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Aronia Melanocarpa (black chokeberry) Branches Biomass as a Source of Valuable Biologically Active Compounds with Antioxidant and Antimicrobial Potential

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Abstract. Aronia melanocarpa is a hardy berry-producing shrub that demands low maintenance and can grow on almost any type of soil. Since the best fruits can be obtained on the branches younger than 7 years, pruning of 1/3 of the shrub is usually performed each winter or after flowering, and also to remove damaged or overgrown branches. For sustainable production of Aronia melanocarpa berries, it is necessary to find a rational use for this pruning lignocellulosic biomass. Some studies available for Aronia melanocarpa berries show that they are very rich in various biologically active substances with proven functional and pharmacological activities including anti-inflammatory, antioxidant, and antimicrobial properties. The chemical composition of Aronia melanocarpa branches is currently almost unknown. The study aimed to evaluate the composition and potential of chokeberry branches as a source of polyphenols. General chemical characterization of the biomass was carried out using the method of analytical pyrolysis. Extraction of branch biomass was carried out using aqueous alcohol solutions. Quantitative analysis of the extracts showed a large amount of oligomeric proanthocyanidins. The most suitable extractant was determined to obtain the highest yield of the dominant polyphenols in the hydrophilic extract. The antioxidant activity of the hydrophilic extracts as well as antibacterial activity against six pathogenic bacteria was evaluated. The results showed the high potential of chokeberry lignocellulosic biomass as a source of valuable biologically active compounds for the creation of preparations for the healthcare, nutrition industry, and cosmetics.

Keywords: Aronia melanocarpa, Black Chokeberry, polyphenols, antimicrobial activity

I. Introduction

One of the most underestimated and little investigated fruit trees is the black chokeberry (also aronia, or black apple berry) – Aronia melanocarpa. Aronia melanocarpa is a deciduous shrub-tree of the Rosaceae family, originated from the eastern parts of North America [1], [2]. The chokeberry tree is a very unpretentious plant, frost-resistant until -35°C, and can grow well in both wet and dry soil, with preferably acidic, but also with alkaline pH [3]. It tolerates well sandy and salty soils. Chokeberries are mostly used for making juice, jam, less often wine [4], and for producing natural food colorants

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with antioxidant properties [5]. According to the Rural Development Service (LAD – in Latvian) data, in Latvia, the black chokeberry cultivation area increased 2.4 times during the last five years, and in 2022, it was 244 ha. The biggest Latvian grower is "Sirpes" Ltd., in Lielvarde (69.8 ha) [6]. As admitted by the Latvian Institute of Horticulture scientists, *Aronia melanocarpa* berries could become an important product of homemakers-gardeners [7]. The European share of the black chokeberry growth was estimated as 33% in 2023 [8]. In a range of studies it was proven that *Aronia melanocarpa* berries are one of the richest plant sources of phenolic and polyphenolic compounds including anthocyanins, procyanidins and proanthocyanidins [2], [9], [10]. *Aronia melanocarpa* contains a lot of vitamins, and is also rich in dietary fibers – pectin and hemicellulose, and water-insoluble dietary fibers, including lignin [2], [11], which are important for the prevention and treatment of atherosclerosis, coronary heart diseases, obesity, and large intestine cancer [11]. The studies recently confirmed anti-inflammatory [12], cardioprotective [13], antidiabetic [9], [14], [15] properties of the *Aronia melanocarpa* pomace and proved it's capabilities in the prevention of degenerative diseases [2]. But *Aronia melanocarpa* needs much wider popularisation and more attention of the growers, producers and consumers. *Aronia melanocarpa* shrub-tree is easy to maintain, it has an annual berry harvest, and, which is very important, no known pest problems [10]. By adding the knowledge about it's pruning lignocellulosic biomass' characteristics and application possibilities, *Aronia melanocarpa* could become a niche export direction for Latvian and other *Aronia melanocarpa*-cultivating countries' gardeners.

The studies of the *Aronia melanocarpa* lignocellulosic biomass are largely missing. In some papers, it was shown that aronia leaves contain a variety of phenolic compounds and have high total phenolics content that confirm their therapeutic potential, including anticancer properties [1], [16], [17]. To the best of our knowledge, there are no studies about aronia branches, although it could be assumed that *Aronia melanocarpa* branches may contain a range of the valuable compounds similar to what are found in the berries and leaves. Our previous comparative study of fruit and non-fruit trees twigs showed great potential of proanthocyanidins obtained from autumn twigs of *Aronia melanocarpa*, they inhibited bacterial biofilm formation by 50% [18]. Since antimicrobial resistance against chemically synthesized antibiotics is one of the biggest problems for human health around the world [19], it is necessary to identify new natural antimicrobial agents for the treatment of infections. The aim of this study was screening of the spring and autumn *Aronia melanocarpa* branches biomass composition by analytical pyrolysis, finding the optimal conditions for the branches' extraction, and evaluation of the antioxidant and antimicrobial properties of the obtained extracts and oligomeric proanthocyanidins against a range of the most frequently detected pathogenic bacteria.

II. Materials and Methods

A. Plant Material

Black chokeberry branches were collected in spring (SP) and autumn (AU) of 2023, in order to evaluate seasonal changes, from Baldone parish, Kekava county of Latvia (DD: 56.77306/24.30162). 7 years old *Aronia melanocarpa* 'Mulatka' branches were cut after the harvesting of the berries. The branches were dried at room temperature and ground in a mill (Cutting Mill SM100, Retsch, Haan, Germany) until the particle size of 1–4 mm. The samples were stored at −8 ˚C.

B. Analytical Pyrolysis of Biomass

Analytical pyrolysis (Py-GC/MC/FID) of biomass samples was performed using a Frontier Lab (Fukushima, Japan) Micro Double-shot Pyrolyzer Py-3030D directly coupled with the gas chromatograph (GC) Shimadzu GC/MS/FID-QP ULTRA 2010 (Japan), as described in details in Andersone et al. [20]. In short, pyrolysis temperature was 500˚C, heating rate: 600 ˚C s^{-1}. Capillary column RTX-1701 (Restec, Metairie, Louisiana, USA) was used. The identification of the individual compounds was performed using Library MS NIST 11 and NIST 11s. The summed molar areas of the relevant GC peaks were normalized to 100%, and the data from four repetitive pyrolysis experiments was averaged.

C. Branches Extraction

Autumn and spring *Aronia melanocarpa* branches extraction was performed by maceration with 96% EtOH and ethanol (EtOH)-distilled water solutions (50% EtOH v/v) and by distilled water, at 60 °C for 60 min. The extracts were freeze-dried using lyophilization equipment Heto Power Dry HS3000 (Thermo Fisher Scientific, Waltham, MA, USA) to yield a dry weight (DW) extract. The yield of the extracts is given as a percentage based on DW. The extracts were stored at −8 °C. Each branches sample was extracted in triplicate, and results were expressed as a percentage per dry branches sample.

D. Proanthocyanidins Separation

Separation of proanthocyanidins from the extracts was performed as described by Andersone et al. [21], using a solvent-resistant column packed with cross-linked dextran-based resin Sephadex LH-20, and sequentially 96% EtOH (v/v) and 70% (v/v) acetone/water solutions as solvents, for low-molecular-weight phenolics and proanthocyanidins, respectively.

E. Determination of Proanthocyanidins Content

To evaluate the potential of *Aronia melanocarpa* biomass for the production of proanthocyanidins, the content of proanthocyanidins in extracts was determined using the Butanol-HCl colorimetric method. The method is based on the hydrolysis of proanthocyanidins in the presence of n-butanol/HCl to the corresponding coloured anthocyanidins [18]. Amounts of 6 mL of acid butanol (5% (v/v) concentrated HCl in n-butanol) and 0.2 mL of iron reagent (w/v) ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 M HCl) were added to 1 mL of the extract aliquots while stirring

the tube without heating and allowing it to be heated in a water bath at 80 °C for 50 min. After 50 min, the absorbance of the mixture was measured against a blank solution at 550 nm using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA). Each extract was analyzed in triplicate, and assay results were expressed as a percentage per dry extract.

F. Determination of Antioxidant Activity.

Extracts and purified proanthocyanidins were tested for their radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl radical DPPH· assay), by measuring the absorbance of the different concentrations of the extracts solutions in DMSO (30 µl) mixed with DPPH· ($1 \cdot 10^{-4}$ mol L^{-1}, 3.0 ml) at 515 nm, using UV/VIS spectrometer Lambda 650 (Perkin Elmer, Shelton, CT, USA) as described in Dizhbite et al. [22]. Each extract and purified proanthocyanidins sample was analyzed in triplicate. The free radical scavenging activity is expressed as the concentration of antioxidant, mg L^{-1}, required for a 50% inhibition of the free radicals (IC_{50}). The lower the IC_{50} value, the higher the antioxidant activity of the sample.

G. Determination of the Antimicrobial Activity

Antimicrobial activity was performed for autumn and spring biomass, 50% and 96% hydrophilic extracts and purified poanthocyanidins, against bacteria strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *S. Pyogenes,* and *C. acnes* as described in [18]. Antimicrobial activity was studied in 96-well plates by the two-fold serial broth microdilution method, which allowed the determination of the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). The MIC was determined as the lowest concentration of the studied sample, which showed no visible growth. From wells where growth was not detected, 4 µL of medium was seeded on an appropriate solidified medium for MBC determination.

H. Statistical Analysis

All analyses were performed in triplicate, except for analytical pyrolysis where four repetitive pyrolysis experiments were done. The results are presented as the mean value. Statistical analysis was done using Microsoft Excel 2016. Confidence intervals (CI) were calculated for a mean using a Student's T distribution at a significance level = 0.05.

III. Results and Discussion

A. Chemical Characterization of Aronia melanocarpa Biomass by Analytical Pyrolysis

Carbohydrates and phenols are the major components of organic part composition of *Aronia melanocarpa* biomass. Biomass phenol-derived pyrolysis products can be divided into phenyl and benzyl derivatives originating from the lignin and extractives (Fig. 1).

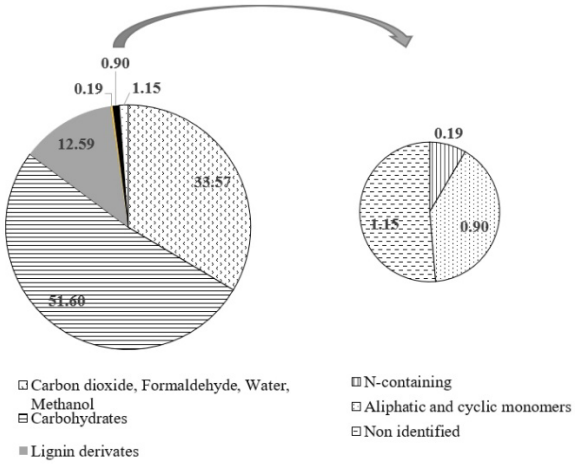

Fig. 1. Py-GC/MS/FID data of *Aronia melanocarpa* biomass-derived volatiles

B. Evaluation of the Extraction Conditions and content of Proanthocyanidins

For evaluation of the potential of *Aronia melanocarpa* as a raw material of valuable biologically active compounds, *Aronia melanocarpa* plant material after grinding and drying was subjected to extraction. The yields of hydrophilic extracts from *Aronia melanocarpa* biomass under study were different and varied from 11.7 % to 17.2 % /DM. The highest yield of hydrophilic extract was obtained by biomass extraction with 96 % (further in the text – 96% EtOH extract) and 50% EtOH (further in the text – 50% EtOH extract). The increased yield of extractives from biomass using 50% EtOH indicates that an ethanol-water solution as an extractant is required to completely isolate hydrophilic extractives. This observation was also confirmed by determining proanthocyanidins in hydrophilic extracts composition.

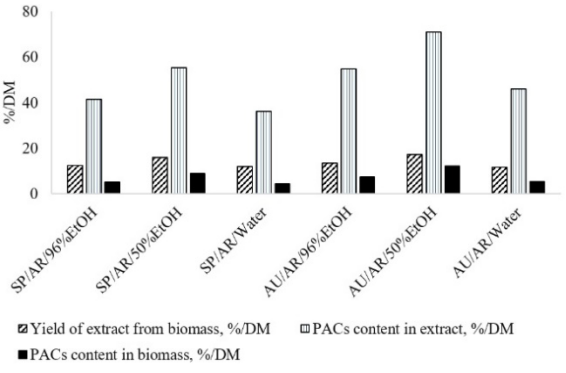

Fig. 2. Chemical characterization of *Aronia melanocarpa* biomass and hydrophilic extracts

The method is based on the hydrolysis of proanthocyanidins in the presence of n-butanol/HCl to the corresponding coloured anthocyanidins. According to the butanol method, the content of proanthocyanidins in the extracts varied from 36.2 to 71.0%/DM. The highest content of proanthocyanidins was in biomass collected in autumn (AU) and in the 50% EtOH extract of this biomass. It was shown that in the above-mentioned

conditions it is possible to obtain from 8.9 to 12.2% proanthocyanidins (PACs) in purified form (Fig. 2).

C. Antioxidant Activity of the Extracts and Proanthocyanidins

In the test with DPPH·, all hydrophilic extracts showed high radical scavenging activity (IC_{50} ranged between 3.8 and 6.9 mg L^{-1}, CI≤0.2 at = 0.05). The 50% EtOH extracts have fairly high antioxidant activity against DPPH· (SP/AR/50%EtOH: IC_{50} = 4.3 mg L^{-1}; AU/AR/50%EtOH: IC_{50} = 3.8 mg L^{-1}, CI≤0.2 at = 0.05) that is higher than that of Trolox, a synthetic water-soluble analogue of vitamin E (alpha-tocopherol), a widely used antioxidant with proven strong antioxidant activity (IC_{50} = 4.7 mg L^{-1}, CI≤0.2 at = 0.05, a lower IC_{50} value corresponds to higher antioxidant activity). The IC_{50} of purified proanthocyanidins, necessary for 50% inhibition of DPPH·, was 4 times lower than for the Trolox.

D. Antimicrobial Activity of Aronia melanocarpa Extracts and Proanthocyanidins

The evaluation of the effect of hydrophilic extracts on antimicrobial activity was studied against the following pathogenic bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus Pyogenes* and *Cutibacterium acnes*. All extracts inhibited both gram-positive and gram-negative pathogenic bacteria. The minimum inhibitory (MIC) and bactericidal concentrations (MBC) of extracts ranged from 0.2 to 3.13 mg mL^{-1} (Table 1, Table 2).

TABLE 1 THE MINIMUM INHIBITORY (MIC) AND BACTERICIDAL CONCENTRATIONS (MBC) OF EXTRACTS AND PROANTHOCYANIDINS, MG ML^{-1}

Samples	*E. coli*		*P. aeruginosa*		*S. aureus*	
	MIC	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***
Proantho-cyanidins	0.08	0.16	0.16	0.31	0.04	0.31
SP/AR/ 96% EtOH	0.20	0.20	0.39	0.78	0.39	0.39
SP/AR/ 50% EtOH	0.20	0.39	0.39	0.78	0.20	0.20
AU/AR/ 96% EtOH	0.20	6.25	0.78	3.13	0.10	0.20
AU/AR/ 50% EtOH	0.10	0.10	1.56	3.13	0.10	0.10
AU/AR/ Water	0.39	0.39	0.39	0.78	0.20	0.39

Confidence interval for a mean is ≤ 0.01 at α=0.05

TABLE 2 THE MINIMUM INHIBITORY (MIC) AND BACTERICIDAL CONCENTRATIONS (MBC) OF EXTRACTS AND PROANTHOCYANIDINS, MG ML^{-1}

Samples	*B. cereus*		*S. pyogenes*		*C. acnes*	
	MIC	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***
Proantho-cyanidins	0.08	0.16	0.08	0.08	2.50	2.50
SP/AR/ 96% EtOH	0.39	3.13	0.20	0.20	3.13	3.13
SP/AR/ 50% EtOH	0.20	0.20	0.39	0.39	1.56	1.56
AU/AR/ 96% EtOH	0.20	0.20	0.05	0.05	1.56	1.56
AU/AR/ 50% EtOH	0.20	0.20	0.05	0.05	0.78	0.78
AU/AR/ Water	0.20	0.39	0.20	0.20	1.56	1.56

Confidence interval for a mean is ≤ 0.01 at α=0.05.

It was reported that oligomeric proanthocyanidins play one of the major roles in the biological activity of the plants extracts [23], [24]. This statement was confirmed by the results of the present research, showing high antibacterial activity of proanthocyanidins isolated from the 50% EtOH extract of *Aronia melanocarpa* branches collected in autumn.

IV. CONCLUSIONS

In this research, *Aronia melanocarpa* collected in autumn and extracted by 50% EtOH solution showed the highest antioxidant activity among all the other *Aronia melanocarpa* extracts, and it was 1.2 times better than of Trolox, a synthetic water-soluble analogue of vitamin E. Antioxidant activity of proanthocyanidins purified from the 50% EtOH extract of *Aronia melanocarpa* branches collected in autumn, in DPPH· tests was even higher than that of extracts, and it was 4 times better than that of Trolox.

All the extracts and proanthocyanidins under study showed the ability to fully stops the bacterial growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and Bacillus cereus*, *Streptococcus Pyogenes* and *Cutibacterium acnes* (with MBC 0.05 – 6.25 mg mL^{-1}). Proanthocyanidins isolated from the 50% EtOH extract of *Aronia melanocarpa* branches collected in autumn had the smallest necessary minimal bactericidal concentration (MBC), and, therefore, the strongest ability to stop the bacterial growth of *Pseudomonas aeruginosa* (MIC/MBC=0.16/0.31 mg mL^{-1}), while the 50%EtOH extrtacts and proanthocyanidins had highest activity against *Escherichia coli* (MBC=0.10-0.16 mg mL^{-1}). Proanthocyanidins, 50% EtOH and 96% EtOH extracts had similar bactericidal concentration levels against *Bacillus cereus* and *Streptococcus Pyogenes* (MBC=0.16–0.20 and 0.05–0.08 mg mL^{-1}), while 50% EtOH extracts were the strongest against *Staphylococcus aureus* (MIC/MBC=0.10 mg mL^{-1}) and *Cutibacterium acnes* (MIC/MBC=0.78 mg mL^{-1}). Thus the results showed the possiblity to use extracts and proanthocyanidins of *Aronia melanocarpa,* or their combination, in fight with pathogenic bacteria.

The study confirmed the high potential of *Aronia melanocarpa* lignocellulosic biomass as a source of valuable biologically active compounds for the creation of antibacterial and antioxidant preparations for the healthcare, nutrition industry, and cosmetics.

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