Bioactive constituents in aronia berries

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Oslo, 2013

Marie Bräunlich

Abstract

The aim of this thesis was to investigate some of the potential health benefits of aronia berries with main focus on the phenolic substances. Extractions and fractionations of aronia berries were performed to obtain several crude extracts and subfractions. In addition, well known constituents of aronia berries (anthocyanins, procyanidins and phenolic acids) were isolated. The different samples obtained were then tested in various *in vitro* bioassays to determine their biological activities as antioxidants, α-glucosidase inhibitors, CYP3A4 inhibitors, and antibacterial agents.

Pure anthocyanins and anthocyanin-rich extracts were found to be potent α-glucosidase inhibitors compared to the positive control acarbose, the active principle in an anti-diabetic drug. α-Glucosidase plays a vital role in digestion of carbohydrates and by inhibition of this enzyme, it might be possible to control postprandial hyperglycemia which is important for diabetic patients. Antioxidant activity was assessed by DPPH radical scavenging and by inhibition of 15-lipoxygenase and xanthine oxidase. In general, the substances tested displayed strong radical scavenging and 15-LO inhibitory effects, but exhibited less activity towards XO.

CYP3A4 is an important-drug metabolizing enzyme, and any change of its activity may lead to pharmacokinetic interactions. Aronia substances were investigated for their CYP3A4 inhibitory effects, and it was shown that procyanidin B5 and subfraction Seph g, rich in proanthocyanidins with a high degree of polymerization, were potent inhibitors of this protein. In addition, the inhibitory activity of proanthocyanidin-containing subfractions was correlated to the degree of polymerization of these substances.

The formation of bacterial biofilms is a well known health problem, as bacteria within such a structure are more difficult to eradicate. Aronia substances were added to planktonic cultures of *Escherichia coli* and *Bacillus cereus* in order to measure their influence on biofilm formation. Results showed that the majority of the samples possessed a biofilm inhibitory activity against the Gram-positive *B. cereus*, but fewer samples were active against the Gram-negative *E.coli*.

Minor differences in chemical structure, for example between procyanidin B2 and B5, gave rise to considerable differences in biological activities such as CYP3A4 inhibition and

inhibition of biofilm formation. Also, biological activity seemed to be influenced by the sugar unit linked to the anthocyanidin.

Four different cultivars of aronia were studied with respect to their phenolic composition and their biological activities. *Aronia prunifolia* contained the highest amount of total phenolics, anthocyanins, and proanthocyanidins. Despite the differences in chemical composition between the cultivars, which could be explained at least partly by unequal growing conditions, only minor differences in biological activity were found.

List of Papers

Paper I Bräunlich, M.; Slimestad, R.; Wangensteen, H.; Brede, C.; Malterud, K. E.; Barsett, H. Extracts, anthocyanins and procyanidins from *Aronia melanocarpa* as radical scavengers and enzyme inhibitors. *Nutrients* **2013**, *5*, 663-678.

Paper II Bräunlich, M.; Christensen, H.; Johannesen, S.; Slimestad, R.; Wangensteen, H.; Malterud, K. E.; Barsett, H. *In vitro* inhibition of cytochrome P450 3A4 by *Aronia melanocarpa* constituents. *Planta Med*. **2013**, *79*, 137-141.

Paper III Bräunlich, M.; Økstad, O. A.; Slimestad, R.; Wangensteen, H.; Malterud, K. E.; Barsett, H. Effects of *Aronia melanocarpa* constituents on biofilm formation of *Escherichia coli* and *Bacillus cereus*. *Molecules* **2013**, *18*, 14989-14999.

Paper IV Wangensteen, H.; Bräunlich, M.; Nikolic, V.; Malterud, K. E.; Slimestad, R.; Barsett, H. Anthocyanins, proanthocyanidins and total phenolics in four cultivars of aronia: Antioxidant and enzyme inhibitory effects. *Submitted to J. Funct. Foods,* **2013**.

List of abbreviations

Amb	Amberlite XAD-7HP fractions from *Aronia melanocarpa* berries
CLSI	Clinical Laboratory Standards Institute
^{13}C NMR	Carbon nuclear magnetic resonance
CYP	Cytochrome P450
DCM	Dichloromethane
DP	Degree of polymerization
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EPS	Extracellular polymeric substance
ET	Electron transfer
EtOH	Ethanol
F-C	Folin–Ciocalteu
FW	Fresh weight
GIT	Gastrointestinal tract
HAT	Hydrogen atom transfer
HDL	High-density lipoprotein
HETE	Hydroxyeicosatetraenoic acid
^{1}H NMR	Proton nuclear magnetic resonance
H_2O	Water
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LC-MS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoprotein
LO	Lipoxygenase

MDZ	Midazolam
MeOH	Methanol
NMR	Nuclear magnetic resonance
OH	Hydroxyl
ORAC	Oxygen radical absorbance capacity
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
Seph	Sephadex LH-20 fractions from *Aronia melanocarpa* berries
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet
XO	Xanthine oxidase

1 Introduction

1.1 Project background

The motivation to start this project was based on observations made in a nursing home for the elderly in Hå, Norway. In 2005 one floor at the Hå elderly care facility started to serve their residents a beverage made from aronia berries as a replacement to other drinks. The drink became popular and they kept on serving it until the end of 2007. During this two year period, employees noticed that the incidents of urinary tract and pneumonia infections were reduced. Also, the residents reported an increased sense of well-being. Another pleasant surprise was the complete disappearance of urine smell at this floor shortly after the introduction of the new beverage. However, this was not a clinical study and no scientific approach was taken to verify the observations (Slimestad, R., personal communication).

This is a collaboration project between Bioforsk Vest Særheim, Tine SA, and the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo. It is part of a project entitled "Potential of black aronia berries (*Aronia melanocarpa*) in treatment of urinary tract infections", funded by the Research Council of Norway.

1.2 Aronia plants

Aronia, also called chokeberry, is a member of the Rosaceae family. The genus is usually considered to contain two species: Black chokeberry, *Aronia melanocarpa* (Michx.) Ell., and red chokeberry, *Aronia arbutifolia* (L.) Pers. (Kokotkiewicz *et al*., 2010; Kulling and Rawel, 2008). Purple chokeberry, *Aronia prunifolia*, is regarded as a hybrid between *A. melanocarpa* and *A. arbutifolia*, it is however ambiguous if it should rather be considered a distinct species. *Aronia prunifolia* (Marshall) Rehder is indexed in The International Plant Names Index (International Plant Names Index, 2013).

Shrubs of the *Aronia* genus are native North American plants that have been traditionally used in Native American medicine, for example in the treatment of colds (Kokotkiewicz *et al*., 2010; Kulling and Rawel, 2008). The aronia shrubs can grow to a height of 2-3 m, they produce in May to June umbels of small white flowers, ripening to red (red chokeberry) or purple-black berries (purple and black chokeberry). In the 20th century, aronia became popular in the Soviet Union and in different parts of Europe, and the plants were cultivated either for their ornamental value or to use the berries as a food ingredient. The aronia plant has become

even more popular in recent years due to its berries having a high content of polyphenols with antioxidant activity (see section 1.2.1 and 1.2.2). Aronia products are well received as nutritional supplements, and the berries are also used as an ingredient for juices, wines, jams, and as a source of natural food colorants (Kulling and Rawel, 2008).

Figure 1. *Aronia melanocarpa*, cultivar "Moskva".

Cultivars used for fruit production are from the species *Aronia melanocarpa*. Commercially important cultivars in Europe and the United States include "Aron", "Nero", "Viking", "Hugin" and "Rubina" (Kulling and Rawel, 2008). In Norway, the cultivar "Moskva" is most common (Figure 1).

1.2.1 Phenolic constituents of aronia berries

It has been suggested that the most important constituents present in aronia, also responsible for many of its putative medical properties are the phenolic compounds (Kokotkiewicz *et al.,* 2010; Kulling and Rawel, 2008). Aronia berries contain high levels of procyanidins, anthocyanins and phenolic acids. Procyanidins have been identified as the major class of polyphenolic compounds in chokeberries (Oszmiański and Wojdylo, 2005). Their content varies from 0.7% to 5.2% dry weight (Kokotkiewicz *et al*., 2010; Oszmiański and Wojdylo, 2005). Aronia plants contain exclusively B type procyanidins with (-)-epicatechin as the main monomer subunit (Kulling and Rawel, 2008; Oszmiański and Wojdylo, 2005). The degree of polymerization (DP) of procyanidins varies from 2 to 23 units in the fruits, with clear domination of >decamers fraction (Kokotkiewicz *et al*., 2010). Free epicatechin is also

present in chokeberries, although its concentration is significantly lower in comparison with polymeric procyanidins. In aronia berries, anthocyanins constitute the second largest group of phenolic compounds, with a concentration range from 0.6% to 2.0% dry weight. The anthocyanins in *A. melanocarpa* are mainly a mixture of four cyanidin glycosides: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and cyanidin 3-xyloside; of those cyanidin 3-galactoside is present in the highest concentration. Also, chokeberries constitute a rich source of phenolic acids. Chlorogenic and neochlorogenic acids were the two dominating phenolic acids identified in the fruits (Slimestad *et al*., 2005). A series of factors, such as habitat/location, harvest date, cultivar, fertilization, and maturation of the berries can affect their content (Jeppsson and Johansson, 2000; Rop *et al.,* 2010; Skupień and Oszmiański, 2007).

1.2.2 Potential health benefits of aronia berries

Many beneficial health effects have been studied for aronia berry juice, nectar, extracts and for some isolated compounds. The berries have a wide range of potential medicinal and therapeutic effects; however, the amount of data on human clinical studies is limited, and most of the reported pharmacological activities were obtained from *in vitro* studies. Nevertheless, there are several papers available from studies in rats (Kokotkiewicz *et al*., 2010; Kulling and Rawel, 2008). This section focuses on the most important *in vitro* and *in vivo* findings reported regarding biological effects of black chokeberries.

The antioxidant properties of aronia juice, extracts and its phenolic constituents have been reported in a series of papers, using different well established *in vitro* assays (Kokotkiewicz *et al*., 2010; Kulling and Rawel, 2008; Valcheva-Kuzmanova and Belcheva, 2006). So far, fresh aronia berries possess the highest antioxidant activity among common berries and fruits when measured with the oxygen radical absorbance capacity (ORAC) assay (Kulling and Rawel, 2008; Zheng and Wang, 2003). A few reports also describe an antioxidant effect in animal models, where chokeberry anthocyanins decreased lipid peroxidation and enhanced the activity of enzymes which are involved in the antioxidant defense system (Faff and Frankiewicz-Jozko, 2003; Kowalczyk *et al*., 2004). An antioxidant activity was also found in humans, as a dietary supplementation with aronia juice limited the exercise-induced oxidative damage to red blood cells in rowers (Pilaczynska-Szczesniak *et al.,* 2005).

Aronia berries may also have a positive influence on several risk factors associated with cardiovascular diseases (Kokotkiewicz *et al*., 2010; Kulling and Rawel, 2008). The lipid-lowering activity of aronia preparations has been well documented with use of rat models. Administration of *A. melanocarpa* juice to hyperlipidaemic rats resulted in substantially decreased levels of total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides in blood plasma (Valcheva-Kuzmanova *et al.,* 2007 a; Valcheva-Kuzmanova *et al.,* 2007 b). In men with a mild hypercholesterolaemia, regular chokeberry juice drinking (250 mL per day) for six weeks resulted in a significant decrease in serum total cholesterol, LDL cholesterol and triglyceride level, whereas the HDL2 cholesterol level was increased (Skoczyńska *et al.,* 2007). Another study showed that combination therapy of statins with a flavonoid-rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction, and a lowering of systolic and diastolic blood pressure in persons supplemented with the extract was also reported (Naruszewicz *et al.,* 2007). In addition, other studies have demonstrated an anti-diabetic activity of aronia juice both in diabetic rats (Valcheva-Kuzmanova *et al.,* 2007 c) and in non-insulin dependent diabetic humans (Simeonov *et al.,* 2002), showing that aronia might be useful in the prevention and control of diabetes mellitus type II and diabetes-associated complications.

Phenolic compounds isolated from berries of *Aronia melanocarpa* showed antimutagenic activities *in vitro* (Gąsiorowski *et al.,* 1997). The Ames test and the sister chromatid exchange assay with cultured human lymphocytes revealed that anthocyanins isolated from black chokeberries possess antimutagenic activity. Also, several reports suggest anti-proliferative or protective effects of chokeberry juice and anthocyanin-rich chokeberry extracts against colon cancer on the basis of *in vitro* studies (Bermudez-Soto *et al.,* 2007; Malik *et al.,* 2003; Zhao *et al.,* 2004) and an animal study (Lala *et al.,* 2006).

Animal studies in rats also report hepatoprotective effects of *A. melanocarpa* (Kowalczyk *et al.,* 2003; Valcheva-Kuzmanova *et al.,* 2004). In one study, anthocyanins from chokeberry decreased the toxicity and accumulation of cadmium in the liver and kidney of rats receiving both these components in their diet. The reduction in harmful effects from cadmium could be explained by the anthocyanins ability to chelate metal ions (Kowalczyk *et al.,* 2003). In another experiment, a hepatoprotective effect of aronia juice was observed in rats after acute exposure to carbon tetrachloride (Valcheva-Kuzmanova *et al.,* 2004). The protective effect of

A. melanocarpa seems to be related to its antioxidative properties and the scavenging of free radicals formed during carbon tetrachloride intoxication.

Antimicrobial properties of phenolic compounds from numerous berry species, such as cranberry, blueberry, and raspberry are well known and have been demonstrated *in vitro*. *Aronia melanocarpa* fruit juice exhibited bacteriostatic activity *in vitro* against *Staphylococcus aureus* and *Escherichia coli*, and possessed an antiviral activity against influenza virus type A (Valcheva-Kuzmanova and Belcheva, 2006).

Besides potential health benefits of natural products, it is equally important to be aware of potential adverse effects associated with consumption of these products. Currently, there are no data in the literature about any unwanted or toxic effects of *Aronia melanocarpa* berries, juice or extracts.

1.2.3 Bioavailability of aronia polyphenols

The extent of the potential benefits associated with the antioxidant effects of polyphenols as well as other positive health effects studied *in vitro* are dependent *in vivo* on the absorption, metabolism, distribution, and excretion of these compounds within the body after ingestion (Denev *et al.,* 2012). Therefore, bioavailability studies allowing the determination of the real exposure of the human organism to the tested compounds are essential. Bioavailability appears to differ greatly among the various phenolic compounds and metabolism is a factor strongly affecting their plasma concentrations. After absorption, polyphenols are usually subjected to three main types of conjugation: methylation, sulfation, and glucuronidation.

The bioavailability of anthocyanins from aronia has been investigated more thoroughly than the bioavailability of the other phenolic constituents, because of their simple profile (Denev *et al.,* 2012). Only small amounts of the anthocyanins are absorbed in the small intestine, thus significant amounts are likely to enter the colon where bacterial degradation occurs (Pascual-Teresa *et al.,* 2010). Anthocyanins are some of the few polyphenols that can be detected unmetabolized (e.g. as glycosides) in plasma. However, bioavailability data of aronia anthocyanins give controversial information (Denev *et al.,* 2012). There are big differences in the number of recovered metabolites in blood and urine and the amounts of recovered parent anthocyanins in the human and animal studies. The major finding in the bioavailability studies of aronia anthocyanins was the determination of nanomolar concentrations in blood and urine. They can undergo glucuronidation and methylation, whereas sulfation was not observed. It

appears likely that these metabolic products are partly responsible for the reported health benefits associated with the consumption of anthocyanins.

So far, neither the flavanols nor the proanthocyanidins isolated from aronia have been subjected to bioavailability studies. The intake of monomeric flavanols with aronia berries is low and it is not very likely that they have a significant influence on plasma polyphenol concentrations (Denev *et al.,* 2012). Generally, low molecular weight oligomeric proanthocyanidins (DP $\leq$ 3) are absorbed intact in the gastrointestinal tract (GIT), but polymerization greatly impairs intestinal absorption (Denev *et al.,* 2012; Pascual-Teresa *et al.,* 2010). Most pass to the colon unaltered where they are catabolized by the colonic microflora, yielding a diversity of phenolic acids including 3-(3-hydroxyphenyl)-propionic acid and 4-O-methylgallic acid. Those can be absorbed into the circulatory system and excreted in urine. Despite their poor absorption, proanthocyanidins may exert a local activity in the gastrointestinal tract or an activity mediated by phenolic acids produced through microbial degradation. Their local action may nevertheless still be of importance because the intestine is for example particularly exposed to oxidizing agents and may be affected by inflammation and numerous diseases, including cancer.

Phenolic acids, when ingested in the free form, are rapidly absorbed from the small intestine (Denev *et al.,* 2012). However, the main representatives in aronia, chlorogenic and neochlorogenic acids are naturally esterified and this impairs their absorption.

Ironically, the polyphenols which are present in the highest amounts in chokeberries (anthocyanins and proanthocyanidins) are the least well absorbed. Nevertheless, there is accumulated experimental evidence of the effectiveness of aronia products in a broad range of pathological conditions (see section 1.2.2) in many cases mediated by uncontrolled oxidative processes. The first site of action of chokeberry polyphenols is the gastrointestinal tract where they and their metabolites released by the gut microflora could act for example as antioxidants.

1.3 Chemistry of isolated compounds

1.3.1 Polyphenols

Polyphenols are common constituents of plant-based foods (Tsao, 2010). Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of polyphenols. They are chemically characterized as compounds with a polyphenol

structure (e.g. several hydroxyl groups on aromatic rings), but molecules with one phenol ring, such as phenolic acids and simple phenols, are also often included (D'Archivio *et al.,* 2007). This group of natural products is very diverse and contains several sub-groups of phenolic compounds, which differ by the number of phenol rings that they contain and by the structural elements that bind these rings to one another (D'Archivio *et al.,* 2007; Tsao, 2010). The main groups of polyphenols are: flavonoids, phenolic acids, simple phenols, stilbenes and lignans.

1.3.2 Flavonoids

Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three carbon bridge, hence C6–C3–C6 (Figure 2). They are the most numerous of the phenolics and are found throughout the plant kingdom (Crozier *et al.,* 2009). They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents, and screens against UV irradiation (Alzand and Mohamed, 2012). Flavonoids occur widely in the fruits and vegetables that are part of the human diet and it has been estimated that at least one gram of flavonoids is consumed daily (Pascual-Teresa *et al.,* 2010). A number of biological and pharmacological activities have been reported for flavonoids, including antioxidant, cytotoxic, anticancer, antiviral, antibacterial, cardioprotective, hepatoprotective, neuroprotective, and antimalarial properties (Alzand and Mohamed, 2012). The main sub-classes of dietary flavonoids are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones, while those that are comparatively minor components of the diet are dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones and aurones (Crozier *et al.,* 2009). Each group of flavonoids possesses unique chemical properties and has a particular distribution in plants (Alzand and Mohamed, 2012).

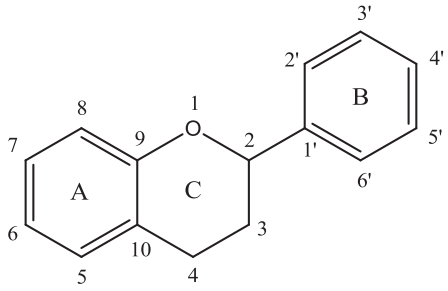


Figure 2. Flavonoid skeleton.

1.3.3 Flavanols and proanthocyanidins

Catechins are classified as flavan-3-ol monomers and are characterized by a C6-C3-C6 skeleton with a hydroxyl group in position three of the C-ring, two chiral centers on the molecule (one on carbon two and one on carbon three) and no oxygen atom linked to carbon four (Pascual-Teresa *et al.,* 2010). The two compounds catechin and epicatechin are well known flavonoids with a widespread distribution (Figure 3). Catechin, epicatechin, gallocatechin, epigallocatechin and their galloyl substituted derivatives are commonly found in plant-derived foods and food products. Monomeric flavanols are the biosynthetic precursors of proanthocyanidins, also called condensed tannins.

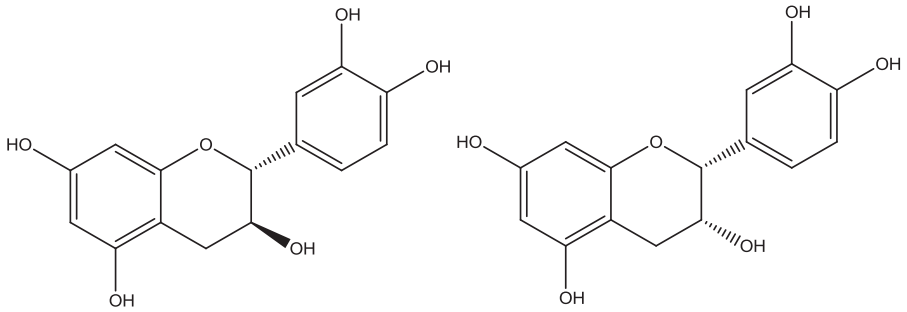


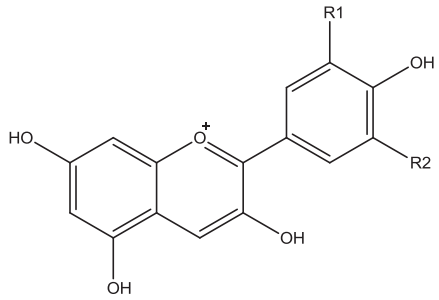
Figure 3. Structures of catechin and epicatechin.

Proanthocyanidins are found mainly in fruits, berries, beans, nuts, cocoa, and wine (Rasmussen *et al.,* 2005). Through the formation of complexes with salivary proteins, proanthocyanidins are responsible for the astringent character of fruits and beverages (e.g. wine) and for the bitterness of chocolate. Condensed tannins are oligomers (dimers to pentamers) or polymers (six or more units) of flavan-3-ols (Pascual-Teresa *et al.,* 2010). Proanthocyanidins that exclusively consist of (epi)catechin units are designated procyanidins, whereas proanthocyanidins containing (epi)afzelechin or (epi)gallocatechin subunits are called propelargonidins or prodelphinidins, respectively (Rasmussen *et al.,* 2005). The most common oligomers are the B series, B1 to B8, formed by two flavanol units, either catechin or epicatechin, joined by a C4-C8 linkage (B1 to B4), or C4–C6 linkage (B5 to B8). The least frequent dimers are the A series, characterized by the presence of double linkages between the two flavanol units, one C4-C8 or C4-C6 and an additional one between C2 and C5 or C7.

Type A proanthocyanidins are less common in food plants; however their presence in peanuts, almond skins and in some berries such as cranberries has been reported (Pascual-Teresa *et al.,* 2010).

1.3.4 Anthocyanins

Anthocyanins are water-soluble pigments responsible for the blue, purple, red and orange colors of many fruits and vegetables (Pascual-Teresa *et al.,* 2010). More than 500 different anthocyanins have been described in the literature, and many reports of new anthocyanin structures have been published during the last decade. This can partly be explained by the use of improved analytical techniques, but also due to their impact on the sensorial characteristics of food products and their potential use as health beneficial compounds. The anthocyanins consist of an aglycone (anthocyanidin), sugar(s), and in many cases acyl group(s). They are positively charged at acidic pH, and this equilibrium form is called flavylium cation (2-phenylbenzopyrylium). Several anthocyanidins (aglycone forms) have been reported with hydroxyl and methoxyl groups present at different positions on the basic structure. Six of them are commonly found in fruits and vegetables: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Figure 4).



Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 4. Structures of the most common anthocyanidins.

1.3.5 Phenolic acids

Phenolic acids are aromatic secondary plant metabolites, widely spread throughout the plant kingdom (Robbins, 2003). In general, the name phenolic acid describes phenols that possess one carboxylic acid functionality. However, when describing plant metabolites, it refers to a distinct group of organic acids. These naturally occurring phenolic acids contain two common carbon frameworks: the C6-C3 hydroxycinnamic and C6-C1 hydroxybenzoic structures,

which constitute some of the main non-flavonoid polyphenolic compounds of dietary significance. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety. The most common hydroxycinnamates are p-coumaric acid, caffeic acid, ferulic acid and sinapic acid, with caffeic acid dominating (Crozier *et al.,* 2009). These often occur as conjugates, for example with tartaric acid or quinic acid. The quinic acid conjugates are usually referred to as chlorogenic acids. Among the hydroxybenzoic acids, gallic acid is the most common.

1.4 Biological activities

1.4.1 Free radicals and antioxidant activity

A free radical is any species capable of independent existence that contains one or more unpaired electrons in its outer orbital (Lobo *et al.,* 2010). Many radicals are unstable and highly reactive, and can cause oxidative damage to biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. Free radicals and other reactive oxygen species (ROS) are continuously produced *in vivo* as a consequence of both enzymatic and non-enzymatic reactions, but are also derived from external sources such as exposure to radiation, cigarette smoking, air pollutants, and industrial chemicals. The term oxidative stress is used to describe the condition of oxidative damage resulting when the balance between free radical generation and antioxidant defenses is disrupted. Oxidative stress is a physiological state, which has been suggested to be an important factor for the development of many diseases including cardiovascular diseases, stroke, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Denev *et al.,* 2012).

An antioxidant has been defined as a substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Frankel and Meyer, 2000). Testing of antioxidant activities of natural products and their potential beneficial effects on health has received much attention in recent years. The antioxidant capacity of natural substances can be assessed with numerous assays. Due to the complexity of the composition of phytochemicals and of the oxidative processes, it is recommended to use more than one method in order to evaluate the total antioxidant activity (Denev *et al.,* 2012; Frankel and Meyer, 2000; Wangensteen *et al.,* 2004).

1.4.1.1 Scavenging of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

Scavenging of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a very rapid and easy to perform method for antioxidant activity testing (Denev *et al.,* 2012). The assay is based on the use of the stable free DPPH radical (Figure 5) which has a deep violet color with a UV–visible absorption maximum in methanol or ethanol solutions at around 515-520 nm (Huang *et al.,* 2005; Molyneux, 2004). Upon scavenging of the radical by an antioxidant, the solution color fades and the reaction progress is conveniently monitored by a spectrophotometer. On the basis of the chemical reactions involved, antioxidants can deactivate radicals by hydrogen atom transfer (HAT) or by electron transfer (ET) reactions (Denev *et al.,* 2012). The DPPH assay was believed to involve hydrogen atom transfer reactions (Huang *et al.,* 2005; Molyneux, 2004), but other studies have suggested otherwise (Foti *et al.,* 2004; Litwinienko and Ingold, 2004). On the basis of the kinetic analysis of the reaction between phenols and DPPH, it was suggested that an ET mechanism is favored in the reaction between phenols having low pKa values and DPPH in strong hydrogen-bond-accepting solvents, such as methanol and ethanol.

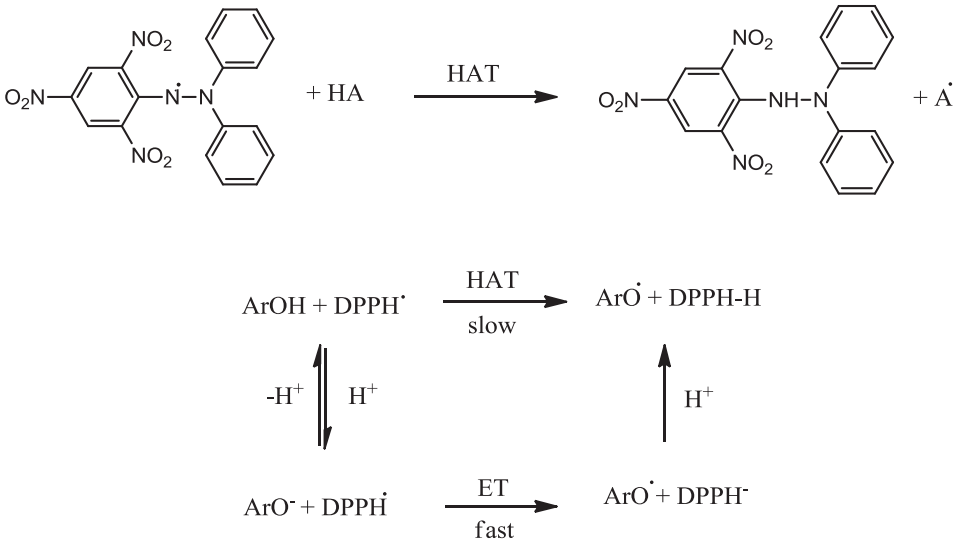


Figure 5. Scavenging of the DPPH radical. Both, the hydrogen atom transfer (HAT, upper picture) and electron transfer (ET, lower picture) have been proposed as scavenging reaction mechanisms (Foti *et al.,* 2004; Litwinienko and Ingold, 2004).

1.4.1.2 Inhibition of 15-lipoxygenase (15-LO)

Lipoxygenases (LOs) are non-heme iron-containing enzymes that catalyze specific lipid peroxidations and are found in plants, animals, and some bacteria (Funk, 2006; Kühn *et al.,* 2005). Mammalian lipoxygenases may be classified into four different subfamilies (Figure 6), originally divided into 5-, 8-, 12-, and 15-LO, and named for the numbered carbon where they oxygenate arachidonic acid (Dobrian *et al.,* 2011; Kühn *et al.,* 2005; Takahashi *et al.,* 2005). Based on the phylogenetic relatedness an alternate classification for mammalian lipoxygenases is proposed, comprising 5-LO, 12/15-LO, platelet-type 12-LO, and epidermal-type LO (Kühn *et al.,* 2005). Among these subclasses, 5-, 12- and 15-LO have been implicated in the development of atherosclerosis. When arachidonic acid is metabolized, all of the different LO isoforms generate lipid hydroperoxides (HPETEs) as the primary product (Dobrian *et al.,* 2011). Those are rapidly reduced intracellularly to their corresponding hydroxides (HETEs). In humans, 12/15-LOs create a number of important lipid mediators, such as 12- and 15-HPETEs and 12- and 15-HETEs. These lipid products have a variety of functions in human tissues, for example they are involved in monocyte binding in the vasculature, an early event in atherogenesis. Other potential mechanisms for the atherogenic promoting role of 15-LO is its ability to oxidize LDL and HDL (Funk, 2006; Kühn *et al.,* 2005). It has also been shown that 12- and 15-LO and their metabolites are important in a variety of other pathological conditions, such as diabetes (both types I and II), renal disease, obesity, and various diseases of the central and peripheral nervous systems (Dobrian *et al.,* 2011). The development of selective LO inhibitors may be useful to treat these disorders in the future. However, the precise role of these enzymes is still under discussion and an active area of investigation, as conflicting biological functions of 12- and 15-LO have been reported (Dobrian *et al.,* 2011; Kühn *et al.,* 2005). Those enzymes were shown to possess both pro-atherogenic (in mice) and anti-atherogenic (in rabbits) effects. Also, 15-lipoxygenase may generate lipoxins with anti-inflammatory activity.

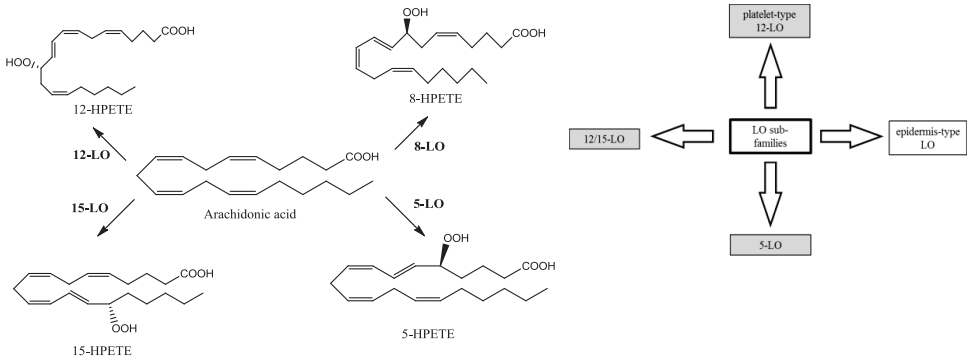


Figure 6. Subclassification of mammalian lipoxygenase isoforms, originally divided based on their ability to insert molecular oxygen at the corresponding carbon position of arachidonic acid (left). Recently, an alternate classification according to their phylogenetic relatedness has been proposed (right). Isoforms on gray background have been implicated in cardiovascular diseases (Kühn *et al.,* 2005; Takahashi *et al.,* 2005).

1.4.1.3 Inhibition of xanthine oxidase (XO)

Xanthine oxidase (XO) is a form of xanthine oxidoreductase, found mainly in the liver and gastrointestinal tract, but also in the kidney, brain and throughout the cardiovascular system (Higgins *et al.,* 2009; Pacher *et al.,* 2006). The primary role of this enzyme is the conversion of hypoxanthine to xanthine, and xanthine to uric acid (Figure 7). Elevated levels of uric acid in the blood can cause hyperuricemia, which is associated with gout and kidney stones (Akowuah *et al.,* 2006), but has also been linked to other pathological states such as cardiovascular diseases (Higgins *et al.,* 2009). The purine analogue allopurinol is a clinically used XO inhibitor in the treatment of gout and hyperuricemia, but this drug suffers from many side effects such as hepatitis, nephropathy, and allergic reactions (Nguyen *et al.,* 2004; Pacher *et al.,* 2006). Thus, novel XO inhibitors with increased therapeutic activity and fewer side effects are desired. The action of XO enzymes also generates superoxide radicals and hydrogen peroxide, which can add to or initiate oxidative stress (Higgins *et al.,* 2009; Pacher *et al.,* 2006). Hence, the inhibition of xanthine oxidase may not only be beneficial to treat gout and hyperuricemia but could also be useful to combat other diseases associated with oxidative stress.

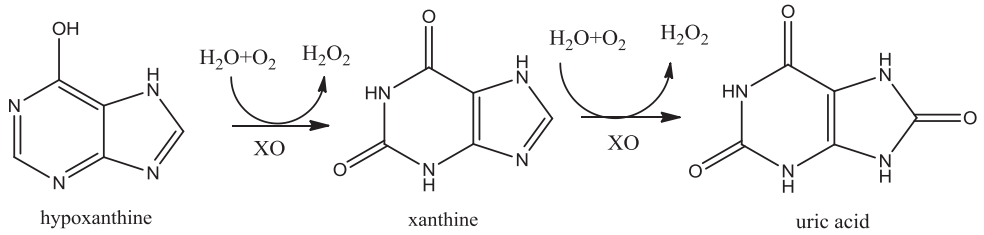


Figure 7. Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid.

1.4.2 α-Glucosidase inhibitory activity

Carbohydrates constitute an important part of the human diet, however, only monosaccharides, such as glucose and fructose, can be absorbed by the small intestine (Coniff and Krol, 1997). Polysaccharides and disaccharides (e.g. starch and sucrose) undergo rapid enzymatic degradation to monosaccharides in the upper intestine through the action of pancreatic α-amylase and α-glucosidase. α-Glucosidase is a membrane bound enzyme located in the brush border of the small intestine (Huang *et al.,* 2010). It is the key enzyme during the final step in the digestive process of carbohydrates, as it catalyzes the cleavage of glucose from oligosaccharides and disaccharides. Inhibition of α-glucosidase in the intestine decreases the rate of hydrolytic cleavage of saccharides and the process of carbohydrate digestion spreads to the lower part of the small intestine (Kumar *et al.,* 2011). This spreading of the digestive process delays the overall absorption rate of glucose into the blood and has proved to be a good strategy to reduce postprandial plasma glucose levels and suppress postprandial hyperglycemia, which might help to prevent the onset of diabetes or long-term diabetic complications. The α-glucosidase inhibitor acarbose is the active principle in an anti-diabetic drug used to treat type II diabetes mellitus and is effective in patients having a diet relatively rich in carbohydrates (Coniff and Krol, 1997). In recent years, many efforts have been made to identify effective α-glucosidase inhibitors from natural sources in order to develop a physiologic functional food or lead compounds for use against diabetes (Kumar *et al.,* 2011).

1.4.3 Cytochrome P450 3A4

Cytochrome (CYP) P450 enzymes are responsible for the metabolism of endogenous components and xenobiotics, such as pharmaceuticals and environmental compounds (Shimada *et al.,* 1994). There are several drug-metabolizing CYPs, but the most abundantly

expressed isoform is CYP3A4, which is responsible for the metabolism of more than 50% of medicinal products on the market, for example calcium-channel blockers, statins, and cyclosporine (Ansede and Thakker, 2004; Kimura *et al.,* 2010). CYP3A4 is located in the small intestine (found in the apical enterocytes) and liver, which makes it well suited to play a significant role in presystemic drug metabolism (Dresser and Bailey, 2003). Serious interactions have occurred as a result of the induction or the inhibition of the cytochrome P450 enzymes, since coadministration of multiple CYP3A4 substrates with inducers or inhibitors may alter pharmacokinetic parameters of many commonly prescribed drugs (Dresser and Bailey, 2003; Harris *et al.,* 2003). Interactions between components in food or herbal supplements and medicines have come into focus as there are numerous examples of metabolic interactions involving modulation of CYP activity by dietary substances. A prominent example of food-drug interactions mediated by CYP3A4 is provided by the inhibitory effects of furanocoumarins in grapefruit juice on presystemic metabolism, particularly in the intestine, leading to increased drug bioavailability. Also, the interaction between components of St. John's wort (*Hypericum perforatum*) and various pharmaceuticals by induction of CYP3A4 leading to reduced drug concentrations in blood is well known (Dresser and Bailey, 2003; Harris *et al.,* 2003; Kimura *et al.,* 2010). Similarly to the grapefruit juice interaction, the St John's wort interaction may be more profound with orally administered compounds that undergo significant presystemic metabolism.

1.4.4 Antimicrobial activity

Pathogenic microorganisms can cause infection and disease in humans, animals and plants. Most microbes belong to four major groups: bacteria, viruses, protozoa or fungi. The development of antibiotics has been an important achievement in medicine; however, infectious diseases are still of major medical and scientific concern (Yoneyama and Katsumata, 2006). There is a pressing need for new antibiotics due to the inevitable development of resistance that follows the introduction of antibiotics to the market. Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources (Cowan, 1999). Compounds such as plant metabolites that serve as a defense mechanism against the attack of bacteria and fungi also constitute a potential source of new antimicrobial drugs. Nevertheless, the development of antimicrobial agents from plant products has been extremely limited.

1.4.4.1 Disk diffusion method

A variety of laboratory methods can be used to measure the *in vitro* susceptibility of microorganisms to different test compounds (CLSI document M2-A9; Cos *et al.,* 2006; Jorgensen and Ferraro, 2009). The disk diffusion assay is simple and practical and has been well-standardized for common, rapidly growing pathogens, and also for certain fastidious ones. Currently, the Clinical Laboratory Standards Institute (CLSI) is responsible for updating and modifying this procedure through a global consensus process to ensure uniformity of technique and reproducibility of results. In the diffusion method, a reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period. The diameter of the zone is related to the susceptibility of the pathogen and to the diffusion rate of the compound through the agar medium. Disk diffusion testing does not provide quantitative results such as minimum inhibitory concentration. Results of this method are qualitative, in that a category of susceptibility (susceptible, intermediate or resistant) is derived from the test. This assay is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar. The relative antimicrobial potency of different substances is not always comparable, mainly because of differences in physical properties (e.g. solubility, volatility and diffusion characteristics in agar).

1.4.4.2 Inhibition of biofilm formation

Bacterial biofilms are sessile communities with high cell density embedded within a self-produced matrix of extracellular polymeric substance (EPS) that are ubiquitous in natural, medical, and engineering environments (Estrela and Abraham, 2010; Ren *et al.,* 2005). Biofilm formation is a key factor for microbial survival in hostile environments, representing a protected mode of growth that allows cells to survive and disperse. Deleterious biofilms are problematic in the industry since they cause fouling and corrosion in systems such as heat exchangers, oil pipelines, and water systems. Biofilms have been found to be involved in a wide variety of microbial infections in the body. They can colonize indwelling medical devices (e.g. cause catheter-related infections), but are also involved in common problems such as urinary tract, middle-ear and oral (e.g. dental plaque and gingivitis) infections and have been linked to chronic diseases such as lung infections in cystic fibrosis (Parsek and Singh, 2003). Bacteria in a biofilm are more likely to survive antibiotics than planktonic (free swimming) cells and are often responsible for reoccurring symptoms and medical treatment

failure (Estrela and Abraham, 2010; Ren *et al.,* 2005). There are many reasons for the higher antibiotic resistance of bacteria in a biofilm, for example their genetic program (gene expression) within such a structure is fundamentally changed. They grow slower and the reduced metabolic activity renders them less prone against most antibacterial drugs. Also, some protection may be conferred by the physical barrier provided by the presence of the EPS that covers the biofilm and may prevent sufficient antibiotic exposure to kill the cells. Recently, there has been a tremendous increase in biofilm research, most of it aiming at the prevention, control, and eradication of biofilms. An important focus of this research has been the development of alternative approaches, either to avoid the use of antimicrobial drugs altogether, or to combine alternative treatments with more traditional antimicrobial drugs.

2 Aims of the study

A consensus has developed over the last few decades that the health promoting effects associated with a diet rich in fruits and vegetables may be derived, in part, from the intake of natural antioxidants, such as vitamin C and polyphenols. Interest in the health benefits of aronia berries is based on their very high levels of flavonoids and other phenolic compounds, in addition to the surprisingly positive effects observed at the Hå nursing home after aronia berry juice was served instead of other beverages (see section 1.1).

The main objectives of the thesis were:

1. To isolate and elucidate the structure of some of the main bioactive polyphenols in aronia berries (paper I).

2. To measure *in vitro* antioxidant activity of aronia extracts, fractions and compounds. A large proportion of the ingested polyphenols will not be taken up into the circulation and passes through the upper gastrointestinal tract to the large intestine. Investigation of their effects in the gut is therefore highly relevant. The inhibitory activities of black chokeberry substances were also tested on the digestive enzyme, α-glucosidase, which is present in the small intestine (paper I and IV).

3. To study the effects of aronia substances on the activity of the metabolic enzyme CYP3A4 in order to provide more information on the food-drug interaction potential (paper II).

4. To investigate the inhibition of biofilm formation and the antibacterial activity of aronia berry extracts, fractions and compounds thereof on *Escherichia coli* and *Bacillus cereus* (paper III).

5. To compare four cultivars of *Aronia* with respect to their phenolic composition as well as their biological activities (paper IV).

3 Summary of papers

Paper I. Extracts, anthocyanins and procyanidins from *Aronia melanocarpa* as radical scavengers and enzyme inhibitors

In this study the four main anthocyanins (cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and cyanidin 3-xyloside) and three procyanidins (dimers B2 and B5 and trimer C1) were isolated from *Aronia melanocarpa*, cultivar "Moskva". The compounds were identified on the basis of their chromatographic and spectroscopic data (TLC, HPLC, LC-MS). In addition, crude extracts and subfractions were obtained from the berries. NMR analysis revealed that proanthocyanidins were present in the 50% EtOH extract. Subfractions of this extract were further shown to consist mainly of proanthocyanidins with epicatechin stereochemistry. The substances were investigated for their antioxidant and α-glucosidase inhibitory activities. Subfractions rich in procyanidins were found to be potent α-glucosidase inhibitors; they possessed high radical scavenging properties, a strong inhibitory activity towards 15-LO and a moderate inhibitory activity towards XO. Trimeric procyanidin C1 showed higher activity in the biological assays compared to the dimeric procyanidins B2 and B5. Also, the activity of the anthocyanins was found to be influenced by the sugar units linked to the anthocyanidin.

Paper II. *In vitro* inhibition of cytochrome P450 3A4 by *Aronia melanocarpa* constituents.

The aim of this work was to investigate the effects of crude extracts, subfractions, and compounds found in aronia on CYP3A4 activity in order to determine the potential risk of food-drug interactions. Midazolam was used as the probe substrate and recombinant insect cell microsomes expressing CYP3A4 as the enzyme source. In this assay, several constituents of *A. melanocarpa* possessed CYP3A4 inhibitory activity. Procyanidin B5 was a considerably stronger CYP3A4 inhibitor *in vitro* than the isomeric procyanidin B2 and comparable to bergamottin, a known CYP3A4 inhibitor from grapefruit juice. The inhibitory activity of proanthocyanidin-containing subfractions was correlated to the degree of polymerization. For the anthocyanins, the ability to inhibit CYP3A4 *in vitro* seemed to be influenced by the sugar

unit linked to the anthocyanidin. The phenolic acids (chlorogenic and neochlorogenic acid), however, did not inhibit CYP3A4-mediated midazolam metabolism.

Paper III. Effects of *Aronia melanocarpa* constituents on biofilm formation of *Escherichia coli* and *Bacillus cereus*

This study aimed to evaluate the ability of *A. melanocarpa* extracts, subfractions and compounds to prevent biofilm formation on PVC microtitre plates and to inhibit bacterial growth of *Escherichia coli* and *Bacillus cereus*. It was found that the majority of the samples displayed biofilm inhibition against the Gram-positive *B. cereus* at concentrations of 1 mg/mL, but exhibited less activity against the Gram-negative *E. coli*. However, the dichloromethane (DCM) extract and epicatechin that showed no anti-biofilm activity against *B. cereus* were effective against *E. coli*. In fact, epicatechin was the most active against biofilm-producing *E. coli,* whereas the 50% EtOH extract displayed the most potent biofilm inhibition against *B. cereus*. This might be due to the presence of unknown active compounds in this extract, or to synergistic effects. The antibacterial activity of aronia substances was investigated by the disk diffusion method, and it was shown that they were not toxic to the species screened. This non-toxic inhibition may confer a lower potential for resistance development compared to conventional antimicrobials.

Paper IV. Anthocyanins, proanthocyanidins and total phenolics in four cultivars of aronia: Antioxidant and enzyme inhibitory effects

The aim of this paper was to compare four cultivars of *Aronia* (*A. melanocarpa* "Moskva", "Hugin", "Nero" and *A. prunifolia*) with respect to their phenolic composition, antioxidant and α-glucosidase inhibitory activity. The content of anthocyanins in the cultivars was determined by HPLC using a reverse phase chromolith column instead of other commonly used reverse phase columns. Due to its faster flow rate, separation was achieved much quicker. In this study, the separation was accomplished in less than four minutes, which is a considerable advantage compared to anthocyanin analysis performed previously. Variations in chemical composition between the cultivars were found, which could be explained at least partly by unequal growing conditions. Cyanidin 3-galactoside was the major anthocyanin in all cultivars, with the highest content in *A. prunifolia* (497 ± 20 mg/100 g FW). Also, *A. prunifolia* had the highest content of polyphenols (2996 ± 172 mg gallic acid equivalents/100 g FW) and proanthocyanidins (4.79 g procyanidin B2 equivalents/100 g FW). For the food

industry and also for the herbal supplement industry, *A. prunifolia* should be the most interesting species as a high content of polyphenols is usually desirable. Anthocyanin fractions from all cultivars were shown to be potent α-glucosidase inhibitors. However, despite their variations in chemical composition only minor differences in biological activity between the cultivars were found.

4 Results and discussion

4.1 Extraction and isolation procedures

The aronia berries studied were obtained fresh and then kept at -20 ˚C until extraction, which was performed using different apparatus and procedures, as described in this chapter.

For paper I and II, the freeze-dried and pulverized aronia berries from cultivar "Moskva" were extracted with DCM followed by EtOH in a Soxhlet apparatus. The plant residue was further extracted with 50% EtOH at 70 ˚C (paper I and II), followed by extraction with distilled H_2O at 50 ˚C and 100 ˚C (paper II). This extraction procedure gives a rough separation of constituents with different polarity. The phenol–sulfuric acid method and ^{1}H NMR analyses revealed that carbohydrates were present in the 50 ˚C and 100 ˚C H_2O extracts, whereas ^{1}H and ^{13}C NMR analyses revealed the presence of proanthocyanidins in the 50% EtOH crude extract. In order to separate the tannins and pigments from other non-phenolic compounds, adsorption chromatography (Amberlite XAD-7HP) and adsorption and size exclusion chromatography (Sephadex LH-20) were performed with eluents of decreasing polarity (Figure 8) as described in paper I.

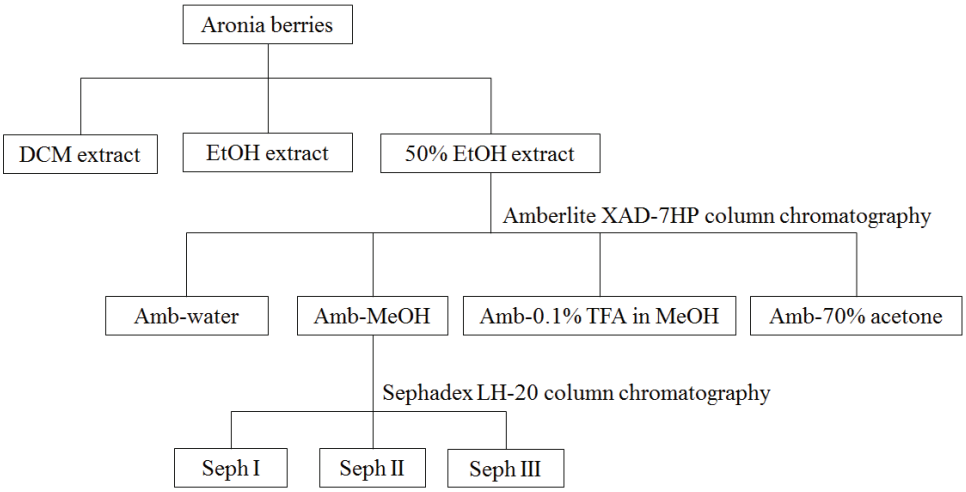


Figure 8. Illustration of the extraction and fractionation procedure presented in paper I.

Due to lack of material, the extraction and fractionation had to be rerun for the studies presented in papers II and III. This time, column chromatography on Sephadex LH-20 without prior absorption on XAD-7HP was chosen (Figure 9) as described in paper II. For proper separation, a longer column (80 cm for paper II versus 30 cm for paper I) was used. Sephadex LH-20 columns can separate substances of different molecular weights; however, aromatic compounds such as proanthocyanidins can have a higher affinity to the column material than to the eluent leading to potential failure of size-based separation. Nevertheless, thiolysis of Sephadex fractions d and g indicated that the DP of proanthocyanidins found in subfractions Seph d-g increases with the elution volume. After a chromatographic run, collected fractions were combined as indicated by TLC. Spots were visualized by UV-irradiation (254 and 366 nm) and by spraying with $Ce(SO_4)_2$ in aqueous sulfuric acid followed by heating to detect organic compounds.

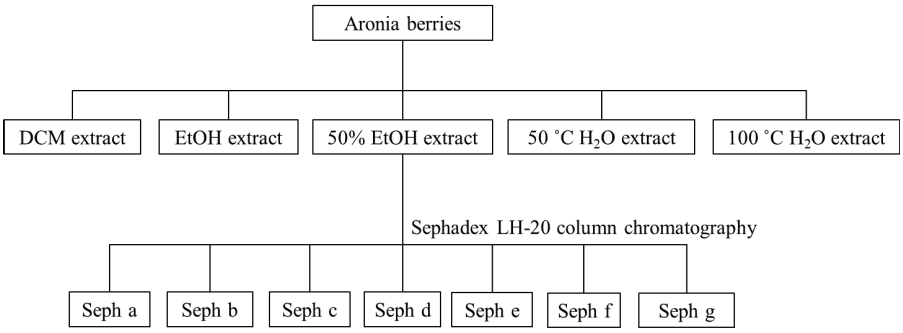


Figure 9. Illustration of the extraction and fractionation procedure presented in paper II.

In parallel to this procedure, pure compounds were isolated by different extraction methods performed at room temperature since the Soxhlet system could cause thermal degradation of anthocyanins and procyanidins. Four anthocyanins (paper I) were isolated from aronia berries by extraction with 0.5% TFA in MeOH, removal of MeOH, and partitioning between ethyl acetate and water (Figure 10). The aqueous phase was purified by column chromatography (Amberlite XAD-7HP and Sephadex LH-20) and collected fractions were analyzed by HPLC. In addition, cyanidin aglycone was obtained by acidic hydrolysis of cyanidin 3-galactoside (paper III). The oligomeric procyanidins B2, B5, and C1 are present in black chokeberries (Kulling and Rawel, 2008); however, instead of using berries as the source, bark was chosen. Bark was indeed found to be a simpler source for isolation since the complexity with respect

to total phenolic structures was lower (e.g. no anthocyanins, paper I). Procyanidin dimers B2 and B5 and trimer C1 (Figure 10) were extracted from aronia bark with 70% acetone, followed by defatting of the extract with DCM, partitioning against ethyl acetate and column chromatography (Sephadex LH-20). The obtained fractions were analyzed by TLC and HPLC.

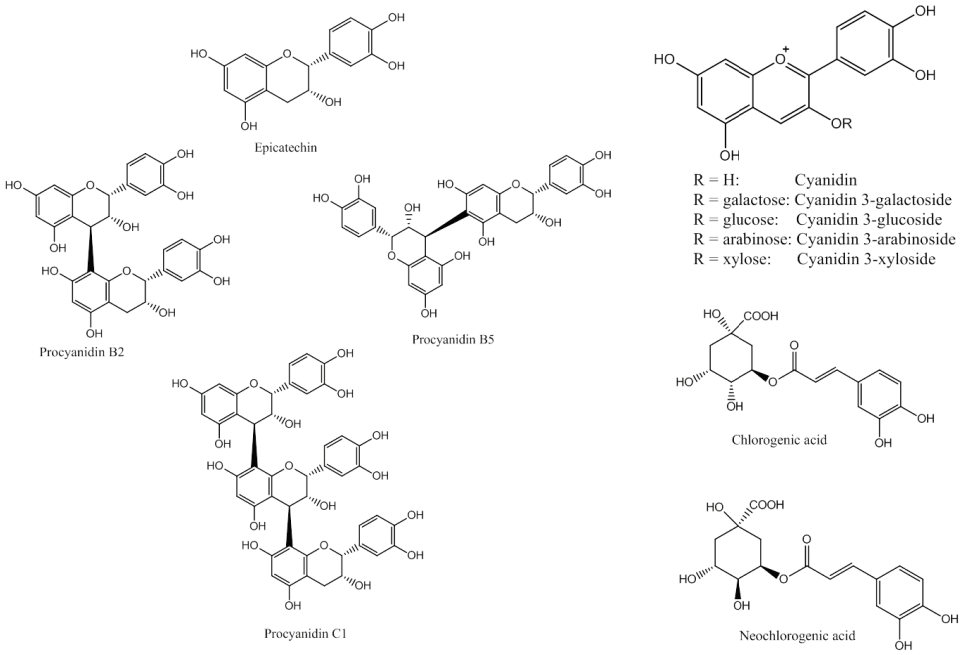


Figure 10. Compounds present in aronia berries. Cyanidin, anthocyanins and oligomeric procyanidins were isolated from aronia, whereas epicatechin and the phenolic acids were purchased from Sigma-Aldrich.

Isolated compounds were identified on the basis of their chromatographic and spectroscopic data such as TLC, HPLC and LC-MS. The optical rotation was also recorded as part of the characterization of the compounds. For stereoisomers in particular (e.g. chlorogenic and neochlorogenic acid), the optical activity is an important information to fully identify the compounds. Anthocyanins could not be measured due to their high absorbance at the sodium D wavelength. It has not been possible to find literature values for those, suggesting that others might have had a similar issue.

After isolation, anthocyanins and procyanidins were kept in the dark, at low temperature and in a dry state. However, long time storage sometimes led to degradation, for example loss of

the sugar unit was observed for anthocyanins. Therefore, compounds were analyzed by HPLC prior to biological activity testing. The isolation, purification and structure determination of pure compounds are relatively time consuming processes. Hence, substances present in aronia that are commonly and easily available, such as epicatechin, chlorogenic and neochlorogenic acid, were purchased from Sigma-Aldrich.

4.2 Chemical characterization (paper IV)

The content of total phenolics and proanthocyanidins in 80% EtOH extracts of the four cultivars of aronia (*Aronia melanocarpa* "Moskva", "Hugin", "Nero" and *Aronia prunifolia*) were determined by using the Folin–Ciocalteu (F-C) method and the acid butanol assay, respectively. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes which can be measured spectroscopically at 765 nm (Ainsworth and Gillespie, 2007). Results are typically expressed as gallic acid equivalents. The chemistry is non-specific and based on redox reactions, hence, other oxidation substrates in a given extract sample can interfere. Consequently, the method is not specifically detecting phenolic compounds since F-C reagent can also be reduced by other readily oxidized substances such as ascorbic acid. However, the F–C assay is simple, reproducible and has been widely used for approximating the total phenolic content in samples. The acid butanol assay is commonly used to determine condensed tannins (Hagerman, 2011; Amarowicz and Pegg, 2006). In this method, anthocyanidins are liberated from proanthocyanidins and they can be measured spectroscopically at 550 nm. Samples from aronia were colored (due to pigments) before the assay was employed, therefore absorbance values prior to hydrolysis were subtracted from absorbance values after hydrolysis in order to correct the contribution of natural occurring anthocyanins.

HPLC is the method of choice for the accurate determination of both the composition and the concentration of anthocyanins in a given sample (Merken and Beecher, 2000). Anthocyanins are traditionally extracted and analyzed in acidic medium as the red flavylium cation is the most stable form (Crozier *et al.,* 2009). Extracts of anthocyanins were therefore prepared from cultivars using 0.5% TFA in MeOH. HPLC equipped with a diode array detector using a reverse phase chromolith column was applied to identify and quantify the major pigments. In this study, the separation was accomplished much faster (less than four minutes) compared to anthocyanin analysis performed with other commonly used reverse phase columns due to a

faster flow rate in the chromolith column used. Our results with respect to total anthocyanin content were in accordance with previous investigations (Kulling and Rawel, 2008). Cyanidin 3-galactoside was found to be the main anthocyanin in all cultivars, with the highest content in *A. prunifolia* (497 ± 20 mg/100 g FW). Also, *A. prunifolia* had the highest content of polyphenols (2996 ± 172 mg gallic acid equivalents/100 g FW) and proanthocyanidins (4.79 g procyanidin B2 equivalents/100 g FW). In this study, the phenolic composition did vary significantly among the various aronia cultivars. They were, however, neither grown under identical conditions, nor in the same locality, nor harvested on the same date. This may have had major impacts on the results. Nevertheless, Rop *et al.* (2010) showed that differences between cultivars of aronia were caused by both their geographic origin and genetic predispositions. The results of our study indicate that berries from *A. prunifolia* constitute the richest source of anthocyanins and proanthocyanidins among the cultivars investigated and might be the species of choice for the food and herbal supplement industry, as a high content of polyphenols is often desired.

4.3 Biological activities

4.3.1 Antioxidant activity

In paper I and IV antioxidant activity was assessed using three different methods: scavenging of the DPPH radical, inhibition of 15-LO and inhibition of XO.

In paper I, crude extracts of *A. melanocarpa* "Moskva" were inactive as antioxidants, except for the 50% EtOH extract, which showed moderate DPPH radical scavenging activity levels. In contrast, fractions (Amb-MeOH and Seph I-III) of the 50% EtOH extract and isolated compounds (anthocyanins and procyanidins) possessed high radical scavenging properties, strong inhibitory activity towards 15-LO and moderate inhibitory activity towards XO. Among the anthocyanins, cyanidin 3-arabinoside possessed the strongest radical scavenging and enzyme inhibitory activities and cyanidin 3-xyloside the weakest ones. These results may indicate that the antioxidant effects are influenced by the sugar units linked to the anthocyanidin. Also, trimeric procyanidin C1 showed higher activity levels in the biological assays compared to the dimeric procyanidins B2 and B5. Yokozawa *et al.* (1998) reported that the number and position of hydroxyl (OH) groups were important factors for the scavenging of free radicals by flavonoids and showed that an increase in molecular weight enhanced the

activity of tannins. Since the number of OH groups is correlated to the molecular size of procyanidins, our findings concur with the suggested theory.

The antioxidant activities of both the 80% EtOH extracts and the acidic MeOH extracts of *A. melanocarpa* "Moskva", "Hugin", "Nero" and *A. prunifolia* were investigated (paper IV). Since there was a considerable variation in chemical composition between cultivars of aronia (see section 4.2), it was anticipated that these differences would lead to similarly varied results for antioxidant activity testing. However, only minor differences in antioxidant activity levels were found between the cultivars. In general, all extracts possessed moderate DPPH radical scavenging activity and relatively low 15-LO and XO inhibitory activity compared to the positive control quercetin (Table 1). Polyphenols are well known for their antioxidant activities, and as the extracts of *A. prunifolia* had the highest content of proanthocyanidins, anthocyanins and total polyphenols among the cultivars tested, it was expected that they would also have the highest antioxidant activity. The results showed however no correlation between the content of polyphenols in the extracts and their antioxidant activity level.

Table 1. Antioxidant activity of four cultivars of aronia berries tested by DPPH radical scavenging, inhibition of 15-lipoxygenase and inhibition of xanthine oxidase.

	DPPH	**15-LO**	**XO**
	IC_{50} (µg/mL)	% inh 83.3 µg/mL	% inh 83.3 µg/mL
80% ethanol extract 'Moskva'	35.7 ± 1.5	4.0 ± 2.7	25.5 ± 3.0
80% ethanol extract 'Hugin'	16.2 ± 0.4	10.9 ± 2.9	34.5 ± 2.0
80% ethanol extract 'Nero'	35.5 ± 1.4	8.9 ± 3.1	23.7 ± 4.1
80% ethanol extract *A. prunifolia*	21.2 ± 0.7	5.7 ± 3.4	26.3 ± 2.7
Acidified methanol extract 'Moskva'	61.4 ± 1.6	8.5 ± 4.7	2.7 ± 1.6
Acidified methanol extract 'Hugin'	39.7 ± 1.4	12.8 ± 5.7	14.7 ± 1.8
Acidified methanol extract 'Nero'	54.3 ± 1.6	17.4 ± 5.2	13.0 ± 3.8
Acidified methanol extract *A. prunifolia*	33.2 ± 1.3	14.7 ± 5.0	18.0 ± 5.5
Quercetin (positive control)	3.3 ± 0.6	26.0 ± 2.0 [1]	0.6 ± 0.1 [1]

[1] presented as IC_{50} (concentration to give 50% inhibition)

The DPPH assay is technically simple, but presents some disadvantages (Huang *et al.,* 2005). For example, the DPPH radical is a long-lived nitrogen radical, which has few similarities to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. Also, it was reported that DPPH reacts reversibly with some polyphenols, resulting in altered radical-scavenging values (Denev *et al.,* 2012; Huang *et al.,* 2005). Generally the observed DPPH radical scavenging activity of aronia substances is promising but the limitations of the testing method should be taken into account for assessing the potential impact.

Soybean lipoxygenase was used to measure the inhibition of 15-LO. Most of our knowledge on lipoxygenase structure and function originates from studies on soybean LO isoforms. In several aspects, such as reaction mechanism, kinetic parameters and iron content, the soybean 15-LO constitutes a suitable model for mammalian lipoxygenases. Several investigations have found that there is a significant correlation between inhibitory activities towards the soybean and towards the mammalian 15-LO (Utenova *et al.,* 2007 and references cited therein). However, there are other properties that differ between plant and mammalian lipoxygenases. Thus, it has been suggested that experimental data obtained with the soybean enzyme should be interpreted with care when conclusions to mammalian physiology are drawn (Kühn *et al.,* 2005).

Allopurinol, a purine analogue, is a drug used primarily to treat gout and conditions associated with hyperuricemia (Pacher *et al.,* 2006). Although allopurinol is a very efficient drug, it is a relatively weak XO inhibitor in *in vitro* assays. Therefore, one must be aware that *in vitro* inhibitory activities do not necessarily translate to *in vivo* inhibitory effects and vice versa.

While it is often believed that polyphenols such as proanthocyanidins are unspecific enzyme inhibitors due to their unspecific protein-complex binding properties, previously reported results (Le *et al*., 2012) show that the inhibition of 15-LO and the inhibition of XO by a series of polyphenols are not significantly correlated, and that complex formation between proanthocyanidins and proteins is structure-dependent (Hatano *et al*., 1990; Santos-Buelga and Scalbert, 2000).

Despite some challenges and limitations associated with *in vitro* testing, the DPPH, 15-LO and XO assays cover different aspects of the antioxidant action and the results derived from these tests give a broader view on the antioxidant potential of aronia substances.

The antioxidant activities of these substances *in vivo* would seem to be an important subject for further research. A key issue for *in vivo* activity is whether or not polyphenols reach their targets in the body in sufficient amounts to achieve the beneficial effects (Crozier *et al.,* 2009). Anthocyanins and oligomeric/polymeric proanthocyanidins from aronia appear to have low bioavailability (see section 1.2.3). Their circulating metabolites may differ greatly in structure and potential function compared to the original compounds, which may therefore not be present in sufficient amount in the bloodstream to achieve the beneficial antioxidant effects there. However, the role of antioxidants in the GIT may also be of importance (Denev *et al.,* 2012). A usual diet contains various prooxidants, including metals such as iron, copper and heme, lipid hydroperoxides, aldehydes, and nitrite. In addition, elevated levels of lipid peroxides have been observed in the postprandial state. These prooxidants may induce oxidative stress in the gastrointestinal tract, which could for example be the source of stomach ulcers and induce stomach, colon, and rectal cancers. Due to the metal chelating ability, the radical scavenging activity and other antioxidant properties of polyphenols, it may be possible to suppress such oxidative stress and oxidative stress-mediated diseases in the GIT.

Many of the colonic metabolites of the polyphenols from aronia might still have free OH groups and could maintain their antioxidant activity. When reaching the bloodstream, they may contribute to enhance plasma antioxidant capacity. Therefore, in addition to the original phenolic compounds, their metabolites should also be considered to understand the biological activities and antioxidant functions of polyphenols from aronia berries.

4.3.2 α-Glucosidase inhibitory activity

In paper I and IV, aronia substances were investigated for their abilities to inhibit the digestive enzyme α-glucosidase. The 50% EtOH crude extract, subfraction Amb-MeOH and compounds of *A. melanocarpa* "Moskva" were strong α-glucosidase inhibitors. All were more efficient than the positive control acarbose, the active compound in an anti-diabetic drug. In addition, anthocyanins were more active than the dimeric and trimeric procyanidins. Trimeric procyanidin C1 was also found to possess stronger inhibitory activity compared to the dimeric procyanidins B2 and B5. This concurs with previously reported results showing that the

activity increases with increasing molecular weight of oligomeric flavan-3-ols (Ma *et al.,* 2010). The acidic MeOH extracts of the four aronia cultivars were all much more potent than their respective 80% EtOH extracts and the positive control, indicating that anthocyanins in these extracts might be responsible for the effects (paper IV). Matsui *et al.* (2001) examined the α-glucosidase inhibitory properties of natural anthocyanin extracts and also reported that these compounds showed strong *in vitro* activities. However, McDougall *et al.* (2005) showed that the extent of inhibition is related to the anthocyanin concentration. Based on our results from paper IV, we did not find such correlation between the total anthocyanin content in the extracts and extent of α-glucosidase inhibition. As for the antioxidant activity, only minor differences between the various cultivars were found for the α-glucosidase inhibitory activity.

Since α-glucosidase is located in the brush border of the small intestine (Huang *et al.,* 2010), the inhibition of this enzyme would take place in the GIT and therefore uptake of aronia compounds into the bloodstream is not necessary. Phenolic components in the GIT may influence digestive enzymes and our results indicate that aronia substances could interfere with glucose absorption from the small intestine. However, this is dependent on the stability of the polyphenols at intestinal pH. Investigation of aronia substances on α-amylase activity would be an interesting subject for future research, since this enzyme is also involved in the digestion of carbohydrates (Coniff and Krol, 1997).

4.3.3 CYP3A4 inhibitory activity

Pharmacokinetic interactions often occur as a result of activity changes of drug-metabolizing proteins, especially cytochrome P450 isoenzymes. The inhibitory activities of aronia substances from *A. melanocarpa*, cultivar "Moskva", on CYP3A4 were investigated in paper II. Midazolam was used as the probe substrate, and recombinant insect cell microsomes expressing CYP3A4 were used as the enzyme source. In the initial screening study, a high concentration (226 µg/mL final concentration) of extracts, subfractions, and compounds was applied in order to compare their ability to inhibit CYP3A4. For the active substances (50% EtOH crude extract, subfractions Seph d–g, cyanidin 3-arabinoside and procyanidin B5), measurements were done at lower concentrations and it was found that these samples inhibit CYP3A4 in a concentration-dependent manner. Interestingly, procyanidin B5 (IC_{50} value of 44.6 ± 3.1 µg/mL for inhibition of 1′-OH MDZ) was a considerably stronger CYP3A4 inhibitor *in vitro* than the isomeric procyanidin B2 (IC_{50} value > 226 µg/mL), and comparable to bergamottin, a known CYP3A4 inhibitor from grapefruit juice. Seph g (IC_{50} value of

5.4 µg/mL for inhibition of 1´-OH MDZ) was found to contain the highest concentration of proanthocyanidins with the highest DP and possessed the strongest CYP3A4 inhibitory activity, whereas subfractions Seph e (IC_{50} value of 17.9 µg/mL) and f (IC_{50} value of 10.4 µg/mL) were found to contain less proanthocyanidins and also showed weaker CYP3A4 inhibition. Based on these observations, it appears that the proanthocyanidins in these fractions are the constituents responsible for the observed *in vitro* inhibition. Also, the inhibitory activity of proanthocyanidin-containing fractions was correlated to the degree of polymerization. Among the anthocyanins, cyanidin 3-arabinoside showed stronger CYP3A4 inhibition than cyanidin 3-galactoside and cyanidin 3-glucoside, thus suggesting that the ability to inhibit CYP3A4 *in vitro* is influenced by the sugar unit linked to the anthocyanidin.

As pointed out in section 1.2.3, proanthocyanidins with high DP and anthocyanins appear to have low bioavailability, and metabolism is a factor strongly affecting their plasma concentrations. However, since CYP3A4 is the major CYP enzyme in the intestine, with highest content in the proximal small intestine and located in the apical enterocytes, substances that are able to penetrate the intestinal epithelial cell membrane have the potential to inhibit CYP3A4 even if they have low plasma concentrations and are extensively metabolized by conjugation reactions (Dresser and Bailey, 2003). Anthocyanins, dimers and trimers of flavan-3-ols have been reported to be able to cross the apical membrane of intestinal epithelial cells, but polymerization of proanthocyanidins greatly impairs their absorption (Denev *et al.,* 2012; Hassimotto *et al.,* 2008; Steinert *et al.,* 2008; Wiczkowski *et al.,* 2010). Consequently, the clinical relevance of the present *in vitro* observations needs to be further investigated. It would be interesting to measure the concentration of these compounds in products derived from the berries (e.g. juices, jams) and investigate if the inhibition of CYP3A4 can actually be experienced *in vivo*.

4.3.4 Antimicrobial activity

The formation of biofilms on medical devices such as catheters is a well known health hazard. Once a biofilm is established, it is difficult to eliminate because the bacteria are reinforced and protected by the matrix, making them more resistant to antibiotics (Estrela and Abraham, 2010). In paper III, *A. melanocarpa* (cultivar "Moskva") extracts, subfractions and pure compounds were investigated for their abilities to prevent a biofilm formation and to inhibit bacterial growth of *Escherichia coli* and *Bacillus cereus in vitro*. The Gram-negative *E. coli* is the most common pathogen associated with urinary tract infections, and its biofilm formation

on urinary catheters is a known problem (Costerton *et al.,* 1999). *Bacillus cereus* is a Gram-positive, spore forming bacterium commonly identified as the source of food-borne diseases, but also as the cause for a number of systemic and local infections such as catheter-related bloodstream infections and pneumonia (Bottone, 2010). In general, the majority of the aronia samples displayed biofilm inhibition against the Gram-positive *B. cereus* at concentrations of 1 mg/mL, but exhibited less activity against Gram-negative *E. coli*. The 50% EtOH crude extract was the most potent inhibitor of *B. cereus* biofilm formation, most likely due to the presence of unknown active compounds in this extract, or to synergistic effects. It was initially assumed that the observed anti-biofilm activity of samples was caused by bactericidal or bacteriostatic properties towards these bacteria. However, results derived from the disk diffusion method showed that growth of the species was not affected by aronia samples. This non-toxic inhibition may confer a lower potential for resistance development compared to conventional antimicrobials.

It was planned to evaluate the activity of aronia samples against biofilm formation of two *E. coli* strains, *E. coli* K12 JM109 and uropathogenic *E.coli*. Uropathogenic *E. coli* made a very poor biofilm in this assay and good biofilm-forming conditions were not achieved for this bacterium. There are therefore no results related to this strain in this assay.

An important aspect of research on mature biofilms is the testing of the response of bacteria within a biofilm to antibiotics compared to their response in a single-cell state. However, the purpose of this study was to investigate the potential effects of aronia on the inhibition of biofilm formation with planktonic bacteria as starting material. No antibiotics were added to the system as a positive control because planktonic bacteria would be sensitive to the antibiotics (as observed in the disk assay) and therefore would not form biofilms.

A limitation of this study is that the measurements were made for only one endpoint (biomass) at a single test concentration, and therefore potency values could not established. Additional testing of other endpoints than biomass (e.g. resazurin staining for quantification of bacterial viability (Skogman *et al.,* 2012)), dose-response relationships, and also potential effects of the compounds on mature biofilms would be interesting subjects for future research.

5 Conclusions

Aronia berries already benefit from a positive image which is partly associated with the general acceptance that fruits and vegetables are an important part of the diet and a general trend towards a healthier lifestyle. In addition, in recent years, natural antioxidants have been praised for their beneficial effects on health.

This study provides a better understanding of the potential health benefits of polyphenols from aronia berries. Several constituents from aronia showed activity as antioxidants, as α-glucosidase inhibitors and displayed anti-biofilm activity against *E. coli* and *B. cereus*. It was however also found that some of these substances could inhibit the important drug-metabolizing enzyme CYP3A4. This may potentially lead to undesirable pharmacokinetic interactions.

In this study, the results were derived from *in vitro* experiments. Follow-up testing *in vivo* is necessary to validate the findings and a central subject for further research on the bioactivity of aronia.

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Papers

Paper I Bräunlich, M.; Slimestad, R.; Wangensteen, H.; Brede, C.; Malterud, K. E.; Barsett, H. Extracts, anthocyanins and procyanidins from *Aronia melanocarpa* as radical scavengers and enzyme inhibitors. *Nutrients* **2013**, *5*, 663-678.

Paper II Bräunlich, M.; Christensen, H.; Johannesen, S.; Slimestad, R.; Wangensteen, H.; Malterud, K. E.; Barsett, H. *In vitro* inhibition of cytochrome P450 3A4 by *Aronia melanocarpa* constituents. *Planta Med*. **2013**, *79*, 137-141.

Paper III Bräunlich, M.; Økstad, O. A.; Slimestad, R.; Wangensteen, H.; Malterud, K. E.; Barsett, H. Effects of *Aronia melanocarpa* constituents on biofilm formation of *Escherichia coli* and *Bacillus cereus*. *Molecules* **2013**, *18*, 14989-14999.

Paper IV Wangensteen, H.; Bräunlich, M.; Nikolic, V.; Malterud, K. E.; Slimestad, R.; Barsett, H. Anthocyanins, proanthocyanidins and total phenolics in four cultivars of aronia: Antioxidant and enzyme inhibitory effects. *Submitted to J. Funct. Foods,* **2013**.

Paper I

Extracts, anthocyanins and procyanidins from *Aronia melanocarpa* as radical scavengers and enzyme inhibitors

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Article

Extracts, Anthocyanins and Procyanidins from *Aronia melanocarpa* as Radical Scavengers and Enzyme Inhibitors

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Abstract: Extracts, subfractions, isolated anthocyanins and isolated procyanidins B2, B5 and C1 from the berries and bark of *Aronia melanocarpa* were investigated for their antioxidant and enzyme inhibitory activities. Four different bioassays were used, namely scavenging of the diphenylpicrylhydrazyl (DPPH) radical, inhibition of 15-lipoxygenase (15-LO), inhibition of xanthine oxidase (XO) and inhibition of α-glucosidase. Among the anthocyanins, cyanidin 3-arabinoside possessed the strongest and cyanidin 3-xyloside the weakest radical scavenging and enzyme inhibitory activity. These effects seem to be influenced by the sugar units linked to the anthocyanidin. Subfractions enriched in procyanidins were found to be potent α-glucosidase inhibitors; they possessed high radical scavenging properties, strong inhibitory activity towards 15-LO and moderate inhibitory activity towards XO. Trimeric procyanidin C1 showed higher activity in the biological assays compared to the dimeric procyanidins B2 and B5. This study suggests that different polyphenolic compounds of *A. melanocarpa* can have beneficial effects in reducing blood glucose levels due to inhibition of α-glucosidase and may have a potential to alleviate oxidative stress.

Keywords: *Aronia melanocarpa*; anthocyanins; procyanidins; DPPH; 15-lipoxygenase; xanthine oxidase; α-glucosidase

1. Introduction

Aronia, *Aronia melanocarpa* (Michx.) Elliott, syn. *Photinia melanocarpa* (Michx.) K. R. Robertson and J. B. Phipps, sometimes called black chokeberry, belongs to the Rosaceae family and is cultivated as a decorative shrub, as a source of berries for juices, wines and jams and as a rich source of natural food colorants [1,2]. In recent years, black chokeberries have gained popularity due to their high content of polyphenols with antioxidant activity [3]. In fact, they possess the highest antioxidant activity among berries and other fruits investigated so far as measured with the oxygen radical scavenging capacity (ORAC) assay [2,4]. The Aronia berries contain high levels of flavonoids, mostly anthocyanins and proanthocyanidins. The formation of free radicals is strongly associated with lipid peroxidation and has also been implicated in the development of a variety of diseases, including cellular aging, mutagenesis, inflammation, carcinogenesis, coronary heart disease and diabetes [5]. Accumulated evidence has suggested that diabetic patients are under oxidative stress, with an imbalance between the free radical generating and radical scavenging capacities [6–11]. 15-Lipoxygenase (15-LO) and xanthine oxidase (XO) are peroxidative and prooxidative enzymes, respectively, and sources of reactive oxygen species (ROS) in vascular cells [12,13]. An overproduction of ROS may be involved in endothelial dysfunction. Thus, substances that inhibit the production of ROS could have a positive effect on cardiovascular function. It has been suggested that *A. melanocarpa* fruit juice and its anthocyanins might be useful in the prevention and control of diabetes mellitus type II and diabetes associated complications [14,15]. α-Glucosidase, which is a membrane-bound enzyme located at the epithelium of the small intestine, plays a vital role in digestion of carbohydrates, as it catalyzes the cleavage of glucose from disaccharides and oligosaccharides. It might be possible to prevent the onset of diabetes by controlling postprandial hyperglycemia through the inhibition of α-glucosidase and α-amylase activities, resulting in a delay of carbohydrate digestion to absorbable monosaccharide [16]. Studies have revealed that anthocyanins potentially inhibit intestinal α-glucosidase, and Adisakwattana *et al*. [16] reported that cyanidin 3-rutinoside retards absorption of carbohydrates by that mechanism of action. To our knowledge, no systematic investigation on chokeberry anthocyanins and procyanidins as radical scavengers, lipoxygenase inhibitors, xanthine oxidase inhibitors and α-glucosidase inhibitors has been reported previously.

The present paper reports *in vitro* antioxidant activity of extracts, subfractions, anthocyanins and procyanidins isolated from *A. melanocarpa* measured by scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibition of the enzymes 15-LO and XO. Furthermore, the polyphenol-rich extracts were tested for their ability to inhibit α-glucosidase.

2. Experimental Section

2.1. Plant Material

Aronia berries, *A. melanocarpa* (Michx.) Elliott var. Moscow, were harvested at Særheim, Klepp, Norway (58°47′N, 5°41′E) in August 2010. The berries were kept at −20 °C until extraction. A voucher specimen (MB201201) is deposited in the Pharmacognosy section, School of Pharmacy, University of Oslo, Norway. Bark, as a source of procyanidins, was sampled from the same plants. Branches with a diameter of 1–2 cm were chosen, and the bark was carefully removed. In total 1500 g fresh weight (FW) was sampled, giving 732 g dry weight (DW) upon lyophilization. The plant material was cut in pieces and kept at −20 °C until extraction.

2.2. Chemicals

Diphenylpicrylhydrazyl (DPPH) radical, linoleic acid, 15-LO from soybeans, hypoxanthine, XO from bovine milk, α-glucosidase from baker's yeast, 4-nitrophenyl α-D-glucopyranoside (PNP-G), quercetin, acarbose, sodium potassium phosphate, benzyl mercaptan, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), ethyl acetate (EtOAc), dichloromethane (DCM), acetone, ethanol (EtOH) and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Procyanidin B2 and B5 reference compounds were obtained from Plant Polyphenols LLC (Boyce, LA, USA). All other reagents were of the highest purity available.

2.3. Extraction and Fractionation

Aronia berries (5.5 kg FW) were freeze-dried, pulverized and extracted with DCM (5 L) followed by EtOH (4.5 L) in a Soxhlet apparatus. The plant residue was further extracted by stirring with 3 × 3 L 50% EtOH at 70 °C for 2 h. All extracts were concentrated *in vacuo*, yielding 26 g, 250 g and 80 g, respectively. The 50% EtOH extract (2.5 g) was fractionated on an Amberlite XAD-7HP (3 × 30 cm; Sigma-Aldrich, St. Louis, MO, USA) column by elution with 400 mL water followed by 300 mL MeOH, 200 mL 0.1% TFA in MeOH and 200 mL acetone-water (70:30). The procedure was repeated until a total amount of 10 g had been applied on the column. The MeOH fraction (Amb-MeOH) was concentrated to dryness *in vacuo*, and a yield of 2 g was obtained. Of this, 920 mg was dissolved and further fractionated over Sephadex LH-20 (3 × 30 cm; GE Healthcare, Uppsala, Sweden) with MeOH-water (from 20:80 to 100:0) as eluent. This gave three subfractions, namely Seph I (418 mg; eluted with 20% MeOH), Seph II (44 mg; 40% MeOH) and Seph III (278 mg; 100% MeOH).

2.4. Isolation of Anthocyanins

In parallel to the work described in section 2.3., Aronia berries (2 kg FW) were extracted by maceration with 2 × 6 L MeOH (0.5% TFA v/v) for 24 h at ambient temperature and most of the solvent evaporated *in vacuo*. The concentrated water-enriched extract (0.5 L) was partitioned against EtOAc (4 × 0.5 L). The organic phase was discarded, whereas the water-phase was concentrated and purified on a bed of Amberlite XAD-7HP (5 × 50 cm column) by use of water until the eluate had

pH 6 followed by elution with 1 L MeOH (0.5% TFA). The anthocyanin-enriched extract was then eluted through a Sephadex LH-20 column (5 × 100 cm) by use of a step gradient of 15% (3 L) and 30% (4 L) MeOH (0.1% TFA v/v). Fractions of 200 mL were collected and analyzed by HPLC. Purity acceptance was defined as the analyte peak area representing >96% of total area as detected at 520 and 280 nm. Overlapping fractions were combined and reapplied to the same column, and five column runs gave approximately 200 mg cyanidin 3-galactoside (**1**), 200 mg cyanidin 3-arabinoside (**3**) and 20 mg cyanidin 3-xyloside (**4**). Only minute amounts of pure cyanidin 3-glucoside (**2**) were obtained.

2.5. Isolation of Procyanidins

Instead of using berries as a source of oligomeric procyanidins, bark was chosen, as this is a richer source and as this gave a simpler polyphenolic composition (e.g., no anthocyanins). A bark sample of 466 g DW was extracted with 2 × 10 L 70% acetone at room temperature for 24 h. After concentration *in vacuo*, the 0.5 L extract was defatted with 2 × 0.5 L DCM. The organic phase, which contained 6.4 g dry matter, was discarded. The sample was further partitioned against 3 × 0.5 L EtOAc, and the organic phase was concentrated *in vacuo*, followed by lyophilization. The EtOAc part yielded 7.8 g DW. Of this, 2 g was applied to a 5 × 100 cm column of Sephadex LH-20, and separation was achieved by use of a step gradient of 50% (2 L), 80% (3 L) and 100% (10 L) MeOH. Fractions of 1 L were collected and analyzed by thin layer chromatography (TLC) and HPLC. About 200 mg procyanidin B2 (**5**), 20 mg B5 (**6**) and 50 mg of C1 (**7**) were obtained. Purity acceptance was defined as the analyte peak area comprising >96% of total chromatographic area at 280 nm and at fluorescence detection (see section 2.6.).

2.6. Analysis

2.6.1. TLC

TLC analysis of procyanidins was performed with silica gel on polyethylene terephthalate (PET)-foils (Sigma-Aldrich, St. Louis, MO, USA) in the mobile phase system EtOAc-HCOOH-HOAc-H_2O (75:2:3:20 v/v), upper layer. Mobile-phase distance was 10 cm. Spraying with vanillic acid-HCl-MeOH (1:4:100, w/v/v), followed by heating with a hair dryer, was used for spot detection.

2.6.2. HPLC

An Agilent 1100-system, Agilent Technologies (Santa Clara, CA, USA), was used for the purity check of the individual anthocyanins, and they were identified by comparison with in-house reference compounds [17]. Separation took place over an Eclipse XDB-C8 (4.6 × 150 mm, 5 µm) column (Agilent Technologies, Santa Clara, CA, USA) by use of a binary solvent system consisting of (A) HCOOH-H_2O (1:9 v/v) and (B) HCOOH-MeOH-H_2O (10:50:40, v/v). The gradient (B in A) was isocratic, with 0% B for 2 min, linear from 0% to 70% in 18 min, from 70% to 100% for the next 2 min, from 100% to 0% in 2 min, followed by recondition of the column for 2 min. All HPLC samples were filtered through a 13 mm syringe filter (Nylon 0.45 µm, VWR International, Radnor, PA, USA) prior to injection. The flow rate was 0.8 mL/min, 10 µL samples were injected on the column and separation took place at 30 °C. Chromatograms were obtained at 280 and 520 nm.

HPLC analyses of procyanidins were performed on the same instrument and column with the solvent system (A) 0.05% TFA and (B) 0.05% TFA in MeCN. The gradient (B in A) was 5% (10 min), from 5% to 15% (10 min), from 15% to 20% (10 min), from 20% to 100% (6 min), from 100% to 5% (4 min) and finally recondition of the column for 2 min. The flow rate was 0.5 mL/min, column temperature 30 °C, and aliquots of 10 μL were injected. Fluorescence detection was achieved with excitation at 276 nm and emission at 316 nm (HP 1046A detector, Hewlett-Packard, Palo Alto, CA, USA), whereas UV-absorbance was detected at 280 nm.

2.6.3. UV Measurements

For UV measurements used in bioassay, a Biochrom Libra S32 PC (Biochrom Ltd, Cambridge, UK) was employed.

2.6.4. Thiolysis

About 10 mg crude extract was dissolved in 5% benzyl mercaptan in MeOH containing 1.1% HCl (v/v) and kept at 50 °C for 30 min. Procyanidin B1 and B2 were used as standards, with terminal units catechin and epicatechin, respectively. The products following the thiolysis reactions were analyzed by the method described for procyanidins on HPLC.

2.6.5. Mass Spectrometry

Crude extracts of berries (MeOH-extract) and bark (70% acetone), together with the isolated procyanidins, were further characterized by liquid chromatography coupled with mass spectrometry (LC-MS) using a nanoAcquity ultra performance liquid chromatography (UPLC) (Waters, Milford, MA, USA) coupled with a quadrupole time-of-flight (QTOF) micro hybrid mass spectrometer (Waters) equipped with nanoLockspray mass calibration. Reversed phase separations were achieved on a 600 mm long and 0.05 mm ID in-house prepared porous polymer monolithic column. The mobile phase consisted of (A) 0.1% formic acid and 0.05% ammonium hydroxide in water mixed with (B) 0.1% formic acid in acetonitrile. Injection of 2 μL sample diluted in water was done with a high flow rate (3 μL/min) for 5 min on a short trap column (50 mm) of a similar type as the analytical column, using a mobile phase composition of 1% B. A linear gradient from 0 to 10 min was performed by varying B from 2% to 10% at an elevated flow rate of 400 nL/min, in order to compensate for the large dwell volume. Then, the flow rate was reduced to 200 nL/min and the compounds eluted with a gradient reaching 30% of B at 30 min and 80% of B at 40 min. Electrospray ionization in the positive mode (ESI+) was used with a capillary voltage at 3 kV and a cone voltage at 35 V. Mass spectra were collected in the *m/z* range of 200–2000 with 1 s scantime. Instrument control, data acquisition and data processing were done by using MassLynx 4.1 software (Waters, Milford, MA, USA).

2.6.6. NMR

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy of extract and fractions were conducted on Varian Gemini 200 (Varian, Palo Alto, CA, USA), Bruker DPX 300 or Bruker AVII 400

(Bruker, Rheinstetten, Germany) instruments and performed in $CDCl_3$ or CD_3OD with tetramethylsilane (TMS) as an internal standard.

2.7. DPPH Radical Scavenging

Scavenging activity towards the DPPH radical was carried out as previously described [18]. Briefly, to 2.95 mL of a methanolic solution of DPPH (A_{517} 1.0), 50 µL of the test compound (dissolved in DMSO or MeOH) was added. The mixture was stirred, and the decrease in UV absorbance at 517 nm was measured over a period of 5 min. Percent radical scavenging was calculated as $100 \times (A_{start} - A_{end})/(A_{start})$, where A_{start} is the absorbance before addition of the test compound and A_{end} is the absorbance value after 5 min of reaction time. Values were corrected for absorbance of the test substances. Quercetin was used as a positive control.

2.8. Inhibition of 15-Lipoxygenase (15-LO)

Soybean lipoxygenase was used to measure inhibition of 15-LO. To a solution of linoleic acid (134 µM) in borate buffer (0.2 M, pH 9.00, 2.90 mL) was added 50 µL of test substance dissolved in DMSO or MeOH or (for blanks) DMSO or MeOH alone. A solution of 15-LO in 50 µL borate buffer (10,000 U/mL) was added, and the increase in absorbance at 234 nm from 30 to 90 s after addition was measured. Percent enzyme inhibition was calculated as $100 \times [(\Delta A_1 - \Delta A_2)/\Delta A_1]$, where ΔA_1 and ΔA_2 are values for increase in A_{234} for sample without test substance and with test substance, respectively [18]. Quercetin was used as a positive control.

2.9. Inhibition of Xanthine Oxidase (XO)

The XO inhibitory activity with hypoxanthine as the substrate was measured spectrophotometrically based on the procedure of Noro *et al.* [19], with some modifications. The assay mixture consisting of 50 µL of test compound (dissolved in DMSO or MeOH) or (for blanks) DMSO or MeOH alone, 1.85 mL of 50 mM sodium-potassium phosphate buffer (pH = 7.5) and 100 µL of enzyme solution (1.8 U/mL in 50 mM sodium-potassium phosphate buffer, pH = 7.5) was prepared immediately before use. Substrate solution (1.0 mL, 20 µg/mL hypoxanthine in distilled water) was added. The mixture was stirred, and the increase in absorbance at 290 nm was measured over a period of 5 min. Percent enzyme inhibition was calculated as $100 \times [(\Delta A_1 - \Delta A_2)/\Delta A_1]$, where ΔA_1 and ΔA_2 are values for the increase in A_{290} for the sample without test substance and with test substance, respectively. Quercetin was used as a positive control.

2.10. α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was determined by a slight modification of the procedure reported by Matsui *et al.* [20]. The enzyme solution was set at 800 mU/mL of α-glucosidase in a 50 mM phosphate buffer (pH = 7.0) containing 100 mM sodium chloride. For each assay, 20 µL of the test solution in DMSO or MeOH and 80 µL of the enzyme solution were preincubated at 37 °C for 5 min. The reaction was started by adding 1.9 mL of substrate solution (0.7 mM PNP-G in the buffer), and the solution was then incubated at 37 °C for 15 min. After the reaction had been stopped by adding

2.0 mL of a 0.5 M Tris solution, the absorbance of PNP released from PNP-G at 400 nm was measured. Percent enzyme inhibition was calculated as $100 \times (A_B - A_S)/(A_B)$, where A_B and A_S represent the absorbance of the blank and sample, respectively. Acarbose was used as a positive control.

2.11. Statistics

Samples for DPPH, 15-LO, XO and α-glucosidase assays were analyzed in triplicate and results are given as averages ± SD. Student's *t* test was used for statistical evaluation and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Extraction and Chemical Characterization

In chokeberry fruits, anthocyanins constitute the second largest group of phenolic compounds [1]. The anthocyanins in *A. melanocarpa* are mainly a mixture of four cyanidin glycosides: 3-galactoside, 3-glucoside, 3-arabinoside and 3-xyloside, of which cyanidin 3-galactoside is the main one (Figure 1) [17]. In order to isolate the major anthocyanins from Aronia berries, extraction with MeOH containing TFA was performed, since direct alcoholic extractions provide very poor yield, not keeping anthocyanins in the stable flavylium cationic form. The anthocyanins (**1**–**4**) were isolated as pure compounds, and their structures are shown in Figure 2. Their chromatographic and spectral characteristics were in agreement with previous observations [17]. Aronia berries were also extracted as shown in Figure 3.

Figure 1. High-performance liquid chromatography (HPLC) chromatogram of the isolated anthocyanins: cyanidin 3-galactoside (**1**), cyanidin 3-glucoside (**2**), cyanidin 3-arabinoside (**3**) and cyanidin 3-xyloside (**4**).

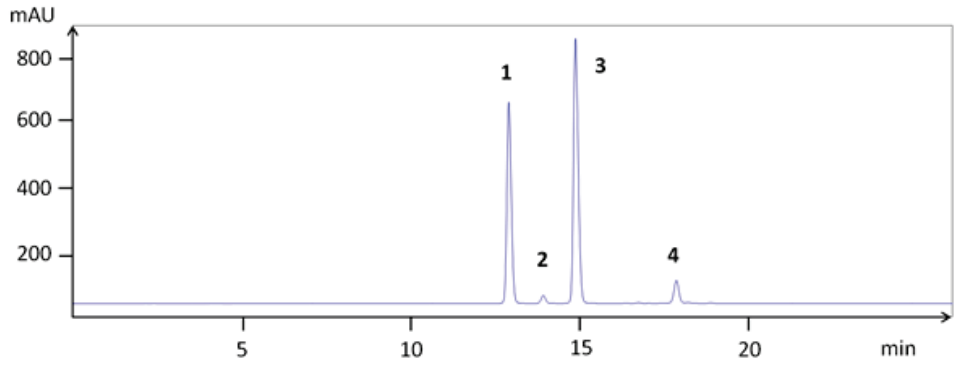
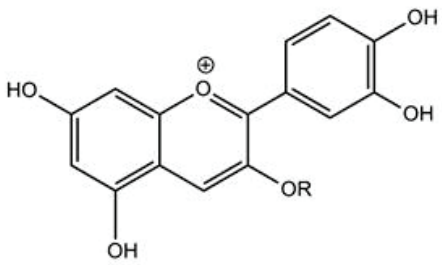
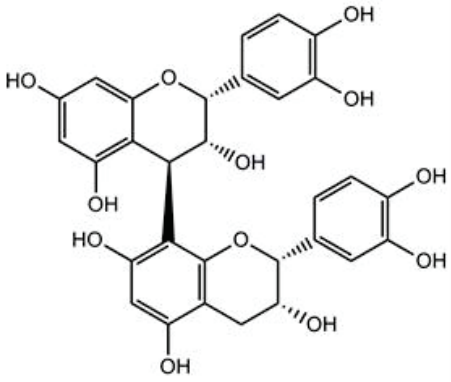


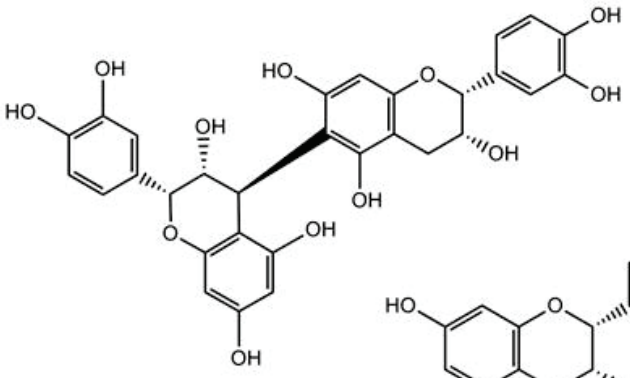
Figure 2. Chemical structures of compounds isolated from berries and bark of Aronia.



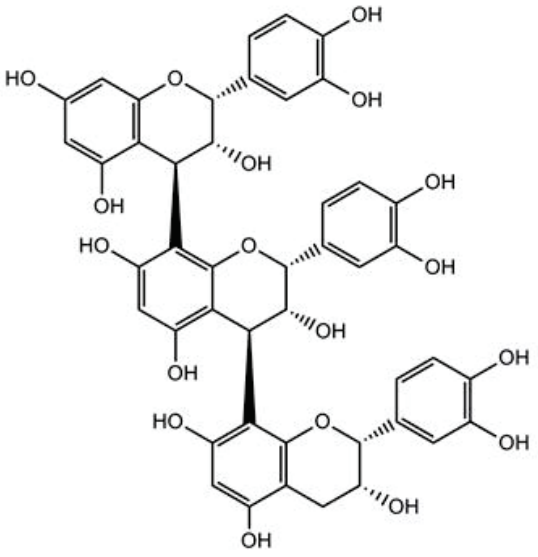
1. R = galactose: Cyanidin 3-galactoside
2. R = glucose: Cyanidin 3-glucoside
3. R = arabinose: Cyanidin 3-arabinoside
4. R = xylose: Cyanidin 3-xyloside



5. Procyanidin B2

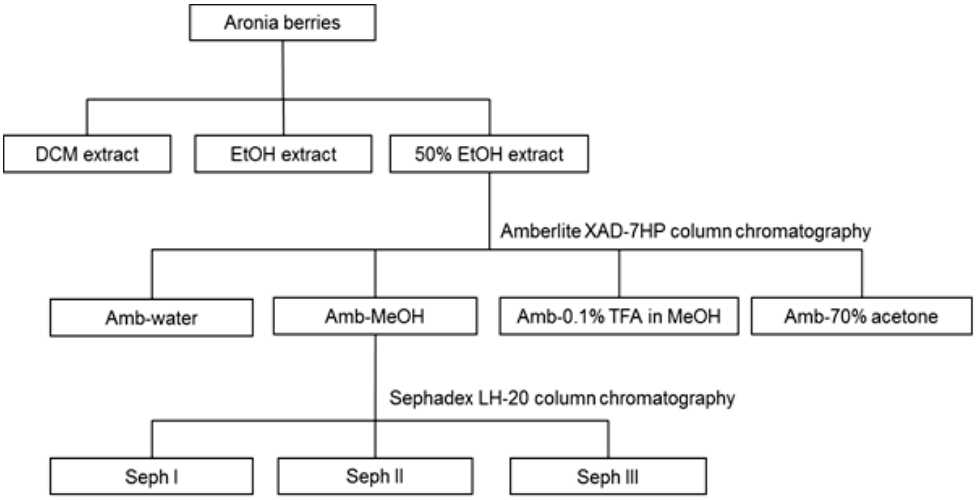


6. Procyanidin B5



7. Procyanidin C1

Figure 3. Procedure for extraction and fractionation from berries of Aronia. Anthocyanins (section 2.4.) and procyanidins (section 2.5.) were extracted by different procedures.



^{1}H NMR and ^{13}C NMR analysis revealed that proanthocyanidins were present in the 50% EtOH extract (70 °C). Subfractions of the extract were further shown to consist mainly of proanthocyanidins with epicatechin stereochemistry (^{1}H NMR: B-ring protons: *ca*. 6.8 ppm, A-ring protons: *ca*. 6.0 ppm [21]; ^{13}C NMR: C-2 at *ca*. 77 ppm, no signals at 80–82 ppm [22]). However, fraction Seph II (eluted with 40% MeOH) did not contain proanthocyanidins.

In parallel to this procedure, dimeric (**5** and **6**) and trimeric (**7**) procyanidins (Figure 2) were isolated from Aronia bark. The bark was found to be a simpler source for isolation of procyanidins compared to berries, as the complexity with respect to total phenolic structures was lower (e.g., no anthocyanins). The concentration of procyanidins was higher in Aronia bark, as well. It has previously been shown that procyanidins B2, B5 and C1 are present both in bark and berries of the Aronia plant [2]. The isolated compounds were identified by co-chromatography against authentic substances (B2 and B5) and by mass spectrometry (Figure 4, Table 1). Thiolysis of crude extracts revealed that epicatechin was the major monomeric unit of the procyanidins, both as the starter and extender unit. Only minor amounts of catechin were detected. This might be due to epimerization of epicatechin.

Figure 4. HPLC chromatogram of a bark extract of *Aronia melanocarpa* showing procyanidins B2 (**5**), B5 (**6**) and C1 (**7**).

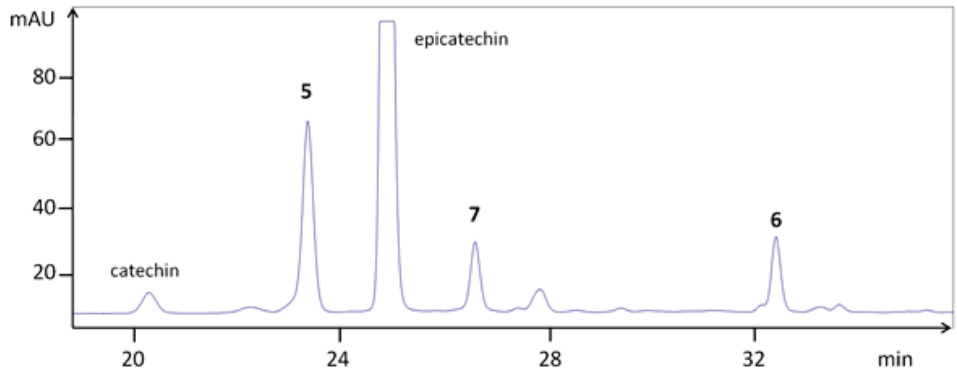


Table 1. Chromatographic and spectral characterization of anthocyanins and procyanidins from *Aronia melanocarpa.*

	Compounds	TLC rR_F	HPLC t_R (min)	LCMS t_R (min)	$[M + H]^+$	Fragments (amu)
1	cyanidin 3-galactoside		12.9	22.0	449.104	287.044
2	cyanidin 3-glucoside		13.9	22.2	449.102	287.044
3	cyanidin 3-arabinoside		15.0	22.7	419.084	287.039
4	cyanidin 3-xyloside		17.8	24.3	419.085	287.040
	epicatechin	79	25.1	28.5	291.078	
5	epi-(4β→8)-epi (B2)	58	23.4	29.9	579.157	291.078
6	epi-(4β→6)-epi (B5)	67	32.5	35.6	579.168	291.084
7	epi-(4β→8)-epi-(4β→8)-epi (C1)	51	26.7	32.7	867.216	579.143, 291.080

It has previously been reported that polymeric procyanidins, composed predominantly of (−)-epicatechin units, are the major class of polyphenolic compounds in chokeberry [1,3]. The degree of polymerization of procyanidins varies from 2 to 23 units in the fruits, with clear domination of >decamers fraction and with flavan-3-ol subunits connected mainly with C4–C6 and C4–C8 bonds (B-type bonds) [1]. Free epicatechin is also present in black chokeberries, although its concentration is significantly lower in comparison with polymeric procyanidins. Previous investigations have reported anthocyanin concentrations of 0.6%–2% (DW) and procyanidin concentrations of 4%–5% (DW) in Aronia berries [2].

3.2. Biochemical Activities

The activity of crude extracts, subfractions and isolated compounds as DPPH scavengers, 15-LO and α-glucosidase inhibitors is shown in Table 2. The 50% EtOH crude extract showed high radical scavenging activity, and the effect was strengthened in the subfractions enriched in procyanidins (Amb-MeOH, Seph I and Seph III fraction). Trimeric procyanidin (compound **7**) showed higher radical scavenging activity than the dimeric procyanidins (compound **5** and **6**). The radical scavenging

ability of compound **5** and **7** is in good accordance with the literature [23,24]. Compound **6**, however, seems to be previously unreported as a DPPH scavenger. Anthocyanins also possessed high radical scavenging capacity. IC_{50} values could not be established for compounds **1**–**4**, since an increase in sample concentration resulted in a strongly colored mixture that influenced the UV-absorbance. For this reason, percent scavenging at a sample concentration of 10.4 µg/mL was measured. Compound **1**–**3** were found to have the strongest DPPH scavenging capacity among the anthocyanins. The activity of compound **4** differed from the activity of the other anthocyanins, having the weakest DPPH radical scavenging capacity. Hence, sugar units linked to the anthocyanidin might have an influence on the biological effect. The radical scavenging activity of the anthocyanins is in fair accordance with previous investigations, although compound **3** has been reported to be slightly less active than **2** [25].

Table 2. Scavenging of the diphenylpicrylhydrazyl (DPPH) radical, 15-LO and α-glucosidase inhibitory activity of Aronia extracts, fractions and compounds.

Material	DPPH IC_{50} [a] (µg/mL) [#]/ % scavenging at 10.4 µg/mL [^]	α-Glucosidase IC_{50} [a] (µg/mL)	15-Lipoxygenase IC_{50} [a] (µg/mL)
DCM	>167 [#]	Inactive	>83
EtOH	>167 [#]	Inactive	>83
50% EtOH	25.0 ± 5.0 [#]	3.5 ± 0.1	>83
Amb-MeOH	3.8 ± 0.2 [#]	0.55 ± 0.01	56.7 ± 0.7
Seph I	3.1 ± 0.5 [#]	nt [b]	30.3 ± 0.7
Seph II	12.0 ± 2.8 [#]	nt [b]	91.0 ± 4.8
Seph III	4.0 ± 0.5 [#]	nt [b]	33.0 ± 2.0
Compound **1**	39.0 ± 2.9 [^]	1.54 ± 0.1	71.5 ± 1.8
Compound **2**	37.0 ± 0.9 [^]	0.87 ± 0.2	73.3 ± 2.1
Compound **3**	40.0 ± 0.4 [^]	0.37 ± 0.08	58.7 ± 2.5
Compound **4**	25.0 ± 5.0 [^]	5.5 ± 1.6	>83
Compound **5**	4.7 ± 0.3 [#]	4.7 ± 0.2	65.1 ± 2.6
Compound **6**	5.2 ± 0.1 [#]	5.5 ± 0.1	72.3 ± 5.7
Compound **7**	3.2 ± 0.1 [#]	3.8 ± 0.2	57.6 ± 2.0
Quercetin (control)	3.0 ± 0.2 [#]	nt [b]	26.0 ± 2.0
Acarbose (control)	nt [b]	130.0 ± 20.0	nt [b]

[a] IC_{50}: Concentration to give 50% scavenging or inhibition; [b] nt: Not tested; DCM: dichloromethane.

The 50% EtOH crude extract, the Amb-MeOH fraction and compound **1**–**7** showed high activity in the α-glucosidase assay compared to the positive control acarbose, an anti-diabetic drug. In addition, the purified anthocyanins were more active than the isolated dimeric and trimeric procyanidins (compound **5**–**7**). It is known that some anthocyanin extracts from plants exert a potent *in vitro* α-glucosidase inhibitory effect [26]. Also, McDougall *et al.* [27] found that the extent of inhibition of α-glucosidase is related to the anthocyanin content in different soft fruits. Among the anthocyanins, compounds **2** and **3** showed the highest activity and compound **4** the lowest. The activity of substance **1** is consistent with the literature [28]. To our knowledge, α-glucosidase inhibitory activity of substances **2**–**7** has not been reported previously. Ma *et al.* [29] showed that the α-glucosidase inhibitory activity of flavan-3-ol monomer and oligomers increased as the molecular weight increased,

with a significant difference in potency between the strongest ones (pentamers) and the weakest one (monomer). The Amb-MeOH fraction appeared to contain polymeric procyanidins, and this could explain its strong effect towards α-glucosidase. Trimeric procyanidin (compound **7**) possessed stronger α-glucosidase inhibitory activity compared to the dimeric procyanidins (compound **5** and **6**). It appeared that the activity increased with increasing molecular weight, which is in good accordance with previously reported results [29]. For the anthocyanins, we found a highly significant correlation between α-glucosidase inhibition and DPPH radical scavenging activity ($p < 0.005$, $R^2 = 0.997$). This is in good accordance with the literature [6]. For the crude extracts, the Amb-MeOH fraction and the procyanidins, the correlation was not significant. The Sephadex LH-20 fractions (Seph I–III) could not be tested for α-glucosidase inhibitory activity due to lack of material.

The Amb-MeOH fraction showed high inhibitory activity toward 15-LO, and the effect was strengthened in the subfractions enriched in procyanidins (Seph I and Seph III fraction). Differences in activity between isolated anthocyanins and procyanidins were relatively small. Both groups of compounds possessed high 15-LO inhibitory ability, with compound **3** and **7** being the most active ones. The 15-LO inhibition of **1**, **2**, **3** and **7** is in accordance with previous investigations [23,30]. Substances **4**, **5** and **6**, however, seem to be previously unreported as 15-LO inhibitors.

The activity of crude extracts, subfractions and isolated compounds as XO inhibitors is presented in Table 3.

Table 3. Xanthine oxidase inhibitory activity of extracts, fractions and compounds from Aronia berries.

Material	% inhibition at a concentration of 42 µg/mL
DCM	Inactive
EtOH	Inactive
50% EtOH	Inactive
Amb-MeOH	26.3 ± 3.4
Seph I	32.2 ± 7.8
Seph II	19.9 ± 7.6
Seph III	46.5 ± 5.9
Compound **5**	12.7 ± 2.8
Compound **6**	6.6 ± 0.7
Compound **7**	15.5 ± 1.5
	% inhibition at a concentration of 17 µg/mL
Compound **1**	11.9 ± 4.4
Compound **2**	20.9 ± 3.4
Compound **3**	39.1 ± 2.1
Compound **4**	11.4 ± 2.8

The Amb-MeOH fraction possessed modest activity in the XO assay, and the effect was again strengthened in the subfractions enriched in procyanidins (Seph I and Seph III fraction). Due to absorbance above the upper detection limit of the spectrometer (sample concentrations >42 µg/mL for crude extracts, subfractions and procyanidins and sample concentrations >17 µg/mL for anthocyanins), higher concentrations of extracts and compounds could not be tested. Compound **7** was the strongest inhibitor among the isolated procyanidins, and compound **3** was the strongest among the anthocyanins.

However, all were less efficient than the positive control quercetin (IC_{50} 0.6 ± 0.1 μg/mL). To our knowledge, inhibition of XO of Aronia berry extracts and substances **1**, **3**, **4** and **6** have not been reported previously. The 50% EtOH crude extract showed no inhibitory activity toward XO.

Both the DCM and the EtOH crude extract were inactive as DPPH radical scavengers, 15-LO, XO and α-glucosidase inhibitors. Among the isolated anthocyanins, compound **3** possessed the strongest and compound **4** the weakest radical scavenging and enzyme inhibitory activity. These effects seem to be influenced by the sugar units linked to the anthocyanidin. Trimeric procyanidin (compound **7**) showed higher activity in the biological assays compared to the dimeric procyanidins (compounds **5** and **6**), and it appeared that the activity increased with increasing molecular weight. In addition, there was a difference in activity between the two dimeric procyanidins, with compound **5** being the most active one. Reactive oxygen species can be generated by the prooxidative enzyme, XO, and the peroxidative enzyme, 15-LO, in vascular cells [31]. Components isolated from Aronia berries demonstrated inhibitory activity towards 15-LO and XO and may have a potential to alleviate oxidative stress. Until recently, anthocyanins were believed to have a very low bioavailability, but it has been demonstrated that the bioavailability of anthocyanins was underestimated [32,33]. In addition, anthocyanins are some of the few polyphenols that can be detected unmetabolized (e.g., as glycosides) in plasma [32]. It has to be taken into consideration that the bioavailability of flavanols varies depending on the degree of polymerization. Low molecular weight oligomeric procyanidins (DP ≤ 3) are absorbed intact in the gastrointestinal tract, but polymerization greatly impairs intestinal absorption [32–34]. In order to act as 15-LO and XO inhibitors *in vivo*, constituents have to be absorbed from the gastrointestinal tract. In view of the known bioavailability of tested compounds, Aronia products and extracts containing anthocyanins and oligomeric procyanidins (DP ≤ 3) may have biologically relevant 15-LO and XO effects. Inhibition of α-glucosidase delays carbohydrate digestion and the absorption of monosaccharides from the intestine [16]. Fractions enriched in procyanidins, the purified procyanidins and anthocyanins from *A. melanocarpa* berries were potent α-glucosidase inhibitors, suggesting that they may have beneficial effects in reducing blood glucose level. In order to act as α-glucosidase inhibitors *in vivo*, compounds do not have to be absorbed from the gastrointestinal tract, since it is a membrane-bound enzyme located at the epithelium of the small intestine [20]. Therefore, anthocyanins and procyanidins with even a high degree of polymerization may exert local effects in the gastrointestinal tract as α-glucosidase inhibitors.

4. Conclusions

We have shown that some extracts, fractions and constituents from *A. melanocarpa* possess activity of potential health benefits as radical scavengers, 15-LO inhibitors, XO inhibitors and α-glucosidase inhibitors *in vitro*. The difference in activity between the trimeric procyanidin C1 and the dimeric procyanidins B2 and B5 has, to our knowledge, not been reported previously. Also, it was found that the activity seems to be influenced by the sugar units linked to the anthocyanidin. No systematic investigations of *A. melanocarpa* extracts and fractions as radical scavengers, 15-LO inhibitors, XO inhibitors and α-glucosidase inhibitors has been reported previously. The activities of these extracts, fractions and compounds *in vivo* would seem to be an important subject for further research. The presence of biologically active compounds in Aronia berries increases the nutritional value of this

plant, as a rich source of radical scavengers and inhibitors of peroxidative and prooxidative enzymes (15-LO, XO) and of α-glucosidase, an enzyme which may be involved in diabetes. This would appear to be of interest to the food industry.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Paper II

In vitro inhibition of cytochrome P450 3A4 by *Aronia melanocarpa* constituents

Paper III

Effects of *Aronia melanocarpa* constituents on biofilm formation of *Escherichia coli* and *Bacillus cereus*

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Article

Effects of *Aronia melanocarpa* Constituents on Biofilm Formation of *Escherichia coli* and *Bacillus cereus*

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Abstract: Many bacteria growing on surfaces form biofilms. Adaptive and genetic changes of the microorganisms in this structure make them resistant to antimicrobial agents. Biofilm-forming organisms on medical devices can pose serious threats to human health. Thus, there is a need for novel prevention and treatment strategies. This study aimed to evaluate the ability of *Aronia melanocarpa* extracts, subfractions and compounds to prevent biofilm formation and to inhibit bacterial growth of *Escherichia coli* and *Bacillus cereus in vitro*. It was found that several aronia substances possessed anti-biofilm activity, however, they were not toxic to the species screened. This non-toxic inhibition may confer a lower potential for resistance development compared to conventional antimicrobials.

Keywords: *Aronia melanocarpa*; biofilm formation; *Escherichia coli*; *Bacillus cereus*; flavonoids

1. Introduction

Bacterial biofilms are sessile communities embedded within a self-produced matrix of extracellular polymeric substance (EPS) that are ubiquitous in natural, medical, and engineering environments [1,2]. Biofilms formed by pathogenic Gram-negative *Escherichia coli* strains can pose serious problems to human health, such as prostatitis, biliary tract infections, and urinary catheter cystitis [3]. Deleterious biofilms are also problematic in industry since they can cause fouling and corrosion in systems such as heat exchangers, oil pipelines, and water systems [1]. *Bacillus cereus* is a Gram-positive, spore forming bacterium closely related to the human and animal pathogen *Bacillus anthracis*, the cause of anthrax. *B. cereus* is frequently identified as the causative agent of food-borne diseases. As such, the interest in this bacterium is growing. This ubiquitous organism can easily contaminate food production or processing systems and forms biofilms that are highly resistant to cleaning procedures [4,5]. In addition to food poisoning, *B. cereus* strains have the potential to cause a number of systemic and local infections in both immunologically compromised and, although less frequent, immunocompetent individuals. Certain groups of individuals are more commonly infected, including neonates, intravenous drug abusers, and patients with traumatic or surgical wounds, or with indwelling catheters. The disease spectrum is wide, including (but not limited to) catheter-related bloodstream infections, central nervous system (CNS) disease (meningitis and brain abscesses), endophthalmitis, and pneumonia [6].

Bacteria in a biofilm are often responsible for reoccuring symptoms and medical treatment failure [7,8]. Biofilm infections are difficult to eradicate because the genetic program (global gene expression pattern) of bacteria within such a structure is fundamentally changed, resulting in increased protection against e.g., macrophages and antibiotics, compared to planktonic (free living) cells [9]. Thus, the eradication of *E. coli* biofilms required 220 times higher antibiotic concentrations than for the same strain in serum. Also, the fact that many cells in a biofilm live for extended periods without going through cell division, contributes to resistance toward antibiotics, which are primarily effective on dividing cells [8]. In addition, some protection may be conferred by the physical barrier provided by the presence of the EPS that covers the biofilm and may prevent sufficient antibiotic exposure to kill the cells. Hence, novel antagonists with the potential to remove and/or prevent the formation of biofilms are needed. Agents which do not directly inhibit bacterial growth may confer a lower selection pressure for resistance development [1,10]. Recently, there has been a tremendous increase in biofilm research. An important focus of this research has been the development of alternative approaches, either to avoid the use of antimicrobials altogether, or to combine alternative treatments with more traditional antimicrobial drugs with the potential to totally eliminate biofilm formation on e.g., indwelling devices [11].

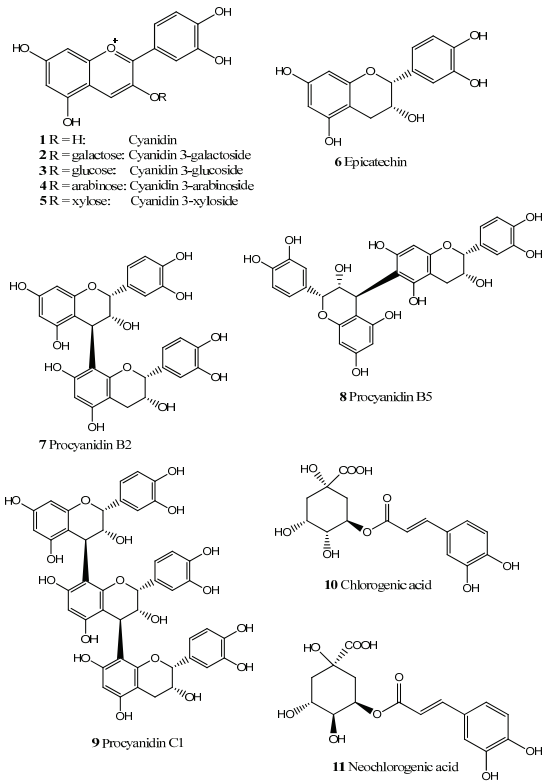
The aronia plant (*Aronia melanocarpa* (Michx.) Elliott var. Moscow (Rosaceae)) has gained popularity in recent years due to its berries with a high content of polyphenols with antioxidant activity [12,13]. Aronia products are used as nutritional supplements, and the berries are an important source for juices, wines, and jams, and constitute a rich source of natural food colorants [12,14]. Aronia berries contain high levels of flavonoids, mostly proanthocyanidins and anthocyanins. Also, chlorogenic and neochlorogenic acids are known constituents [15].

Here we present the first study investigating the effects of aronia constituents on biofilm formation, complementing earlier studies investigating the antimicrobial activity of aronia berry extracts against *Staphylococcus aureus*, *Escherichia coli*, and type A influenza virus [16]. The aim of the present study was to screen extracts, subfractions and compounds from *A. melanocarpa* for their abilities to inhibit biofilm formation of *E. coli* and *B. cereus in vitro*. Effects on preformed biofilms were not investigated. This study represents a continuation of our research on the bioactivity of aronia [17,18].

2. Results and Discussion

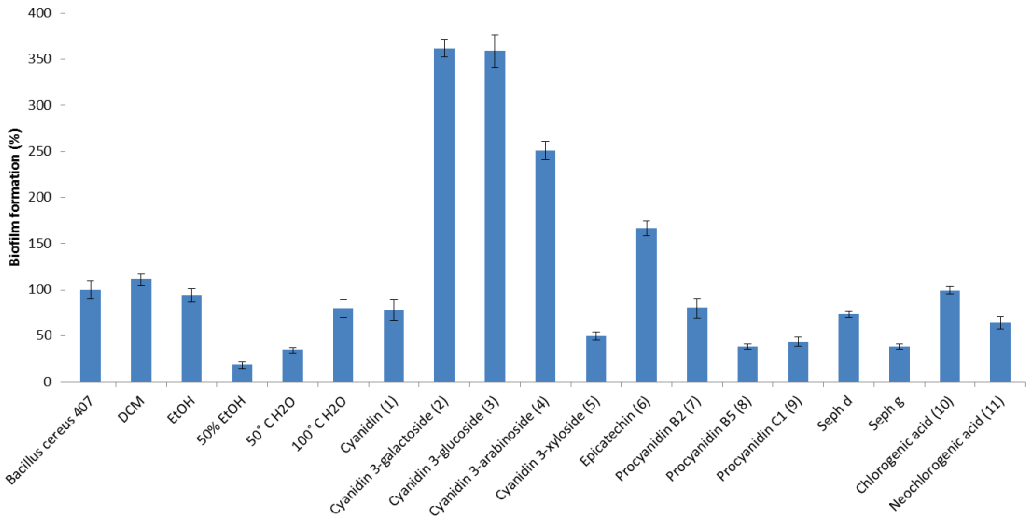
Aronia berries were extracted with dichloromethane (DCM), 96% EtOH, 50% EtOH, and H_2O as previously described [17]. ^{1}H-NMR and ^{13}C-NMR analyses revealed that proanthocyanidins were present in the 50% EtOH extract, which was further fractionated on a Sephadex LH-20 column to yield subfractions Seph a–g. Seph d and Seph g were chosen for further studies since their proanthocyanidin compositions had been well characterized. Seph d contained only trace amounts of proanthocyanidins with an average degree of polymerization (DP) of seven, whereas the majority of proanthocyanidins, with an average DP of 34, was found in Seph g. The proanthocyanidins in the subfractions were found to contain epicatechin as the monomeric unit. Also, compounds **1**–**11**, all well known constituents in aronia berries [14,15], were included in this study (Figure 1). Isolated compounds were identified on the basis of their chromatographic and spectroscopic data (TLC, HPLC, LC-MS) and optical rotations [17].

Figure 1. Chemical structures of compounds **1**–**11**.



Due to the growing interest in antimicrobial agents that can prevent or treat infections caused by biofilm-forming bacteria, aronia extracts, subfractions and compounds were tested for their ability to prevent biofilm production of two clinically relevant bacterial pathogens, *B. cereus* and *E. coli*, at sample concentrations of 1 mg/mL (125 µg per well) in a microtiter plate assay that constitutes a long-established method of measuring biofilm formation *in vitro* [19,20]. Maximum biofilm mass employing this assay was observed after 48 h of incubation for *B. cereus* [21] and after 24 h for *E. coli* (data not shown). These incubation times were therefore applied to investigate the abilities of aronia samples to inhibit biofilm formation. Polyvinyl chloride (PVC) microtitre plates were chosen in this assay since this material is often used in the manufacturing of medical devices, such as catheters [22]. The majority of aronia samples displayed biofilm inhibition against the Gram-positive *B. cereus* strain 407 (Figure 2), but exhibited less activity against Gram-negative *E. coli* JM109 (Figure 3). However, two samples (DCM extract and compound **6**) that showed no anti-biofilm activity against *B. cereus*, were effective against *E. coli*. In fact, compound **6** was the most active against biofilm-producing *E. coli*, whereas the 50% EtOH extract displayed the most potent inhibition of biofilm formation against *B. cereus*.

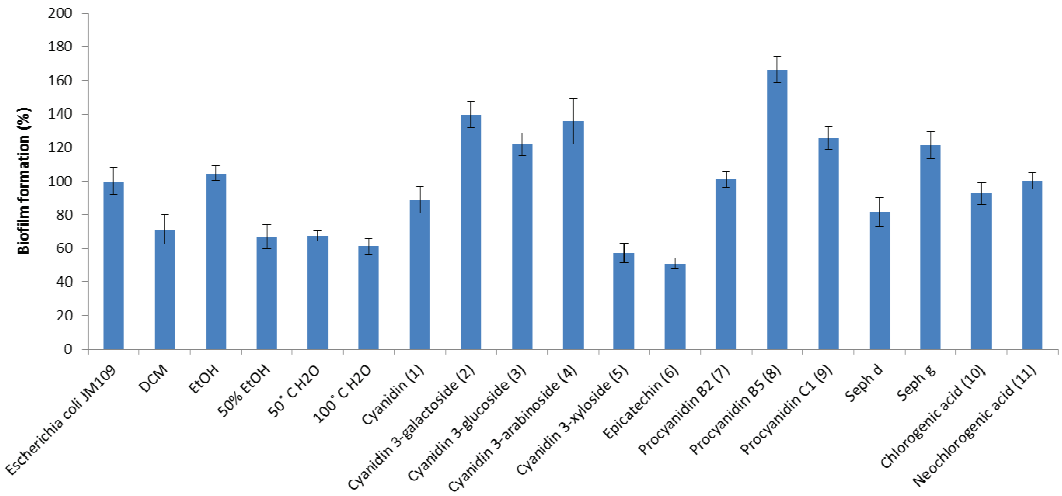
Figure 2. Biofilm formation of *B. cereus* strain 407 incubated with crude extracts, subfractions and compounds from aronia, compared to a non-treated control (*Bacillus cereus* 407, first bar from left) for which average biofilm formation was set to 100%.



Among the anthocyanins, compounds **2**–**4** induced biofilm mass production of the bacteria, however, when *B. cereus* 407 and *E. coli* JM109 were grown in the presence of compound **5**, a 50% decrease in biofilm formation was observed. The cyanidin aglycone **1** possessed weak anti-biofilm activity against both bacteria. Thus, the ability to inhibit biofilm formation appears to be influenced by the sugar unit linked to the anthocyanidin. The largest variations in activity between the Gram-positive and Gram-negative bacterium were observed for the procyanidins. For *B. cereus*, monomeric epicatechin (**6**) increased biofilm mass, but dimeric (compounds **7** and **8**), trimeric (compound **9**) and polymeric (Seph d and Seph g) procyanidin fractions were effective in reducing biofilm production.

Since procyanidin B5 (dimer), C1 (trimer) and fraction Seph g (polymer) appear about equally active, there is no clear correlation between degree of polymerization and inhibition of biofilm formation. On the contrary, monomeric epicatechin decreased biofilm mass of *E. coli*, whereas dimeric, trimeric and polymeric (except for Seph d) procyanidins increased its mass. Interestingly, we observed a considerable difference in anti-biofilm activity between procyanidin B2 (**7**) and the isomeric procyanidin B5 (**8**), indicating that minor structural differences might influence the ability to inhibit biofilm formation. Also, it was found that neochlorogenic acid (**11**) was more active as a biofilm inhibitor against *B. cereus* than the isomeric chlorogenic acid (**10**). However, their effects against *E. coli* were negligible.

Figure 3. Biofilm formation of *E. coli* JM109 incubated with crude extracts, subfractions and compounds from aronia, compared to a non-treated control (*Escherichia coli* JM109, first bar from left) for which average biofilm formation was set to 100%.



The antibacterial activity of aronia substances against biofilm-forming *E. coli* JM109 (K12 strain) and *B. cereus* 407, and the uropathogenic *E. coli* strain CFT073 (which formed biofilm very poorly) was investigated by the disk diffusion method. Growth of the two *E. coli* strains was not affected by any aronia substance, and only 1.0 mg of the 50% EtOH extract showed some activity against *B. cereus* with a zone diameter of 10.2 ± 0.8 mm. However, it was much less efficient than the positive control gentamicin (10 µg) where a zone diameter of 20.5 ± 0.7 mm was observed. Hence, the inhibition of biofilm formation does not seem to be caused by toxicity towards the bacterial strains. Aronia samples might inhibit biofilm formation by interfering with quorum sensing, chemotaxis and/or motility genes that confer on cells a degree of motility in excess of that allowing adequate biofilm formation, as reported for *E. coli* K-12 exposed to ursolic acid by Ren *et al*. [1]. Investigation of the exact mechanism of this non-toxic inhibition, and also of potential effects of the compounds on already formed biofilms, would seem to be important subjects for further research. It has previously [2,23] been suggested that stress induced by the presence of the test substance might lead to increased production of EPS by the bacterial cell. This might be involved in the increase of biomass observed for some of the tested substances. At present, however, it is not clear why some of the test substances have

this effect, while others do not. Conceivably, there may exist a balance between anti-biofilm activity (such as disturbance of quorum sensing) and biofilm stimulating activity, and as shown in our study, this balance would seem to be dependent on minor differences in chemical structure. In our experiments, blanks without bacteria added to the growth medium served as negative controls for biofilm formation. We also included controls without aronia sample added, as negative controls for biofilm inhibition. This study was designed to investigate inhibition of biofilm formation, starting out from planktonic cultures. Thus, no antibiotics were added to the assay system, as the sensitivity of the planktonic bacteria to the antibiotics would not allow growth and formation of a biofilm structure. Future experiments may include testing the chemotolerance of cells within a biofilm, by adding antibiotics to a pre-formed biofilm.

The prevalence of bacterial pathogens living in biofilms, where they are much more resistant to antibiotics and clearance by the immune system compared to planktonic cells, has promoted the search for new strategies to control biofilm infections [9]. Previous studies have included the influence of cranberry extracts on the formation of oral biofilms [24,25], on biofilm produced by *Staphylococcus epidermidis* on contact lenses [26], and by cranberry juice on biofilm produced by uropathogenic *E. coli* strains [27,28]. These studies confirm that cranberry juice can lead to a decrease in the ability of pathogenic bacteria to develop biofilm on inert surfaces such as urinary catheters and contact lenses. Cranberry contains A-type proanthocyanidins, which have been implicated as active constituents responsible for its bacterial anti-adhesive properties that prevent the attachment of *E. coli* onto uroepithelial cells, thereby preventing urinary tract infections [29]. However, it has previously been reported that cranberry juice is more effective than isolated cranberry proanthocyanidins alone in preventing biofilm formation [22,29]. In our study, the 50% EtOH extract was the most potent inhibitor of *B. cereus* biofilm formation. This might be due to the presence of unknown active compounds in this extract, or to synergistic effects. Against *E. coli* biofilm, epicatechin was the most effective substance tested, while effects of oligomeric and polymeric proanthocyanidins were negligible. Interestingly, cyanidin 3-xyloside showed activity against Gram-negative *E. coli* and Gram-positive *B. cereus*, whereas the other anthocyanins were inactive. Recently, a large number of flavonoids have been investigated for anti-biofilm activity towards *Staphylococcus aureus* [30]. In that study, flavones, chalcones, flavonols, flavans, flavanones, isoflavonoids, neoflavonoids and dihydroflavonols were studied. Our work on catechins, anthocyanins and procyanidins would therefore seem to extend the range of active flavonoids. The results presented underscore the need for more research on the anti-biofilm effect of berries and their constituents. This work was intended to investigate whether aronia extracts and constituents can inhibit formation of biofilms. Other aspects such as study of other endpoints than biomass, dose-response relationships, and bacterial viability would be highly relevant subjects for future research.

3. Experimental

3.1. General

Two Gram-negative *Escherichia coli* strains (*E. coli* K12 JM109, and uropathogenic *E. coli* CFT073 (ATCC 700928)) and one Gram-positive *Bacillus cereus* strain (*B. cereus* 407 wild-type [31])

were used in the present study. For each organism, frozen glycerol (20%) stocks in lysogeny broth (LB) medium were prepared and maintained at −80 °C. A HTS 7000 Plus Bio Assay Reader (Perkin Elmer, Waltham, MA, USA) was used for UV-Vis measurements during the biofilm screening procedure. Gentamicin antimicrobial susceptibility test disks were obtained from Oxoid Ltd., (Basingstoke, UK). Epicatechin (**6**), chlorogenic acid (**10**) and neochlorogenic acid (**11**) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Plant Material

Aronia berries (*Aronia melanocarpa* (Michx.) Elliott var. Moscow (Rosaceae)) were harvested at Bioforsk Vest Særheim, Klepp, Norway (58°47'N, 5°41'E) in August 2010 and identified by one of the authors (R. Slimestad). The berries were kept at −20 °C until extraction. A voucher specimen (MB201201) is deposited in the Pharmacognosy section, School of Pharmacy, University of Oslo, Norway. Bark, as source of procyanidins, was sampled from the same plants. Branches with a diameter of 1–2 cm were chosen, and the bark was carefully removed. The plant material was cut in pieces and kept at −20 °C until extraction.

3.3. Extraction, Fractionation and Isolation

Aronia berries were extracted with DCM and 96% EtOH, 50% EtOH, and H_2O followed by chromatography of the 50% EtOH extract to give subfractions Seph a–g, as previously described [17]. In parallel to this, cyanidin aglycone (**1**), four anthocyanins: cyanidin 3-galactoside (**2**), cyanidin 3-glucoside (**3**), cyanidin 3-arabinoside (**4**) and cyanidin 3-xyloside (**5**) and three procyanidins: procyanidin B2 (**7**), procyanidin B5 (**8**) and procyanidin C1 (**9**) were isolated from aronia berries (compounds **1**–**5**) and bark (compounds **7**–**9**). The detailed protocol for compound purification has been described previously [18]. Cyanidin was prepared by acidic hydrolysis of cyanidin 3-galactoside. Cyanidin 3-galactoside (about 100 mg) was dissolved in MeOH (0.5% HCl, 5 mL) and mixed with 2 M HCl (5 mL). Hydrolysis occurred in a capped glass tube at 100 °C for 30 min. Cyanidin was isolated from the mixture by elution with 50% MeOH (0.1% HCl) over a bed of Sephadex LH-20 (3 × 40 cm Pyrex column). Purity was determined to be above 97% by HPLC [18].

3.4. Microtiter Plate Biofilm Formation Assay

Biofilm screening was performed essentially as in Auger *et al*. [4], with some modifications. Microorganisms were streaked from frozen glycerol stocks (−80 °C) onto LB agar plates and incubated overnight (30 °C for *B. cereus*, 37 °C for *E. coli*). After growth on solid medium, an isolated colony was picked and inoculated in 5 mL LB medium for 18 h, at 30 °C for *B. cereus* and 37 °C for *E. coli*, under constant agitation at 225 rpm. Precultures were prepared by inoculating 50 µL of the 18 h culture in 5 mL LB medium (1:100), and incubated as before for 3 h. Further, 6 µL of the preculture was diluted in 1 mL fresh bactopeptone medium and 200 µL of test substance (dissolved in H_2O or 10% MeOH in H_2O) was added. 125 µL of each diluted culture was transferred to eight wells of a 96-well PVC microtiter plate (Falcon 353911). In each plate, eight wells were used as blanks, containing bactopeptone medium only. For coloured samples, blanks contained bactopeptone medium

and the respective substances. After incubation for 48 h at 30 °C for *B. cereus* and 24 h at 37 °C for *E. coli*, which constituted the incubation times for which biofilm formation had been determined to be at its maximum for the given experimental set up, the biofilm density was measured as follows: the microtiter plate wells were washed once with phosphate-buffered saline (PBS) in order to remove non-adherent bacteria. Bacterial cells bound to the walls of the wells were stained with a 1% (w/v) crystal violet solution at room temperature for 20 min. The wells were then washed three times with PBS, followed by solubilization of the dye in an acetone/ethanol (1:4) mixture by slowly and continuously pipetting up and down three times to ensure extraction of the dye from cells. The mixture was immediately transferred to a transparent flat-bottomed microtiter plate and the absorbance at 492 nm of the solubilized dye was subsequently determined. *E. coli* K12 JM109 and *B. cereus* 407 were good biofilm-forming strains. Uropathogenic *E. coli* CFT073 was tested, but made a very poor biofilm under the same conditions. Screening studies of the various test samples on biofilm inhibition were carried out as three individual experiments, with eight technical replicates for each experiment, and the results are presented as averages ± SEM. Test sample concentrations of 1 mg/mL (125 µg per well) were applied. Samples dissolved in 10% MeOH in H_2O had a total MeOH concentration of 1.7% during incubation.

3.5. Antimicrobial Disk Susceptibility Test

Antimicrobial activity was determined according to the guideline *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition* (CLSI document M2-A9) [32]. A small amount of isolated colonies from 18 to 24 h old pure culture was suspended in sterile saline and the turbidity of this suspension was adjusted to a 0.5 McFarland standard. A dipped swab was then used to inoculate a Mueller-Hinton agar plate. Antimicrobial susceptibility blank disks (BBL, 6 mm in diameter), containing either 1.0 mg (50 µL, 20 mg/mL) and 125 µg (50 µL, 2.5 mg/mL) crude extract or 125 µg of compounds **1–11** and fractions Seph d and Seph g, were placed on the agar surface together with a positive control disk (gentamicin 10 µg) and a negative control disk containing 50 µL solvent (H_2O or MeOH). Culture plates were incubated for 18 h at 37 °C, and zone diameters were recorded. All tests were performed in triplicate.

4. Conclusions

The presence of biofilms on medical devices and their role in infections is well known. The tolerance of catheter-associated biofilm-forming organisms toward antimicrobial treatments in the health care environment underscores the importance of alternative treatment strategies. In the present study, we have shown that exposure of biofilm-forming *E. coli* and *B. cereus* strains to several aronia constituents reduced biofilm production. Whether our results can be used in future development of biofilm inhibitors requires further investigation.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.

Paper IV

Anthocyanins, proanthocyanidins and total phenolics in four cultivars of aronia: Antioxidant and enzyme inhibitory effects

Anthocyanins, proanthocyanidins and total phenolics in four cultivars of aronia: Antioxidant and enzyme inhibitory effects

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ABSTRACT

Aronia berries are known for their high content of anthocyanins and proanthocyanidins which contribute to their high antioxidant potential. In the present study, four different cultivars of aronia berries, A*ronia melanocarpa* 'Moskva', 'Hugin', 'Nero' and *Aronia prunifolia*, have been studied with respect to phenolic composition, antioxidant and enzyme inhibitory activities. Cyanidin 3-galactoside was found to be the major anthocyanin in all cultivars, with the highest content in *A. prunifolia* (497 ± 20 mg /100 g FW). *A. prunifolia* was also found to have the highest content of polyphenols (2996 ± 172 mg gallic acid equivalents/100 g FW) and proanthocyanidins (4.79 g procyanidin B2 equivalents/100 g FW). As antioxidants and enzyme inhibitors, the differences between extracts from the tested berries were minor. The results indicate that berries from *A. prunifolia* constitute the richest source of polyphenols and might be the species of choice in order to attain berries with a high content of anthocyanins and proanthocyanidins.

Keywords*: Aronia melanocarpa*; *A. prunifolia;* anthocyanins; proanthocyanidins; antioxidants; α-glucosidase

Abbreviations: DPPH: 1,1-diphenyl-2-picrylhydrazyl; FW: fresh weight; 15-LO: 15-lipoxygenase; PNP-G: para-nitrophenyl-α-D-glucopyranoside; XO: xanthine oxidase

1. Introduction

In recent years, a substantial interest in fruits and berries has developed due to their potential favorable health effects and their high content of polyphenols as flavonoids and anthocyanins (Miller & Shukitt-Hale, 2012; Tsuda, 2012; Wallace, 2011). The anthocyanins are a subgroup of the flavonoids, and they occur naturally in plants as glycosides. The anthocyanins contribute to the blue, red and purple color in many plant species. The aglycone is called anthocyanidin, and six principal types of anthocyanidins exist depending on the substituents on the B-ring: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. Anthocyanins have been suggested to have positive effects against obesity, diabetes and cardiovascular disease, as well as to have a positive effect on cognitive function (Miller & Shukitt-Hale, 2012; Tsuda, 2012). These effects have been attributed to the high antioxidant capacity of the anthocyanins, and the effects can more precisely be ascribed to molecular mechanisms, such as scavenging of free radicals, inhibition of radical forming and peroxidative enzymes, upregulation of antioxidant enzymes, regulation of signaling pathways and vasodilatory mechanisms (Mladenka, Zatloukalová, Filipský, & Hrdina, 2010; Sies, 2010).

Proanthocyanidins or condensed tannins are oligomeric and polymeric compounds which consist of flavan-3-ols as monomeric units (Aron & Kennedy, 2008). The compounds are well known constituents of pine bark and grape seeds and are present in a wide variety of plant derived foods and beverages. They are the second most abundant phenolic compounds in nature after lignin (Gu et al., 2004). Proanthocyanidins have been widely investigated with respect to beneficial effects on cardiovascular diseases. Consumption of proanthocyanidin-rich foods such as red wine and cocoa (chocolate) seems to decrease blood pressure and insulin resistance and reverse endothelial

dysfunction (Grassi et al., 2005; Heiss et al., 2007; Heiss et al., 2005; Taubert, Roesen, Lehmann, Jung, & Schömig, 2007).

The genus *Aronia* (Rosaceae family) includes two species of shrubs, native to eastern North America and East Canada: *Aronia melanocarpa* (Michx.) Ell., known as black chokeberry and *Aronia arbutifolia* (L.) Pers. (red chokeberry) (Kokotkiewicz, Jaremicz, & Luczkiewicz, 2010; Kulling & Rawel, 2008). *Aronia prunifolia* (purple chokeberry) is regarded as a hybrid between *A. melanocarpa* and *A. arbutifolia* (Kokotkiewicz et al., 2010), it is however ambiguous if it is a separate species. *Aronia prunifolia* (Marshall) Rehder is indexed in The International Plant Names Index ("International Plant Names Index," 2012). Cultivars used for fruit production are from the species *Aronia melanocarpa* (e.g. berries are used for juice, jam and wine production). Commercially important cultivars in Europe and the United States include 'Aron', 'Nero', 'Viking', 'Hugin' and 'Rubina'. In Norway, the cultivar 'Moskva' is most common. The aronia berries contain high levels of flavonoids, mostly proanthocyanidins and anthocyanins, and *in vitro* and *in vivo* studies indicate that the berries may have potential health benefits, e.g. hepatoprotective effects, cardioprotective effects, and antidiabetes effect (reviewed by (Denev, Kratchanov, Ciz, Lojek, & Kratchanova, 2012; Kulling & Rawel, 2008). In order to have a systemic biological effect and impact on health, sufficient amounts of bioactive compounds have to be absorbed through the gastrointestinal tract and reach the systemic circulation. The bioavailability of anthocyanins from aronia juice has been investigated in humans (Wiczkowski, Romaszko, & Piskula, 2010), and it was shown that both cyanidin glycosides and their methylated and/or glucuronidated derivatives were present in plasma. Generally, the bioavailability of anthocyanins is rather low. However, anthocyanin metabolites produced by the intestinal microflora are supposed to be absorbed and contribute to the beneficial health effects, as well (Manach,

Williamson, Morand, Scalbert, & Rémésy, 2005; Williamson & Clifford, 2010). Nor is the bioavailability of proanthocyanidins fully understood. However, dimers and trimers of A- and B-type procyanidins have been detected in plasma of rats (Appeldoorn, Vincken, Gruppen, & Hollman, 2009; Shoji et al., 2006). Chokeberries are a rich source of B-type proanthocyanidins with contents above 5 g/100g dry matter (Oszmiański & Wojdylo, 2005).

Increased popularity of aronia products as functional food is expected in the future (Kulling & Rawel, 2008). New functional food products containing aronia berries or aronia juice are popular products due to their assumed health beneficial effects. In a recent study, we found that chokeberries were powerful antioxidants and enzyme inhibitors, and anthocyanins and proanthocyanidin-rich fractions from the berries could be responsible for the effects (Bräunlich et al., 2013). As far as we know, no scientific papers have described the differences in anthocyanin composition and in antioxidant capacity between the *A. melanocarpa* cultivars 'Moskva', 'Hugin', 'Nero' and *A. prunifolia.* In addition, aronia has been reported to ameliorate diabetes in humans (Simeonov et al., 2002). While we have previously shown that some aronia constituents are inhibitors of α-glucosidase (Bräunlich et al., 2013), the difference between aronia cultivars has not been investigated previously. Hence, the aim of this study was to investigate the chemical composition (quantification of anthocyanins and content of total phenolics and proanthocyanidins) as well as the biological activities (1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and inhibition of 15-lipoxygenase (15-LO), xanthine oxidase (XO) and α-glucosidase) of these four berry sources.

2. Materials and methods

2.1. Plant material

Three *A. melanocarpa* cultivars ('Moskva', 'Hugin' and 'Nero') and an *A. prunifolia* cultivar were included in this study. The cultivar 'Moskva' was collected at Klepp, Norway (august 2010), 'Hugin' was collected in Tøyen Botanical garden, Oslo, Norway (August 2011), the cultivar 'Nero' was collected at Obstbau GbR, Coswig, Germany (September 2011) and *A. prunifolia* was collected in a private garden in Schönefeld, Germany (September 2011). The diameter of the berries was measured; results are shown as the average diameter of five berries of each cultivar. The berries were kept at -20 °C until extraction. Voucher specimens are deposited at School of Pharmacy, University of Oslo, Norway ('Moskva' MB201201; 'Hugin' MB201202; 'Nero' MB201203; *A. prunifolia* MB201204).

2.2. Extraction of phenolics

Frozen aronia berries, 'Moskva', 'Hugin', 'Nero' and *A. prunifolia*, (500 g of each) were extracted with boiling 80% ethanol with a reflux condenser for 2 h. The extraction was repeated twice. The extracts were filtered and combined, and then concentrated on a rotary evaporator followed by lyophilisation to give an 80% ethanol crude extract for each of the aronia cultivars.

For anthocyanin analysis, 10 g berries of each cultivar were weighed and frozen at -20 °C. The frozen berries were cut in small pieces, placed into a screw cap tube and freeze dried before extraction with 10 ml of methanol/0.5% trifluoroacetic acid (TFA) for 40 minutes at 4 °C using a magnetic stirrer. The extraction was repeated four times, and the

extracts were combined after filtration. Extraction was done in five parallels for each aronia cultivar.

2.3. HPLC analysis of anthocyanins

Analysis was performed on a LaChrom Elite HPLC system (VWR-Hitachi) equipped with an L-2455 diode array detector. A Chromolith Performance RP18e 100 x 4.6 mm column (Merck, Darmstadt, Germany) was used for separation. Elution was performed using a gradient of mobile phase A (0.5% TFA in water) and mobile phase B (0.5% TFA in acetonitrile) with the following time schedule: 10% B, 0-1 min; 10-20% B, 1-3 min; 20-85% B, 3-4 min; 85-10% B 4-5 min; and finally 10% B, 5-6 min for reconditioning of the column. The flow rate was 3.0 mL/min and injection volume was 10 µL. The anthocyanin absorbance was recorded at 520 nm, and separation took place at 25 °C. Three parallels of each extract were analysed. All samples were filtered (0.45 µm) prior to injection.

Quantification of anthocyanins was based on a calibration curve of pure cyaniding 3-galactoside (> 96%, determined by HPLC) isolated from aronia berries (Bräunlich et al., 2013), assuming the same absorption coefficients for all anthocyanins (Jordheim, Enerstvedt, & Andersen, 2011). The results are expressed as mg cyaniding 3-galactoside equivalents per 100 g of fresh weight (FW).

2.4. Total phenolic content

The total phenolic content was measured in the 80% ethanol berry extracts by use of the Folin-Ciocalteu method after adjustments (Singleton & Rossi, 1965; Wangensteen,

Samuelsen, & Malterud, 2004). The results are expressed as mg gallic acid equivalents (GAE) per 100 g of FW or mg GAE/g extract.

2.5. Acid butanol assay for proanthocyanidins

Proanthocyanidins were hydrolyzed to anthocyanidins by use of an n-BuOH-HCl-Fe(III) mixture, analyzed at 550 nm and content given as equivalents of procyanidin B2 (Hagerman, 2011; Porter, Hrstich, & Chan, 1986). Aliquots (20-40 mg) of the 80% ethanol extracts were diluted to 10 mL in methanol (0.1% TFA). From each aliquot two 1 mL samples were assayed, the first one diluted 1:1 with acidic methanol whereas the second remained non-diluted. Hydrolysis was performed in capped tubes at 100 °C for 50 min. Absorbances prior to hydrolysis were subtracted from absorbances of the hydrolyzed samples in order to adjust for the original anthocyanin content in the samples.

2.6. DPPH radical scavenging

Scavenging activity towards the DPPH radical was carried out as previously described (Wangensteen et al., 2004). Briefly, to 2.95 mL of a methanolic solution of DPPH (A_{517} 1.0), 50 µL of the test compound (dissolved in DMSO) was added. The mixture was stirred and the decrease in UV absorbance at 517 nm was measured over a period of 5 min. Percent radical scavenging was calculated as 100 x ($A_{start} - A_{end}$) / (A_{start}), where A_{start} is the absorbance before addition of test compound and A_{end} is the absorbance value after 5 min of reaction time. Values were corrected for absorbance of the test substances. Quercetin was used as a positive control.

2.7. Inhibition of 15-lipoxygenase (15-LO)

Soybean lipoxygenase was used to measure inhibition of 15-LO. To a solution of linoleic acid (134 μM) in borate buffer (0.2 M, pH 9.00, 2.90 mL) was added 50 μL of test substance dissolved in DMSO or (for blanks) DMSO alone. A solution of 15-LO in 50 μL borate buffer (10.000 U/mL) was added, and the increase in absorbance at 234 nm from 30 to 90 s after addition was measured. Percent enzyme inhibition was calculated as 100 x $[(\Delta A_1 - \Delta A_2) / \Delta A_1]$, where ΔA_1 and ΔA_2 are values for increase in A_{234} for sample without test substance and with test substance, respectively (Wangensteen et al., 2004). Quercetin was used as a positive control.

2.8. Inhibition of xanthine oxidase (XO)

The XO inhibitory activity with hypoxanthine as substrate was measured spectrophotometrically based on the procedure of Noro et al. (1983) with some modifications (Pham, Malterud, Paulsen, Diallo, & Wangensteen, 2011). The assay mixture consisting of 50 μL of test compound (dissolved in DMSO) or (for blanks) DMSO alone, 1.85 mL of 50 mM sodium-potassium phosphate buffer (pH = 7.5), and 100 μL of enzyme solution (1.8 U/mL in 50 mM sodium-potassium phosphate buffer, pH = 7.5) was prepared immediately before use. Substrate solution (1.0 mL, 20 μg/mL hypoxanthine in distilled water) was added. The mixture was stirred and the increase in absorbance at 290 nm was measured over a period of 5 min. Percent enzyme inhibition was calculated as 100 x $[(\Delta A_1 - \Delta A_2) / \Delta A_1]$, where ΔA_1 and ΔA_2 are values for increase in A_{290} for sample without test substance and with test substance, respectively. Quercetin was used as a positive control.

2.9. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined by a slight modification of the procedure reported by Matsui et al. (1996). The enzyme solution was set at 800 mU/mL of α-glucosidase in a 50 mM phosphate buffer (pH = 7.0) containing 100 mM sodium chloride. For each assay, 20 µL of the test solution in DMSO and 80 µL of the enzyme solution were preincubated at 37 ˚C for 5 min. The reaction was started by adding 1.9 mL of substrate solution (0.7 mM *para*-nitrophenyl-α-D-glucopyranoside (PNP-G) in the buffer) and the solution was then incubated at 37 ˚C for 15 min. After the reaction had been stopped by adding 2.0 mL of a 0.5 M Tris solution, the absorbance of PNP released from PNP-G at 400 nm was measured. Percent enzyme inhibition was calculated as 100 x (A_B - A_S) / (A_B), where A_B and A_S represent the absorbance of the blank and sample, respectively. Acarbose was used as a positive control.

2.10. Statistics

Samples for total phenolic content, DPPH, 15-LO, XO and α-glucosidase assays were analysed in triplicate, for proanthocyanidin content in four parallells, and results are given as averages ± SD.

3. Results and discussion

3.1. Anthocyanin composition

The content of four anthocyanins, namely cyanidin 3-galactoside (**1**), cyanidin 3-arabinoside (**2**), cyanidin 3-xyloside (**3**) and cyanidin 3-glucoside (**4**) (Fig. 1), in four cultivars of aronia berries was determined by HPLC in the acidified methanol extracts,

and the identification of the individual anthocyanins was based on comparison with authentic standards previously isolated from aronia berries (Fig. 2) (Bräunlich et al., 2013). The anthocyanins (**1**-**4**) have previously been identified as the major pigments in aronia berries (Kulling & Rawel, 2008); however the present results revealed large variations in content among the cultivars (Table 1). *A. prunifolia* was found to contain almost three times as high content of anthocyanins as *A. melanocarpa,* cv. 'Moskva' and 'Hugin', whereas cv. 'Nero' contained 60% of the anthocyanin content of *A. prunifolia*. Cyanidin 3-galactoside was the major anthocyanin in all cultivars followed by cyanidin 3-arabinoside. Cultivar 'Nero' and *A. prunifolia* were collected in Germany, whereas 'Moskva' and 'Hugin' were collected in Norway, thus, unequal growing conditions may have had an impact on the results. However, the difference in anthocyanin composition that was found between cultivar 'Nero' and *A. prunifolia* indicate that there are large variations in anthocyanin content among different berry sources of aronia. A recent paper (Rugina et al., 2012) reports the total anthocyanin variation between *A. prunifolia* and two cultivars ('Viking' and 'Aron', not investigated by us) of *A. melanocarpa*. Our study describes for the first time the quantitative content of the individual anthocyanins. The total content is in accordance with the previous studies (Kulling & Rawel, 2008). Berries from *A. prunifolia* contain much higher levels of anthocyanins compared to the *A. melanocarpa* cultivars. These results indicate that berries from *A. prunifolia* might be a preferable species when a high content of anthocyanins is favored. The HPLC analysis was accomplished by using a reverse phase (RP) chromolith column. This is a monolithic column with the advantage of having a faster flow rate, thus giving a quicker separation compared to more commonly used RP-HPLC columns. In this study, the separation of four anthocyanins was accomplished in less than four minutes, a considerable advantage compared to anthocyanin analysis performed previously (Rugina et al., 2012) and in

accordance with previous reports by use of this column (Arapitsas & Turner, 2008; Witzell & Lundström, 2007).

3.2. Extraction yield, content of total phenolics and proanthocyanidins

The berries with diameters in the range from 8.4 ± 0.5 mm (*A. prunifolia*) to 12.9 ± 0.6 mm ('Moskva') were extracted with 80% ethanol, and the extraction yields are presented in Table 2. The extraction of cultivar 'Nero' gave an extraction yield of 19.7%, more than twice that of 'Hugin'. *A. prunifolia* berries contained roughly 50% more phenolics (expressed as gallic acid equivalents (GAE)) than the cultivar 'Nero' which had the highest phenolic content among the *A. melanocarpa* cultivars. The same trend was observed in the proanthocyanidin assay; *A. prunifolia* had the highest content of proanthocyanidins, followed by the cultivar 'Nero'. This is probably due to the fact that a high proportion of the phenolics analyzed in the Folin-Ciocalteu assay are proanthocyanidins.

3.3. Antioxidant activities

The antioxidant activities of the acidic methanol extracts and the 80% ethanol extracts were measured by the DPPH radical scavenging method, as well as by inhibition of the peroxidative enzyme 15-LO and inhibition of the prooxidative enzyme XO. The results are shown in Table 3. The 80% ethanol extract from 'Hugin' and *A. prunifolia* showed highest scavenging activities with IC_{50} values of 16.2 ± 0.4 μg/mL and 21.2 ± 0.7 μg/mL, respectively. The DPPH radical scavenging activities of the acidified methanol extracts were generally lower compared to the 80% ethanolic extracts. This could be due to the

presence of proanthocyanidins in the 80% ethanol extract. Proanthocyanidins have high hydrogen donating capacity, a mechanism important for the reaction with DPPH (Yokozawa et al., 1998). Other low molecular weight compounds with phenolic structures might also be involved in the reaction with DPPH. Small differences between the aronia cultivars were observed in the 15-LO assay and the inhibitory activities were moderate. The 15-LO inhibitory activity was generally higher in the acidified methanol extracts than in the 80% ethanol extracts. In the XO inhibition assay, small differences were observed between the cultivars, as well. In general, the 80% ethanol extracts were found to possess higher activities in this assay compared to the anthocyanin-rich extracts; this could be due to the content of proanthocyanidins and other polyphenols in the ethanol extracts.

3.4. Inhibition of α-glucosidase

Inhibition of α-glucosidase delays carbohydrate digestion and the absorption of monosaccharides from the intestine (Adisakwattana, Yibchok-Anun, Charoenlertkul, & Wongsasiripat, 2011). The results from the α-glucosidase inhibition assay show that the acidified methanol extracts exhibited much higher inhibitory effects compared to the 80% ethanol extracts (Table 4). The acidified extracts were composed mainly of anthocyanins (analyzed by HPLC), and we have shown previously (Bräunlich et al., 2013) that these substances are potent inhibitors of α-glucosidase and may have beneficial effects in reducing blood glucose levels.

4. Conclusion

Our study shows that variations in chemical composition exist between different cultivars of aronia berries and between berries from *A. melanocarpa* and *A. prunifolia*. The *A. prunifolia* species has relatively small berries, but they give a large extraction yield with alcohol and show the highest content of anthocyanins among the tested berries. As antioxidants, the differences between extracts from the tested cultivars were less prominent. The anthocyanin fractions were shown to be potent α-glucosidase inhibitors. The presented results show that among the aronia berries tested, *A. prunifolia* constitutes the richest source of polyphenols and might be the best species when a high content of anthocyanins and proanthocyanidins is preferred.

Acknowledgements

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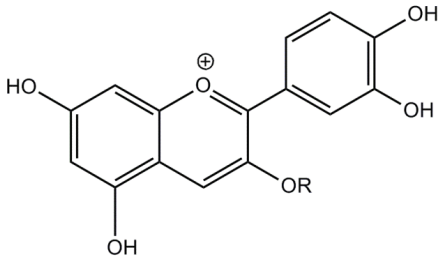
Fig. 1 - Chemical structures of major anthocyanins in aronia berries.



1. R = galactose: Cyanidin 3-galactoside
2. R = glucose: Cyanidin 3-glucoside
3. R = arabinose: Cyanidin 3-arabinoside
4. R = xylose: Cyanidin 3-xyloside

Fig. 2 - RP-HPLC chromatogram of acidified methanolic extracts of berries from aronia: a) 'Moskva'; b) 'Hugin'; c) 'Nero'; d) *A. prunifolia*. The anthocyanins shown are **1**: Cyanidin 3-galactoside, **2**: Cyanidin 3-glucoside, **3**: Cyanidin 3-arabinoside and **4**: Cyanidin 3-xyloside. Y-axis is shown from -100 to 2200 mAU, x-axis from 0 to 5 minutes.

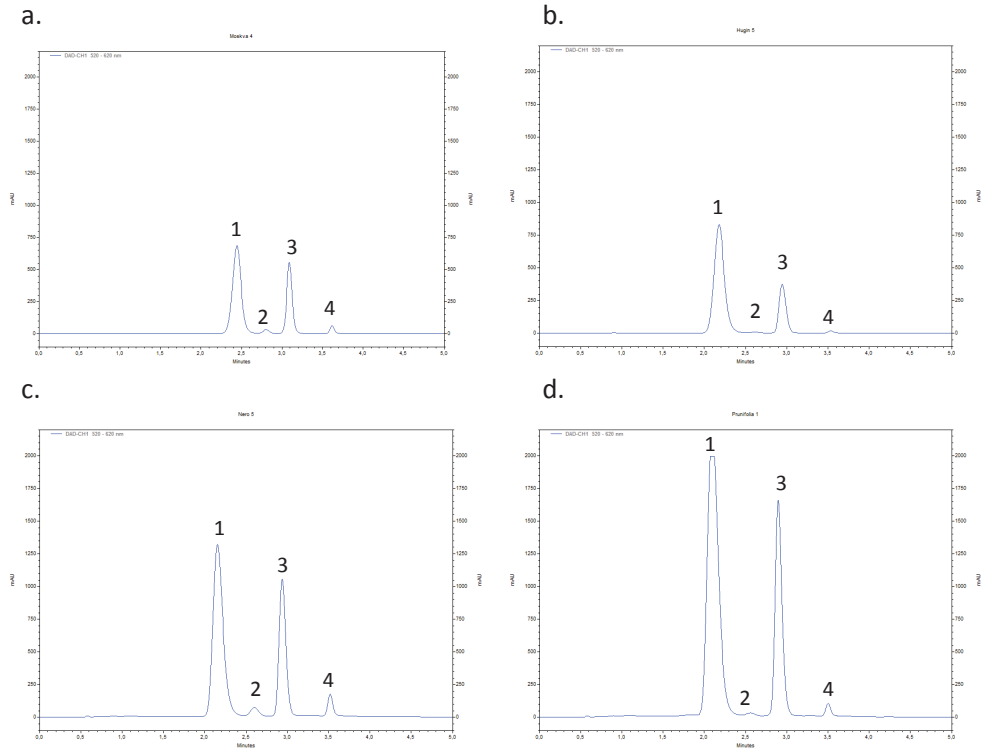


Table 1

Anthocyanin content from four cultivars of aronia berries [1]

	1. Cyanidin 3-galactoside	**2**. Cyanidin 3-glucoside	**3**. Cyanidin 3-arabinoside	**4**. Cyanidin 3-xyloside	Total
'Moskva'	168.4 ± 11.9	tr[2]	83.0 ± 6.6	2.7 ± 0.8	251.5
'Hugin'	196.9 ± 15.5	tr	51.6 ± 5.9	tr	248.5
'Nero'	281.7 ± 25.3	4.7 ± 0.4	149.2 ± 14.7	11.7 ± 1.0	447.3
A. prunifolia	496.8 ± 19.9	tr	236.2 ± 13.2	3.8 ± 0.7	737.3

[1] presented as mg cyanidin 3-galactoside equivalents/100 g FW; [2] trace

Table 2

Diameter, extraction yield, total phenolic content and total proanthocyanidins of four cultivars of aronia berries

	Diameter	Yield of 80% EtOH extract	Total phenolic content[1]		Total proanthocyanidins[3]
	mm	% of FW	mg GAE/g extract[2]	mg GAE/100 g FW	g PCB2/100 g FW
'Moskva'	12.9 ± 0.6	10.7 %	101.1 ± 5.6	1079 ± 60	2.461
'Hugin'	9.6 ± 0.8	9.4 %	147.6 ± 5.6	1389 ± 53	2.820
'Nero'	12.6 ± 0.5	19.7 %	97.5 ± 15.0	1921 ± 296	3.743
A. prunifolia	8.4 ± 0.5	17.1 %	175.3 ± 10.1	2996 ± 172	4.788

[1] presented as mg gallic acid equivalents (GAE); [2] 80% EtOH extract; [3] presented as g procyanidin B-2 equivalents

Table 3

Antioxidant activity of four cultivars of aronia berries tested by DPPH radical scavenging, inhibition of 15-lipoxygenase (15-LO) and inhibition of xanthine oxidase (XO).

	DPPH	15-LO	XO
	IC_{50} (µg/mL)	% inh 83.3 µg/mL	% inh 83.3 µg/mL
80% ethanol extract 'Moskva'	35.7 ± 1.5	4.0 ± 2.7	25.5 ± 3.0
80% ethanol extract 'Hugin'	16.2 ± 0.4	10.9 ± 2.9	34.5 ± 2.0
80% ethanol extract 'Nero'	35.5 ± 1.4	8.9 ± 3.1	23.7 ± 4.1
80% ethanol extract *A. prunifolia*	21.2 ± 0.7	5.7 ± 3.4	26.3 ± 2.7
Acidified methanol extract 'Moskva'	61.4 ± 1.6	8.5 ± 4.7	2.7 ± 1.6
Acidified methanol extract 'Hugin'	39.7 ± 1.4	12.8 ± 5.7	14.7 ± 1.8
Acidified methanol extract 'Nero'	54.3 ± 1.6	17.4 ± 5.2	13.0 ± 3.8
Acidified methanol extract *A. prunifolia*	33.2 ± 1.3	14.7 ± 5.0	18.0 ± 5.5
Quercetin (positive control)	3.3 ± 0.6	26.0 ± 2.0 [1]	0.6 ± 0.1[1]

[1] presented as IC_{50} (concentration to give 50% inhibition)

Table 4

Inhibition of α-glucosidase of extracts from four cultivars of aronia berries

	IC_{50} (µg/mL)
80% ethanol extract 'Moskva'	0.70 ± 0.08
80% ethanol extract 'Hugin'	0.83 ± 0.10
80% ethanol extract 'Nero'	0.88 ± 0.08
80% ethanol extract *A. prunifolia*	0.88 ± 0.08
Acidified methanol extract 'Moskva'	0.049 ± 0.002
Acidified methanol extract 'Hugin'	0.030 ± 0.003
Acidified methanol extract 'Nero'	0.044 ± 0.006
Acidified methanol extract *A. prunifolia*	0.030 ± 0.005
Acarbose (positive control)	130 ± 20