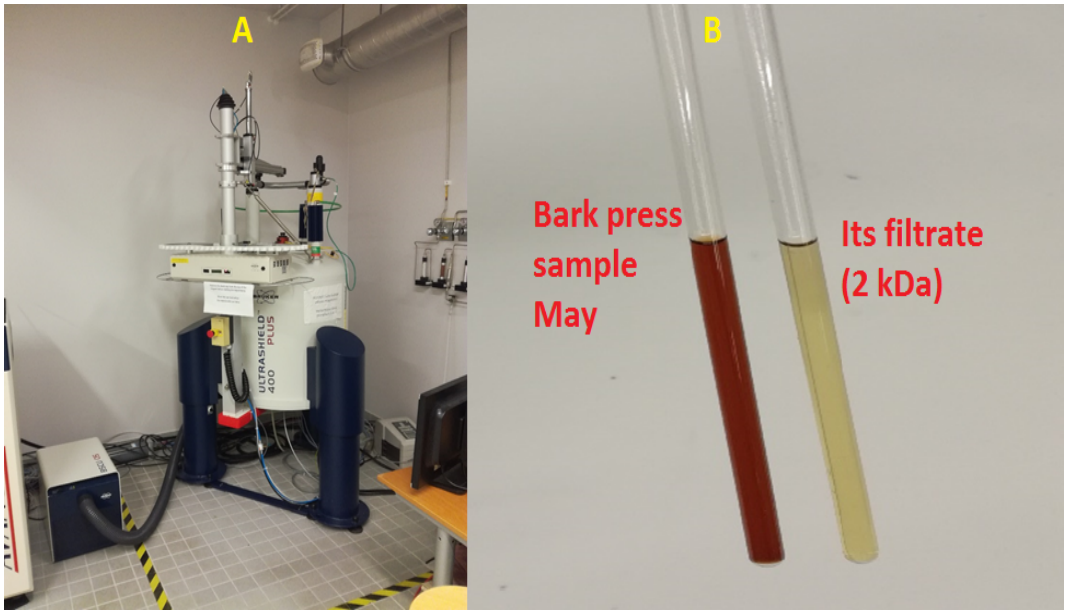

Figure A5: Bruker Avance III NMR spectrometer (A) and samples dissolved in DMSO-d_6/pyridine-d_5 (B).

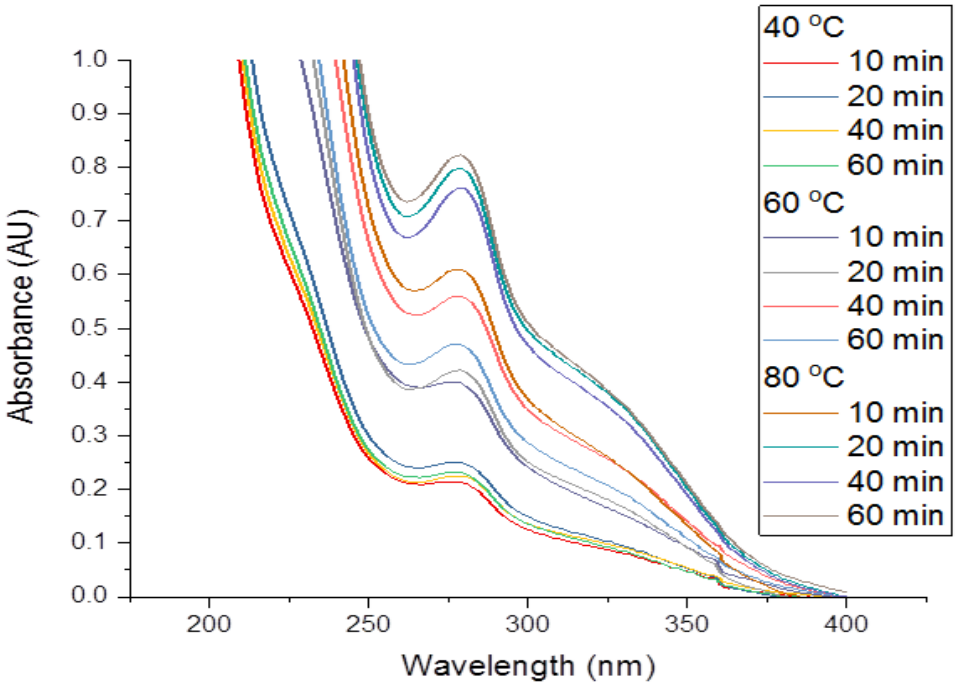

Figure A6: Averaged UV-vis spectra of three crucible filtered hot water extracts from microwave reactor at different temperatures and extraction times. Observed wavelength was 280 nm and 320 nm.

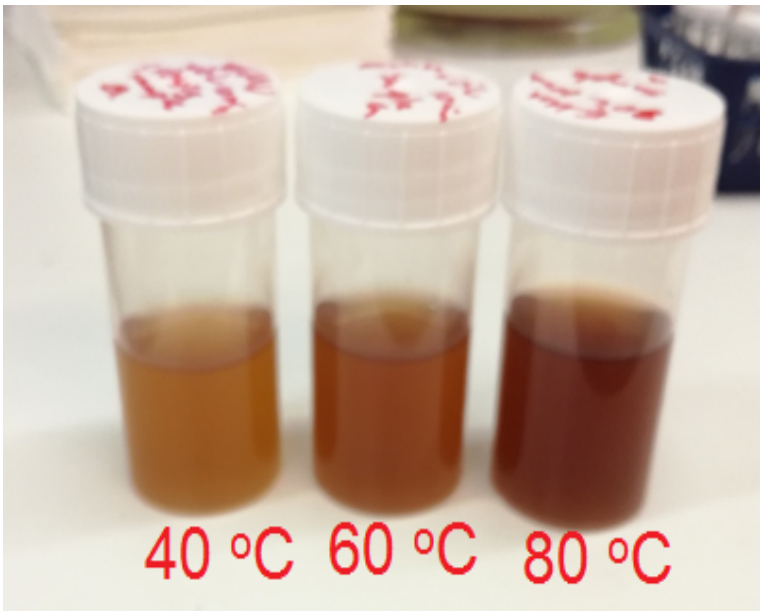

Figure A7: Crucible filtered hot water extracts done with microwave reactor with 10 min extraction time that show intensification of the brown color with the increase of the extraction temperature.

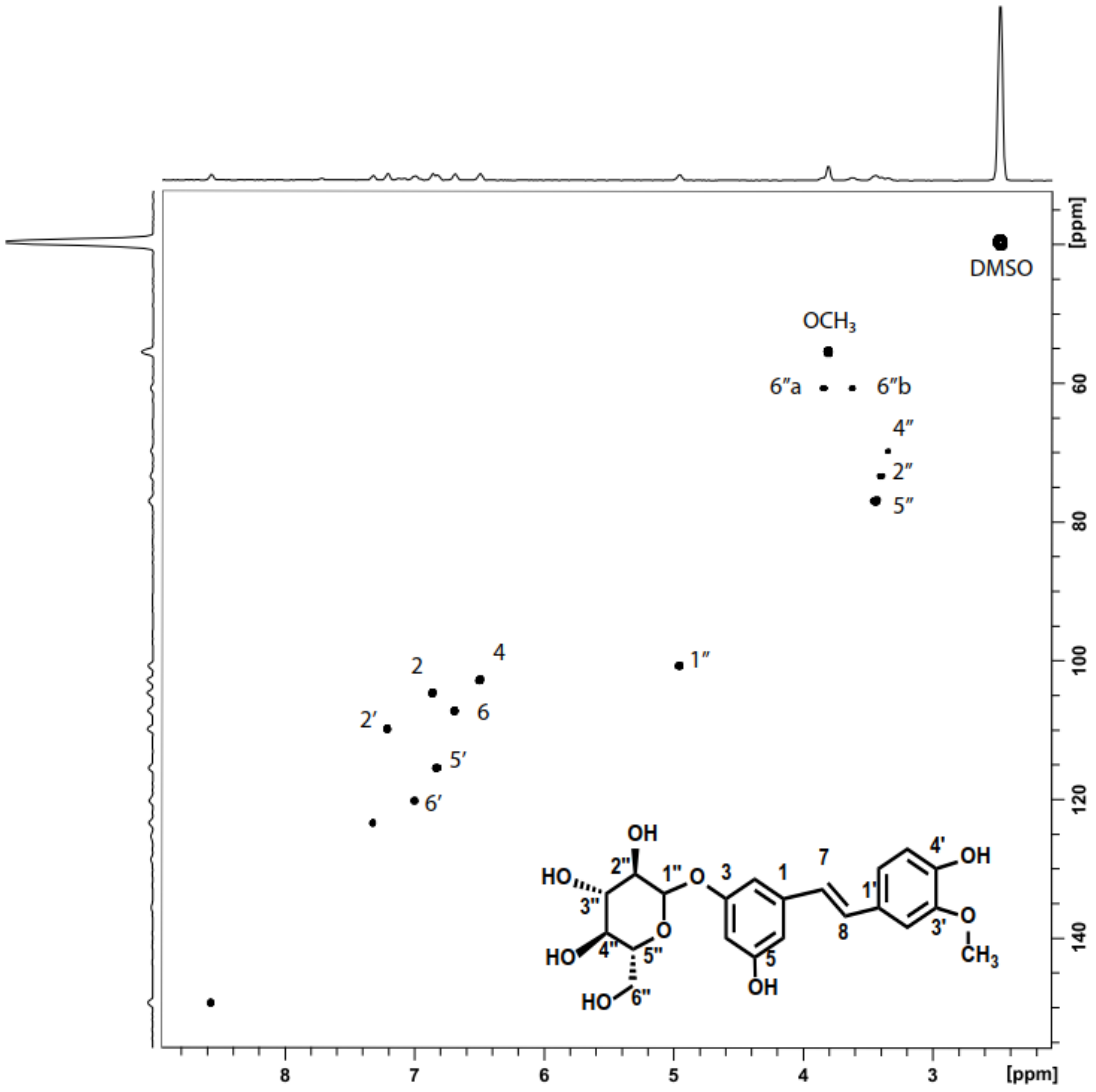

Figure A8: 2D ^{1}H–^{13}C HSQC NMR spectrum of isorhapontin in DMSO-d_6/pyridine-d_5.

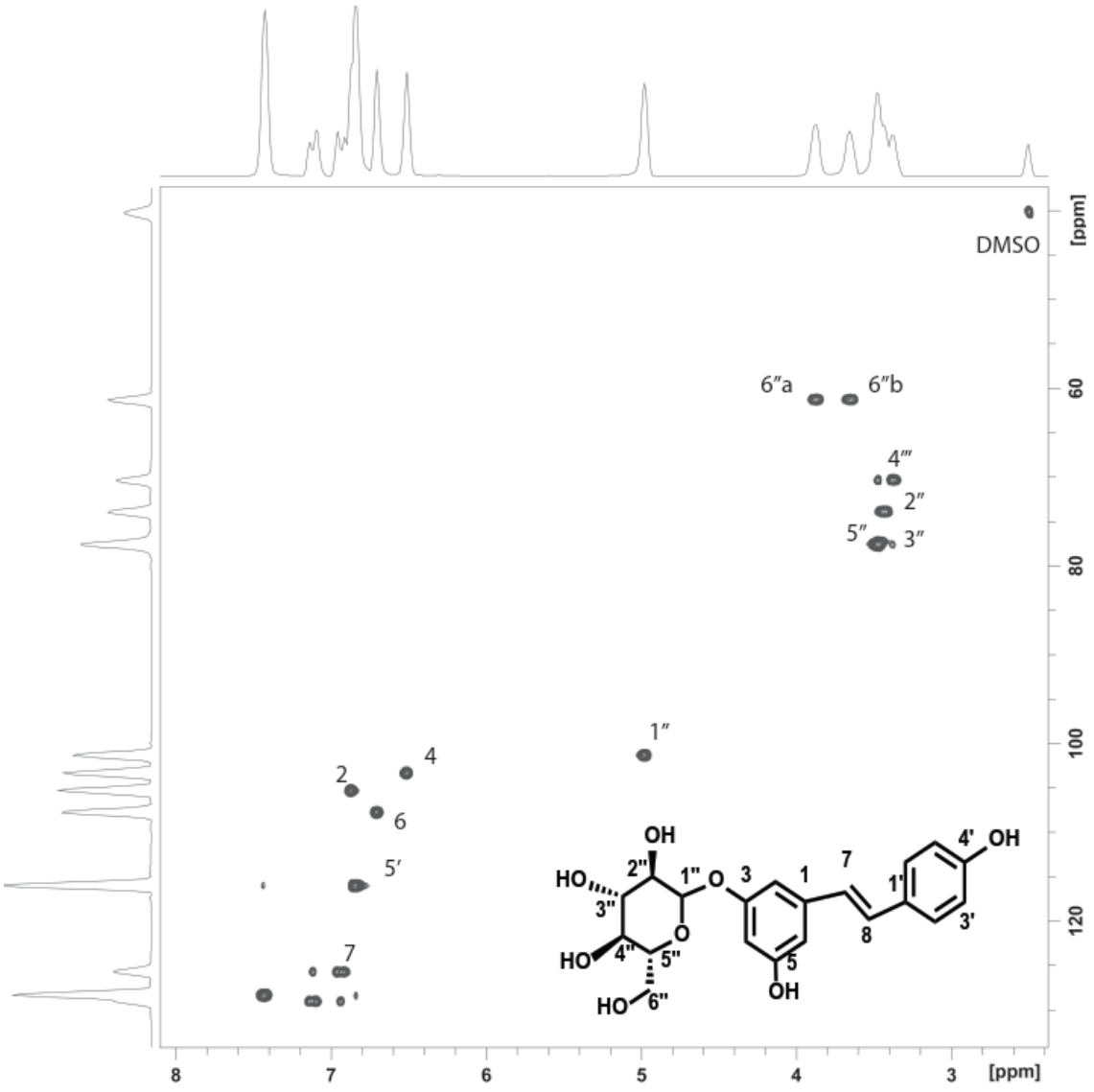

Figure A9: 2D ^{1}H–^{13}C HSQC NMR spectrum of polydatin in DMSO-d_6/pyridine-d_5.

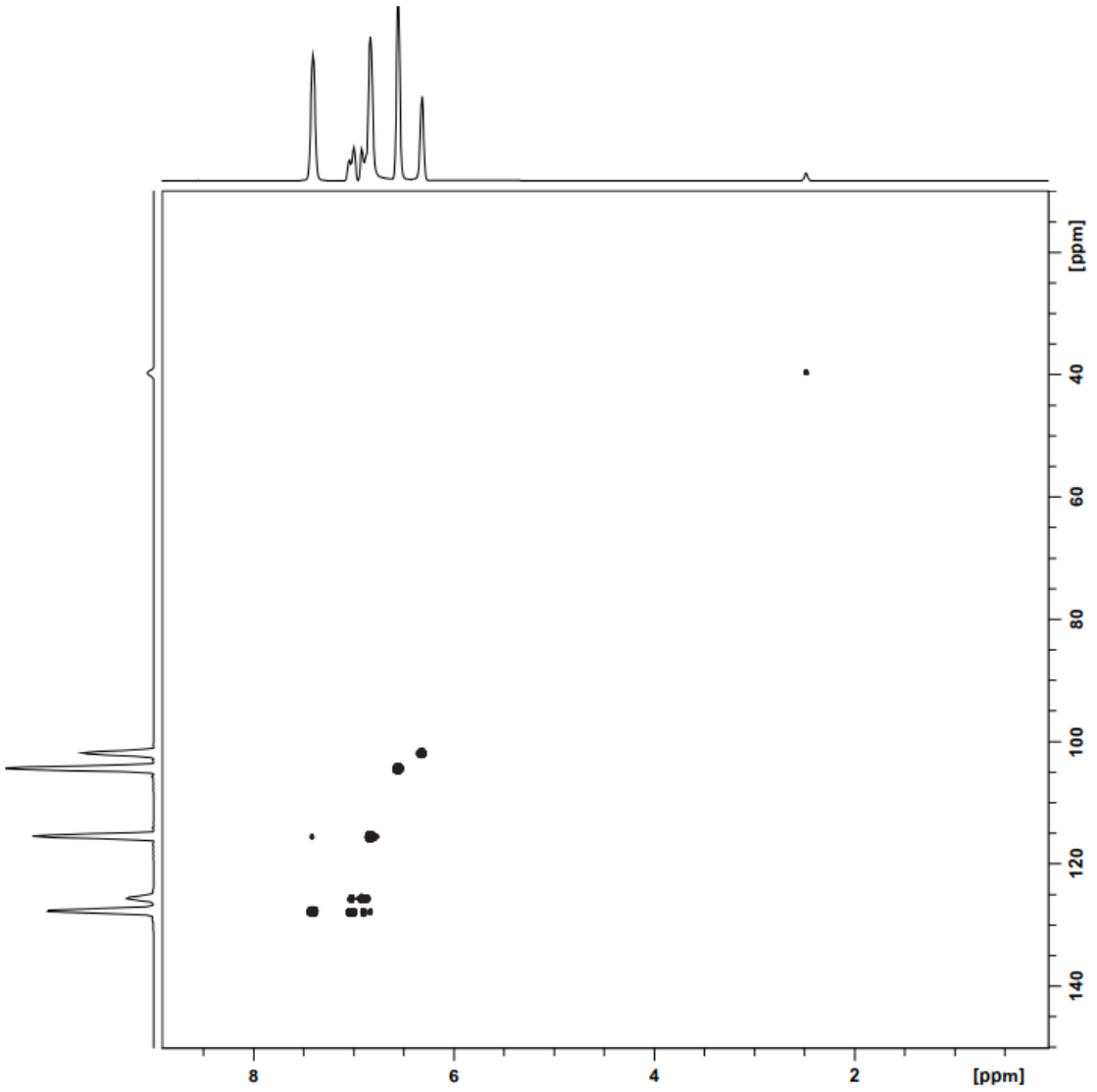

Figure A10: 2D ^{1}H–^{13}C HSQC NMR spectrum of resveratrol in DMSO-d_6/pyridine-d_5.

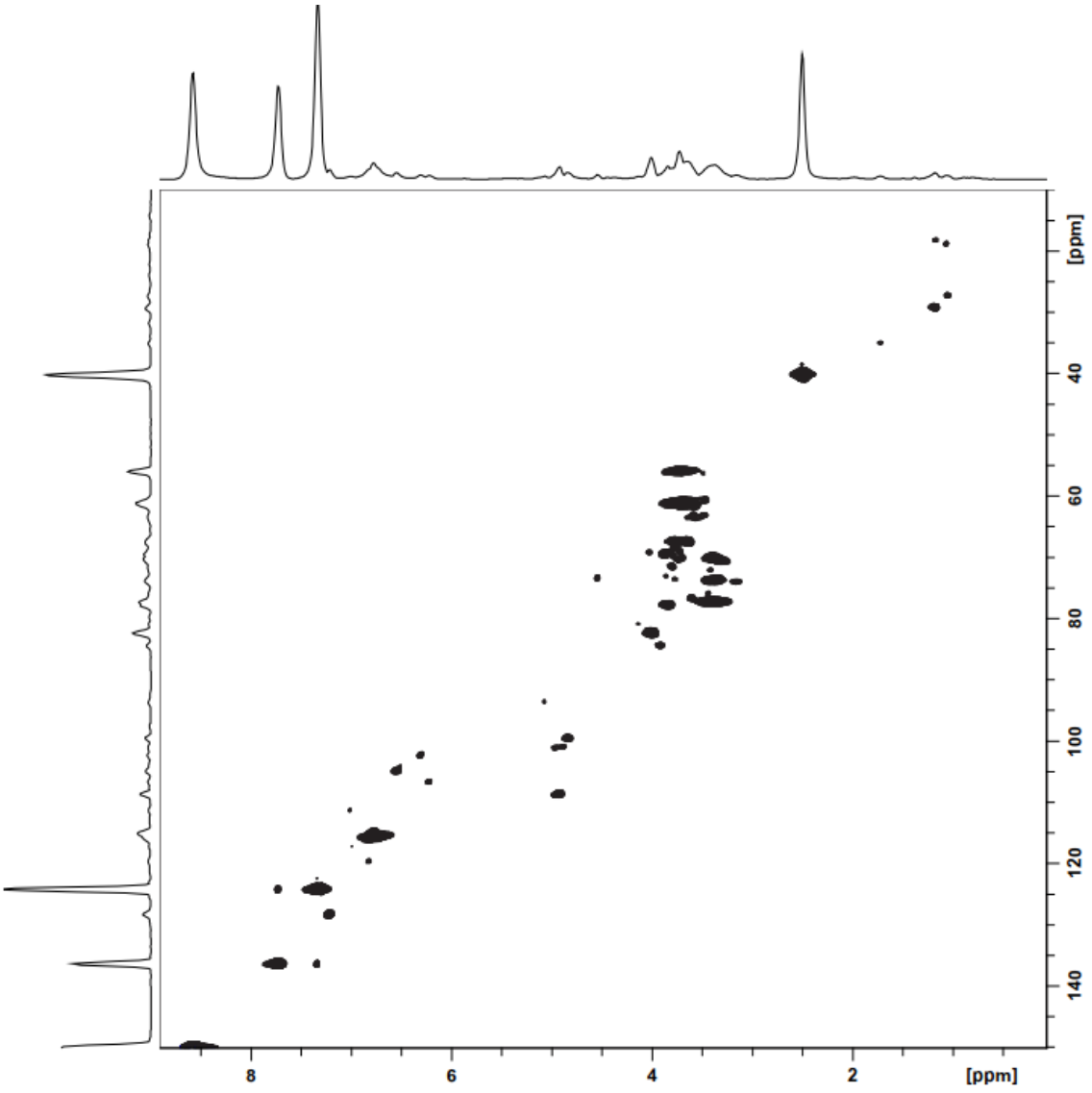

Figure A11: 2D ^{1}H–^{13}C HSQC NMR spectrum of mechanical pressing sample (spruce bark collected in February) in DMSO-d_6/pyridine-d_5.

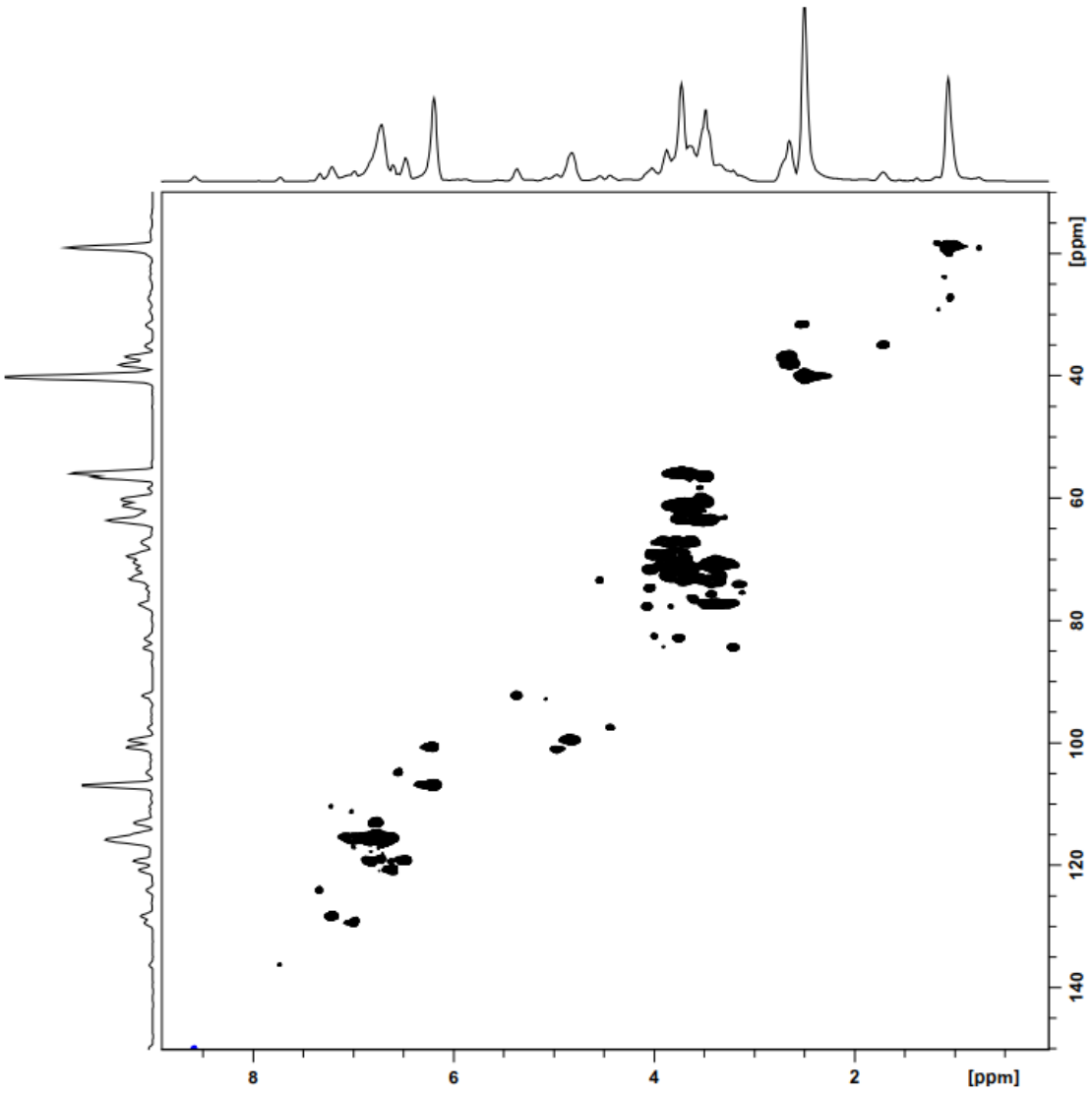

Figure A12: 2D ^{1}H–^{13}C HSQC NMR spectrum of bark press water sample (March) in DMSO-d_6/pyridine-d_5.

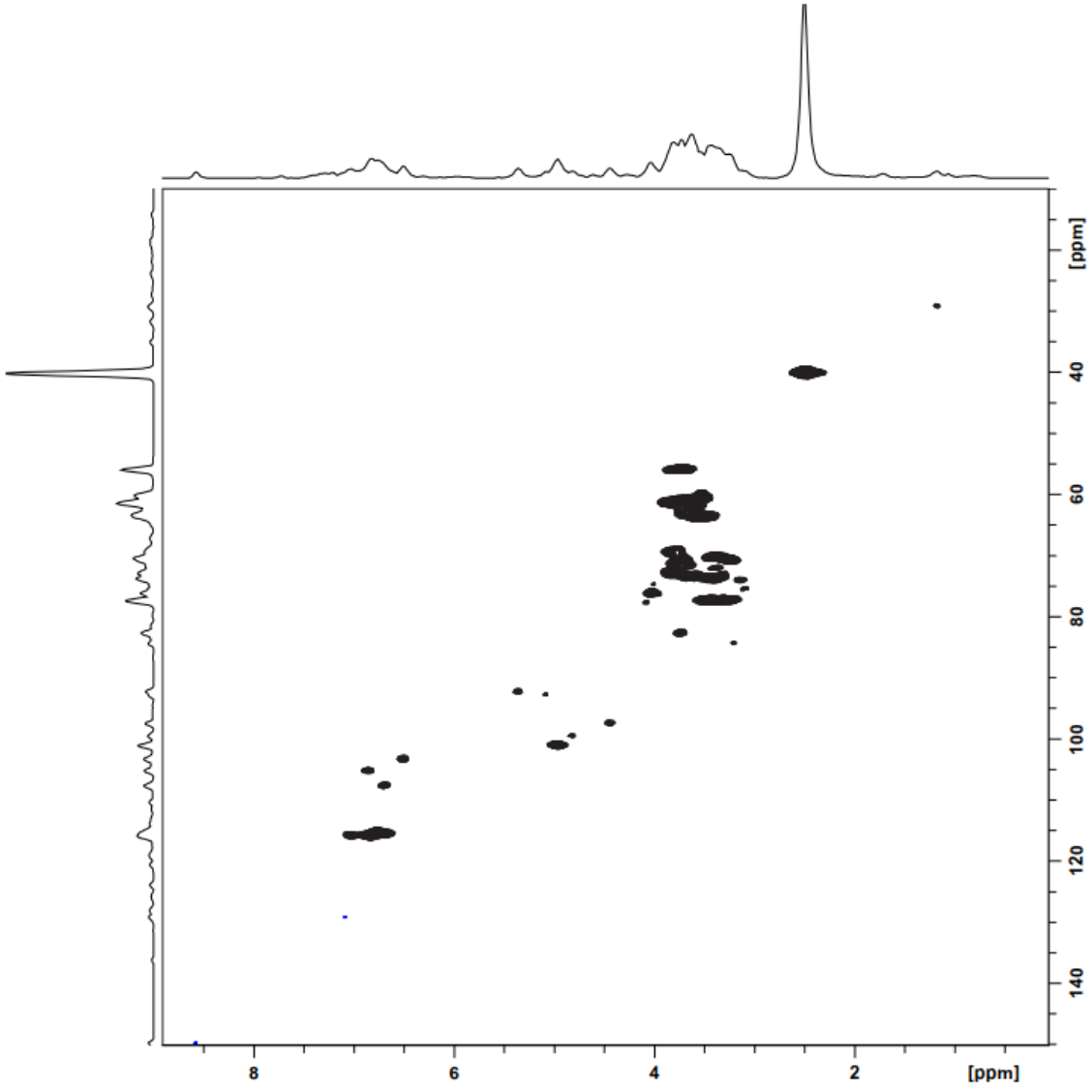

Figure A13: 2D ^{1}H–^{13}C HSQC NMR spectrum of bark press water sample (May) in DMSO-d_6/pyridine-d_5.

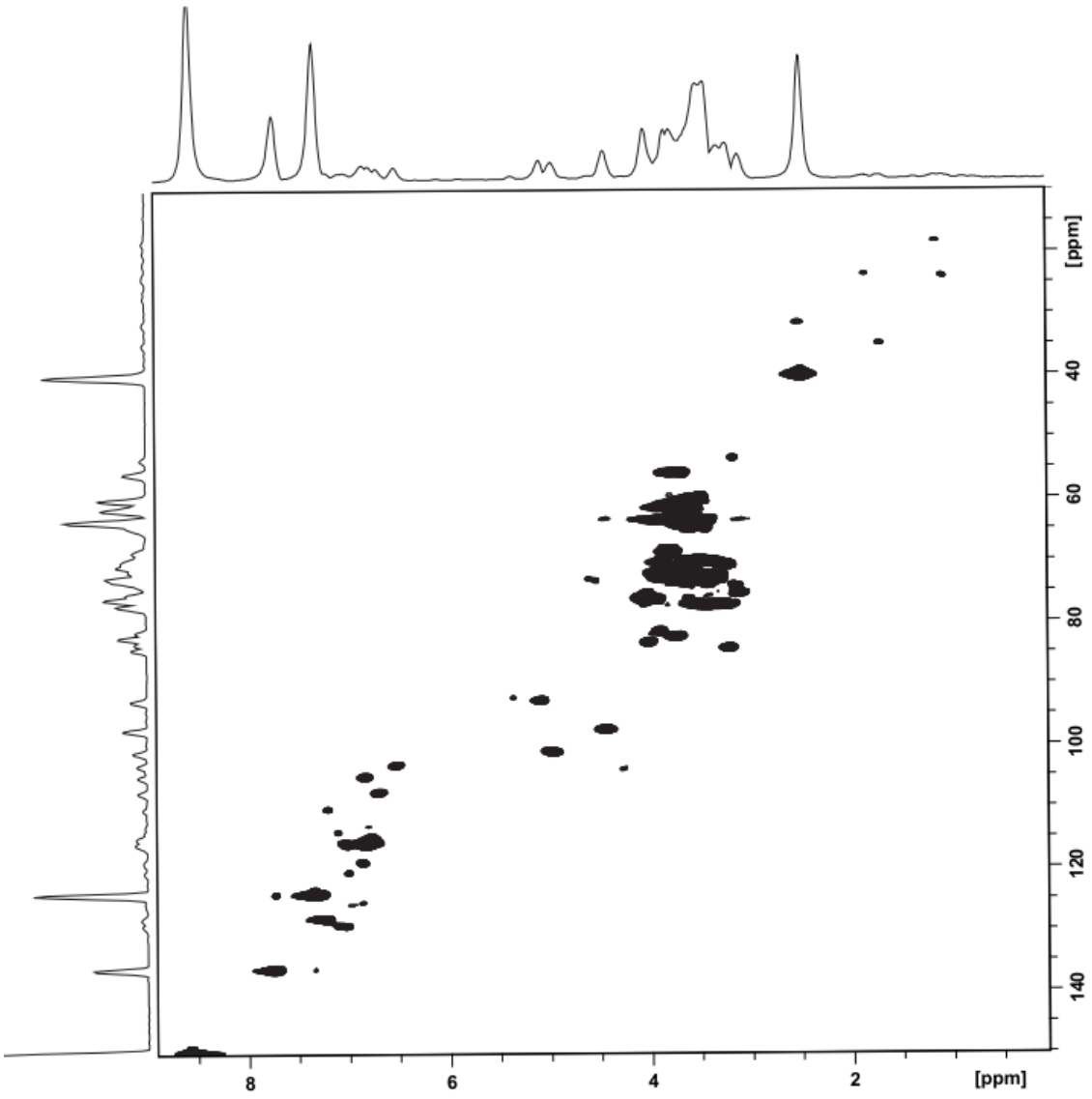

Figure A14: 2D ^{1}H–^{13}C HSQC NMR spectrum of 5 kDa filtrate of bark press water sample (May) in DMSO-d_6/pyridine-d_5.