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Characterization of Antioxidant Activity and Total Phenolic Compound Content of Birch Outer Bark Extracts Using Micro Plate Assay

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Abstract. In modern plants, 2.7 to 2.8 m³ of solid volume veneer blocks are consumed to produce 1 m³ of plywood. After the hydrothermal treatment and debarking of blocks, waste bark is obtained, which makes up 12.5% of the wood mass, while 16-20% of bark is composed of birch outer bark (BOB). Recalculating, BOB makes up 2.0-3.4% of the veneer log mass. Bark is currently burned in boiler houses that is not rational. BOB contains large amount of valuable extractives (up to 34% from o.d. BOB) consisting of various secondary metabolites such as terpenes, flavonoids, hydrocarbons, polyphenols, tannins etc. BOB extractives exhibit antioxidant properties as well as wound-healing and anti-inflammatory activity. The objective of this paper was to compare amount of total phenolic content (TPC) and antiradical activity (ARA) in ethanol extracts from silver birch (Betula pendula Roth.) outer bark using micro plate assay. Among 11 fractions, fraction of average BOB had the highest DPPH free radical scavenging activity with IC50 of 39.28 µg/mL and the highest TPC 7.42 ± 0.52 g GAE/100 g of dry extract.

Keywords: birch outer bark extract, phenolics, antioxidant activity.

I. INTRODUCTION

Betula pendula Roth, the European white birch or Silver birch, is one of the most common deciduous tree species on the Eurasian continent with a wide natural distribution, ranging from the Atlantic to eastern Siberia. [1]. The continuous industrial exploitation of birch wood produces a lot of bark waste [2]. These barks have a chemical richness which could be a potent bioactive material for further use and production of upgraded products, e.g., in the field of functional foods, dietary supplements or cosmetics [1,2].

Birch outer bark has been used for ages as a traditional material and as a provider of medicinal products.

There are therefore many original and review articles that describe research on phytochemicals (e.g., triterpenoids, diarylheptanoids, phenylbutanoids, lignans, phenolics, and flavonoids) in Betula species. Birch outer bark is well-known as the main source of betulin. Its content varies between 10 and 35 % of the total dry weight of the outer bark extract whereas betulinic acid occurs in traces [3]. These phytochemicals have been shown to have antimicrobial, antiviral, antioxidant, immunomodulatory, anti-inflammatory, anti-diabetic, gastroprotective, hepatoprotective, skin protective and wound healing effects [1,4].

The present study was aimed at the characterization of the antioxidant activity and total phenolic compound content of different silver birch (*Betula pendula* Roth.) outer bark extracts. Feedstock for extraction was collected at A/S Latvijas finieris after soaking and debarking operations. While extraction process was carried out in Latvian State Institute of Wood Chemistry, but antioxidative properties were determined in the University of Latvia in the scope of a joint project.

II. MATERIALS AND METHODS

A. Feedstock

Three birch outer bark samples collected at a plywood factory in Latvia, were selected as a representative industrial waste and prepared for the extraction process:

1) birch outer bark from the birch veneer log – BOBL;
2) birch outer bark obtained by floating – BOBF;
3) mechanically separated birch outer bark – BOBM.

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BOBL was collected from the birch log before the soaking and peeling process operation in a plywood factory. For further operations isolated birch outer bark with a moisture content of 35-40 % (determined according to standard EN-14774-3 [5]) was dried at room temperature to a moisture content of 4-7 % and milled in a cutting mill SM 100 (Retsch GmbH & Co) to pass the sieve with holes of diameter 2.00 mm.

BOBF sample was obtained from birch bark after industrial birch veneer log peeling process with the relative moisture content 35-40 %. The collected feedstock was dried at room temperature to a moisture content of 4-7 % and milled in a cutting mill SM 100 (Retsch GmbH & Co) to pass the sieve with holes of diameter 2.00 mm. Milled dry birch bark samples were soaked in deionized water for 24 h by occasional mixing. Birch outer bark, floated on the top of the water surface, was collected and used as a reference raw material for the BOBF sample. After flotation, BOBF was dried and to a moisture content of 2-4 wt% for further operations.

BOBM sample was obtained after industrial birch veneer peeling process by mechanical separation at the plywood factory, where the fraction with the pure birch outer bark was collected after the sieving of birch bark through the fractionation system MUOTOTERA OY classifier using five screens according to SCAN-CM40:01 [6] to decrease the inner bark and woody particles content. For further operations the 10 - 45 mm fraction with a moisture content of 18-20 % (determined according to standard EN-14774-3 [5]) was dried at room temperature to a moisture content of 4-7 % and milled in a cutting mill SM 100 (Retsch GmbH & Co) to pass the sieve with holes of diameter 2.00 mm.

B. Birch outer bark extract

Birch outer bark extract was prepared from Latvian silver birch (*Betula pendula* Roth.) bark collected at the plywood factory as described before. 3 ± 0.15 kg of separated birch outer bark was extracted with 18 ± 0.5 L ethanol or 2-propanol in the 30 L externally heated extraction reactor equipped with a barbotation mixer. After the boiling temperature was reached (82 °C for 2-propanol and 78.1 °C) the first extract 13.5 ± 0.5 L was poured off and collected for further operations. In the reactor was poured in the same amount of clean solvent what was poured off as an extract. The same was repeated also for the third extraction time. Obtained ethanol extract was evaporated using Heidolph Hei-VAP Industrial B Large-Scale equipment and dried in a drying chamber at the 50 °C. For each extraction time (1E, 2E and 3E) extracts were evaporated separately and the yield was calculated, except the average BOBM sample, which was collected from all extraction procedures, mixed together, evaporated and dried. As a result, dry birch outer bark extract was obtained and yields can be seen in Table I. For further analysis dry birch outer bark extract was crushed in a ball mill and fractionated up to 125 µm.

C. Total phenolic content

Total phenolic content (TPC) were determined using Folin-Ciocalteu assay using high-throughput 96-well plate method as described by Herald et al. [7] with slight modifications.

All chemicals used for assays were of analytical grade. The measurement was conducted by mixing working Folin-Ciocalteu solution (1:1 with water), sodium bicarbonate and ethanolic extract or standard solutions. The absorbance was measured after 90 minutes of incubation at 765 nm, along with the blank. TPC was expressed as gallic acid equivalents (g GAE mg/100 g BOB extract), based on gallic acid (GA) calibration curve (range 0.025 – 0.200 mg ml^{-1}, $R^2 = 0.997$).

Analysis were performed on Infinite M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) instrument. Bandwidth 9 mm, temperature 29 °C.

D. Antioxidant activity

The DPPH method is based on the ability to stable free radical 2,2-diphenyl-picrylhydrasyl (DPPH) to react with hydrogen donors. Antiradical activity (ARA) were determined using DPPH assay using high-throughput 96-well plate method as described by Herald et al. [7] with slight modifications.

All chemicals used for assays were of analytical grade The measurements of BOB extracts were done by mixing of 100 µM DPPH solution in ethanol with extract or standard samples. The absorbance was measured at 520 nm, along with the blank. ARA was expressed as ascorbic acid equivalents (AAE mg/ 100 g sample), based on calibration curve (0.03 – 0.09 mg ml^{-1}, $R^2 = 0.998$).

Analysis were performed on Infinite M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) instrument. Bandwidth 9 mm, temperature 28.2 °C. The percentage of radical scavenging activity or BOB average extract was calculated from the following formula (1):

$$\%_{scavenging}\,[DPPH] = [(A_0 - A_1/A_0)] \times 100 \qquad (1),$$

where A_0 was the absorbance of the blank and A_1 was the absorbance in the presence of the average BOB extract.

IC_{50} value of BOBM average extract was determined from the graph obtained using standard ascorbic acid by using the "y =ax + b" formula from the slope of the graph as the amount of ethanolic BOBM extract necessary to decrease the initial DPPH· concentration by 50%.

III. RESULTS AND DISCUSSION

A. Birch outer bark extract

Separated birch outer bark samples was extracted with ethanol and propanol, obtained extracts were evaporated and dried as described in the previous section. As a result, birch outer bark extract was

obtained in the yields, which can be seen in Table I.

Table I
The yield of extracts depending on the solvent and feedstock

Solvent	Feedstock	Abbreviation	Yield (%) o.d.m.
2-propanol	BOBL	Prop-BOBL-1E	13.3 ± 0.2
		Prop-BOBL-2E	9.3 ± 0.1
		Total	22.6 ±0.3
Ethanol		Et-BOBL-1E	19.9 ± 0.2
		Et-BOBL-2E	9.4 ± 0.2
		Et-BOBL-3E	3.5 ± 0.1
		Total	32.8 ± 0.4
	BOBM	Et-BOBM-1E	19.6 ± 0.2
		Et-BOBM-2E	8.5 ± 0.1
		Et-BOBM-3E	3.3 ± 0.1
		Total	31.4 ± 0.3
		Average	30.9 ± 0.3
	BOBF	Et-BOBF-1E	22.1 ± 0.1
		Et-BOBF-2E	6.6 ± 0.1
		Total	28.7 ± 0.2

The total yield of the 2-propanol extract (22.6 %) is lower than that obtained using ethanol as a solvent (28.7-32.8 %). While the highest yield was obtained from the BOBL, which can be explained that the pure birch outer bark without inner bark and woody particle admixture was taken for the extraction process. It was already concluded in the previous study that the extracts yield from the industrial waste is lower due to the elution of monosaccharides and phenolic substances during the soaking of veneer logs. [8]. Thus, the outer birch bark obtained from a plywood factory seems to be a better raw material for the production of triterpenes than that obtained from a freshly cut birch trunk. Despite the fact that the yield of extractives is lower.

At the first extraction time more than 60 % of extractives was obtained from the totally obtained yield, while that for the second extraction time was in the range of 23-28 %. For two samples (BOBL and BOBM) the third extraction time was additionally carried out, which showed that there are small extractive yields (10.7 and 8.4 % from the total yield, respectively) left in the feedstock after two extraction times with ethanol.

B. Total phenolic content

Phenolic compounds, after betulin and betulinic acid, are the next most abundant structures in *Betula pendula* Roth. plants. The phenolic acids and flavonoids present in the plant extracts are natural antioxidants. They have anti-mutagenic and anti-cancerogenic properties, cardioprotective, anti-inflammatory and antimicrobial activity. The corresponding TPC and ARA for different birch outer bark samples are shown in Figures 1; 2 and 3 depending on the used solvents, used feedstock and number of extraction, respectively. The TPC, determined by the Folin-Ciocalteu method are in range of 2.56-7.42 g of gallic acid equivalents per 100 g of birch outer bark extract.

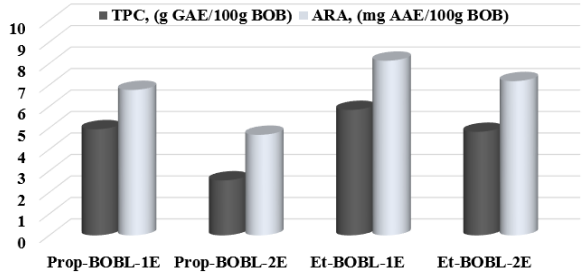

Fig. 1. Dependence of the TPC and ARA of BOBL extracts on the used solvent. TPC expressed as g GAE/100 g extract (λ = 765 nm; b = 0,6 cm). ARA expressed as mg of AAE/100 g extract (λ = 520 nm; b = 0,6 cm). BOBL- Birch outer bark from log.

Fig. 1 shows that there is difference between the BOBL extractives obtained using propanol and ethanol as a solvent. The TPC and ARA are even higher for ethanol extracts, which are more perspective for production of water based cosmetics or dietary supplement, because ethanol is one of the green solvents, which is allowed for obtaining of raw materials for mentioned products [9]. Therefore, for further experiments ethanol was used as a solvent to investigate antioxidant properties of the obtained extracts.

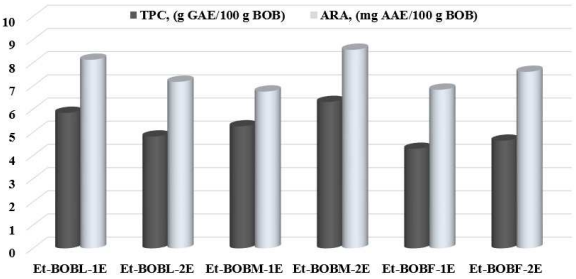

Fig. 2. Dependence of the TPC and ARA of BOB ethanol extract on the used feedstock. TPC expressed as g GAE /100 g extract (λ = 765 nm; b = 0.6 cm). ARA expressed as mg of AAE/100 g extract (λ = 520 nm; b = 0.6 cm). BOBL- Birch outer bark from log; BOBM -birch outer bark mechanically separated; BOBF - birch outer bark obtained by floating.

Fig. 2 shows the differences in TPC and ARA values depending on the feedstock. It is very interesting that for BOBM and BOBF the determined values for second extraction time is higher than that for the first extraction time. While for BOBL it is opposite tendency, which was obtained also using 2-propanol as a solvent (Fig. 1). To check this tendency, BOBM and BOBL two times extracted feedstock was additionally extracted for the third time and compared in the Fig. 3. It is obvious that there is the same tendency, that TPC and ARA values for BOBL

decreases by the number of extraction, while that for BOBM increases.

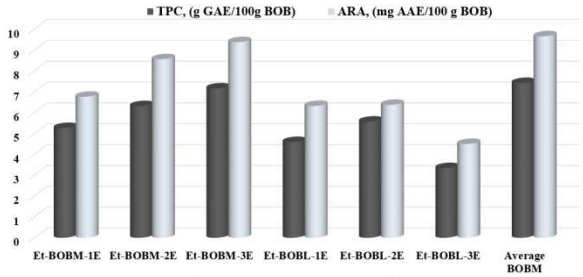

Fig. 3. Dependence of the TPC and ARA of BOB ethanol extract on the extraction time. TPC expressed as g GAE /100 g extract (λ = 765 nm; b = 0.6 cm). ARA expressed as mg of AAE/100 g extract (λ = 520 nm; b = 0.6 cm). BOBL- Birch outer bark from log; BOBM -birch outer bark mechanically separated.

Average sample from BOBM extractions was analysed as well. Surprisingly, the highest quantities between all samples of total TPC and ARA in average extract (7.42 ± 0.52 g GAE/100 g of dry extract and 9.66_± 0.13 mg AAE/100 g BOB, respectively) were found. This means that there is a future in ethanol extracts based on the antioxidant properties, which is a valuable characteristic for cosmetic and dietary supplement.

C. Dilution of ethanol extracts with water

While average dry BOBM extract showed the highest TPC content, it was decided to make measurements of dependence of TPC on sample dilution (Figure 4).

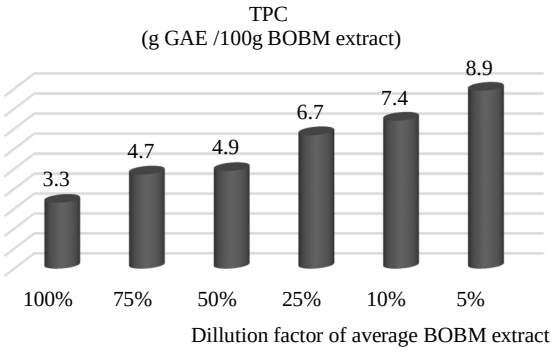

Fig. 4. Total phenolic content dependence on sample dilution of average BOBM extract.

BOB extracts overall don't have very good solubility in alcohol-water media, but it very promising that TPC increases by the increase of dilution percent. These observations of average BOBM extract in ethanol-water solutions can be with high potential for the production of cosmetic preparations and dietary supplement where in most of cases water medium is necessary.

D. Antiradical activity

A number of methods are used to determine the radical scavenging effects of antioxidants. The DPPH method is a preferred method because it is fast, easy and reliable and does not require a special reaction and device.

DPPH radical is a stable organic free radical with an absorption band at 520 nm. It loses this absorption when accepting an electron or a free radical species, which results in discoloration from purple to yellow. Total ARA can be affected by the type and the amount of antioxidants present in BOB extracts [10].

Antioxidant compounds are usually in the phenolic form. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation, and dismutation of radicals [11]. The free radical scavenging activities of extracts depend on the ability of antioxidant compounds to lose hydrogen and the structural conformation of these components [12].

ARA for different BOB samples are shown in Figures 1; 2 and 3 depending on the used solvents, used feedstock and extraction time respectively. ARA in analysed samples ranged from 4.50-9.66 expressed as AAE in mg/100 g BOB extract. The highest ARA was observed for average BOBM extract (9.66 mg AAE/100 g of dry extract).

ARA depending on the dilution percent of analysed extracts were detected as well. Figure 6 shows dependence of ARA content on dilution expressed as AAE mg/100 g average BOBM extract.

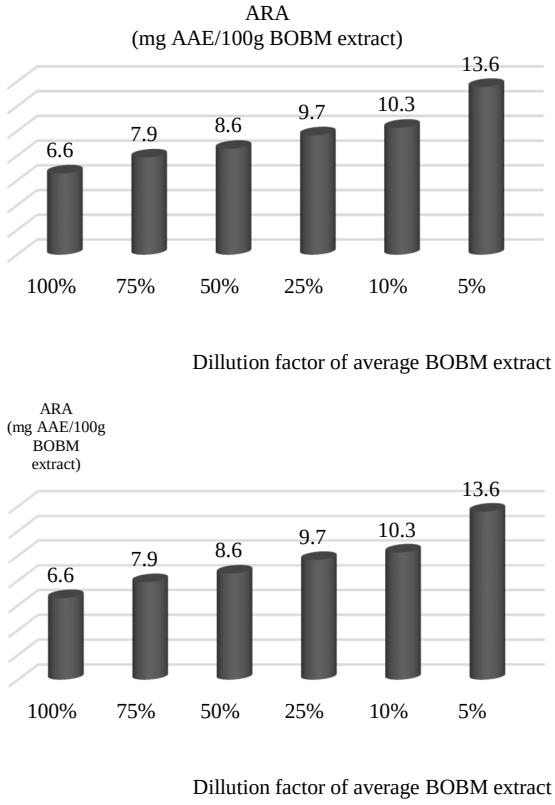

Fig. 5. Antiradical activity dependence on sample dilution of average BOBM extract.

Date from Figure 5 clearly demonstrate that average BOBM extract will not lose its antioxidant

properties by dilution even if will be used in more aquatic solution media.

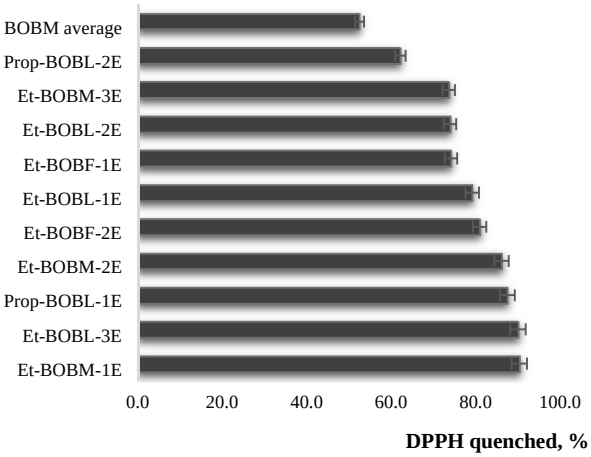

Fig. 6. DPPH radical scavenging activity (%) of birch outer bark extracts from Betula pendula (λ = 520 nm; b = 0.6 cm).

Data from figure 6 shows ability of BOB extracts to quench DPPH· radicals. Lower percentage shows higher ability to quench and reverse. This ability was ranging from 52.5-90.3%. The highest ability to quench free DPPH· radicals (52.5%) was observed for average BOBM extract. It was observed that the radical scavenging effect of the extracts quite positively correlated with their total amount of phenolic compounds (r = 0.628).

Based of obtained data IC_{50} for BOBM average extract and ascorbic acid was determined from the graph by using the "$y = ax + b$" equation from the slope of the graph. IC_{50} shows the inhibitory concentration, defined as the concentration of extract required to scavenge 50% of DPPH· radicals. Since the IC_{50} value of ascorbic acid, which is known to be a potent antioxidant was 6.23 μg/ml, which is significantly low, implies that a very less amount of this antioxidant would give a remarkably high effect in fighting oxidative damage. The average BOBM extract IC_{50} value was 39.28 μg/ml. These values show that average BOBM extract has 6.3 times lower antioxidant activity than ascorbic acid.

IV. CONCLUSION

In this study, total phenolic content, antiradical activity, free radical scavenging activity and total antioxidant level of different silver birch (*Betula pendula* Roth.) outer bark extracts were determined. The high-throughput 96-well plate method proved to be a robust and reproducible method for determining total phenolic content and antiradical activity in BOB extracts. This study demonstrates that all analysed birch outer bark extracts are phenolic-rich materials and very potent sources for natural antioxidants.

As a result, birch bark extracts can be used as well as in pharmaceutical products and cosmetics as a source of natural antioxidants.

V. ACKNOWLEDGMENTS

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