Bioactive compounds in flowers and fruits of *Sambucus nigra* L.

Thesis for the degree of *Philosophiae Doctor* (Ph.D.)

by

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LIST OF PUBLICATIONS

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X. Yuan-Feng Zou; Hilde Barsett, **Giang Thanh Thi Ho**, Kari Tvete Inngjerdingen, Drissa Diallo, Terje Einar Michaelsen & Berit Smestad Paulsen. Immunomodulating pectins from root bark, stem bark and leaves of the Malian medicinal tree *Terminalia macroptera and* structure activity relations. *Carbohydrate Research 2015, 403, 167-173.*

XI. Helle Wangensteen, **Giang Thanh Thi Ho**, Margey Tadesse, Christopher Owen Miles, Nastaran Moussavi, Bertin Mikolo & Karl Egil Malterud. A new benzophenanthridine alkaloid and other bioactive constituents from the stem bark of *Zanthoxylum heitzii*. *Fitoterapia (Milano) 2016, 109, 196-200.*

ABBREVIATIONS

4-O-Me-GlcA	4-O-methylated glucuronic acid
50WSnBe	50 °C water elderberry extract
50WSnFl	50 °C water elderflower extract
100WSnBe	100 °C water elderberry extract
100WSnFl	100 °C water elderflower extract
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AG-I	Arabinogalactan type I (arabino-4-galactans)
AG-II	Arabinogalactan type II (arabino-3,6-galactans)
AH	Weak acid hydrolysis
Ara	Arabinose
ASE	Accelerated solvent extraction
BPII	*Byophytum petersianum* fraction II
^{13}C-NMR	Carbon nuclear magnetic resonance
COSY	Correlation spectroscopy
CVD	Cardiovascular diseases
DA	Dalton
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EB	Elderberries
EF	Elderflowers
EH	Ester hydrolysis
EtOH	Ethanol
f	Furanose
FPLC	Fast protein liquid chromatography
FRAP	Ferric reducing antioxidant power
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography
Glc	Glucose
GlcA	Glucuronic acid

^{1}H-NMR	Proton nuclear magnetic resonance
HepG2	Hepatocellular liver carcinoma cells
HG	Homogalacturonan
HPLC	High performance liquid chromatography
IC_{50}	Concentration to give 50 % inhibition
kDa	Kilodalton
KDO	3-deoxy-D-*manno*-2-octulosic acid
LO	Lipoxygenase
LPS	Lipopolysaccharide
Man	Mannose
Me	Methyl
MeOH	Methanol
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NO	Nitric oxide
NMR	Nuclear magnetic resonance
OA	Oleic acid
OH	Hydroxyl group
ORAC	Oxygen radical absorbance capacity
p	Pyranose
PCA	Protocatechuic acid
PGA	Pholoroglucinol aldehyde
RG-I	Rhamnogalacturonan type I
RG-II	Rhamnogalacturonan type II
Rha	Rhamnose
ROS	Reactive oxygen species
Sn	*Sambucus nigra*
SnBe50	50% EtOH elderberry extract
SnFl50	50% EtOH elderflower extract
SPE	Solid phase extraction
t	Terminal
T2D	Type 2 diabetes

TFA	Trifluoroacetic acid
TMS	Tetramethylsilane
UV	Ultraviolet
WHO	World Health Organization
XO	Xanthine oxidase
XG	Xylogalacturonan
Xyl	Xylose

ABSTRACT

Sambucus nigra L. or black elder has a long history of use in traditional European medicine for treatment of inflammations, infections, diabetes and for boosting the immune system. The aim of this thesis was to investigate the potential health benefits of elderberries and elderflowers with main focus on the immunomodulatory effects and the anti-diabetic potential of pectic polysaccharides and phenolic constituents.

Pectic polysaccharides isolated from the 50% EtOH, 50 °C and 100 °C water extracts from the elderberries and the elderflowers were shown to contain homogalacturonan (HG), rhamnogalacturonan-I (RG-I), arabinogalactan-I (AG-I) and arabinogalactan-II (AG-II), in addition to arabinans. Rhamnogalacturonan-II (RG-II) was only present in some of the elderflower fractions. The distribution of sugar residues, molecular weight, and their linkages varied between the fractions. Weak acid hydrolysis was performed on the most active acidic fractions from the elderberries and the elderflowers. An almost complete loss of Ara was observed, the amount of some linkages to Rha and Gal were also decreased. A negative Yariv test after weak acid hydrolysis indicated a degradation of AG-II. Ester groups in the polysaccharide fractions from the elderflowers were reduced after treatment with NaOH. The de-esterified polysaccharides showed the same distribution of linkages as their respective native polysaccharides. In order to isolate the hairy regions, one acidic fraction from elderberries (SnBe50-I-S3) and four acidic fractions from elderflowers (SnFl-50-I-S2, 50WSnFl-I-S2, 100WSnFl-I-S2, 100WSnFl-I-S3) were treated with endo-polygalacturonase. This led to the isolation of five sub-fractions from the elderberry fraction and two sub-fractions from each of the elderflower fractions. RG-I like structure and side chains of AG-I and AG-II were the predominant part in the isolated sub-fraction-I and sub-fraction-II from the elderberries and in the sub-fractions-I from the elderflowers. These fractions showed high degree of branch points compared to the native fractions. Sub-fractions-II from the elderflowers consisted of RG-I and also small amounts of RG-II structures.

Complement and macrophages are both part of the innate and adaptive immune system. All the acidic polysaccharide fractions from elderberries and elderflowers showed dose-dependent complement fixating activity and stimulated nitric oxide (NO) production in RAW 264.7 macrophages, with elderflower fractions being the most active ones. Removal of Ara and reduced side chain complexity contributed to reduced activity, while removal of ester led to an increased activity compared to the native fractions. The hairy regions isolated after

enzymatic treatment possessed strong complement fixating and macrophage stimulating activity, much stronger than the native fractions, whereas the smooth regions showed a reduced activity.

Type 2 diabetes is one of the most prevalent and serious metabolic diseases, and is associated with hyperglycemia, insulin resistance and dysregulation of glucose- and fatty acid uptake. Elderberry and elderflower crude extracts, flavonoids, phenolic acids and metabolites showed a dose-dependent increase of glucose- and fatty acid uptake in human skeletal muscle cells and human liver cells. A high increase of glucose- and fatty acid uptake observed after exposure to selected intestinal metabolites (protocatechuic acid, phloroglucinol aldehyde, caffeic acid, ferulic acid) is of interest, since they are better absorbed from the intestine compared to the native polyphenols and therefore easier will reach the systemic tissues. Several of the crude extracts and phenolic constituents from elderberries and elderflowers were found to be strong α-amylase and α-glucosidase inhibitors compared to the anti-diabetic drug acarbose. Oxidative stress is associated with the pathogenesis of diabetes and inflammation. In general, the phenolic substances showed strong radical scavenging and 15-lipoxygenase inhibitory effects, but were less active toward xanthine oxidase. The dichlorometane and water extracts from both the elderberries and elderflowers were inactive as antioxidants in contrast to the alcohol extracts, which possessed high or moderate antioxidant activities.

The phenolic-rich extracts from the elderberries and the elderflowers showed potent anti-inflammatory activity as they showed high complement fixating activity and inhibited NO-production in LPS activated RAW 264.7 macrophages and dendritic D2SC/I cells. Flavonoids and phenolic acids were all NO inhibitors, with variation in activity from one compound to the other. The metabolites were inactive in the complement fixating test, but possessed rather strong NO inhibitory activity both in the macrophages and the dendritic cells.

These results showed that polysaccharides and polyphenols from elderberries and elderflowers have potential health promoting effects. Intake of elderberries and elderflowers might help to regulate inflammatory diseases, and might give potential anti-diabetic and immune-stimulating effects.

1. INTRODUCTION

The past decade has witnessed increasing interest in “nutraceuticals” or “functional foods” in which phytochemicals can have health promoting or medicinal properties. Plant food is a good source of a whole range of vitamins and minerals, but the presence of these alone does not seem to explain the health benefits. There is now considerable body of evidence which shows that diets rich in plant food are generally associated with lower disease risk, such as cardiovascular and inflammatory diseases. The search is on for the natural products that convey these health benefits and also identification of the mechanisms of action. Food of plant origin contains many important bioactive substances, both low molecular weight compounds such as phenolic compounds and high molecular weight compounds such as polysaccharides. Within the plant, these bioactive substances have various roles in metabolism and in the interaction with environment. Some provide strength to stems, leaves, flowers and fruits, cell wall extension and plant growth, some protect against insect attacks, while other provide color and smell, and can attract insect for reproduction purposes. Among the vast number of medicinal plants used in Western and non-Western medical approaches, a number have received considerable interest and use in Europe over the past few years, one of them is *Sambucus nigra* L.

1.1 *Sambucus nigra* L. (black elder)

S. nigra. or black elder is a plant native to Europe, America, Northern Africa and Western- and Central Asia. *Sambucus* is a genus of flowering plants in the family Adoxaceae (formerly Caprifoliaceae) that grows on sunlight-exposed places. Black elder is a deciduous shrub or small tree that can grow up to 10 m. They produce umbels of cream-white flowers in early summer and blue-black berries with a diameter up to 6 mm which ripen in late summer (Figure 1) [1, 2]. The elderflowers (Sambuci flos) and the berries (Sambuci fructus) have a long history of use in traditional European medicine - internally (fruits as tea, jelly, juice, or syrup; flowers as tea or syrup) for treatment of disorders of the respiratory tract, gastrointestinal tract, rheumatism, inflammation, diabetes and for viral infections, fever, colds and influenza [1, 2].

A) **B)**

Figure 1. *Sambucus nigra* **A)** Elderberries **B)** Elderflowers. Illustration photos: Colourbox.no

The elderberries contain several components that may contribute to pharmacological activities. Large amounts of anthocyanins such as cyanidin-3-glucoside and cyanidin-3-sambubioside, in addition to rutin and chlorogenic acid are present in fresh fruits. Small amounts of other types of anthocyanins, procyanidins, flavonols and phenolic acids have also been identified [1]. Other ingredients are vitamins and minerals in small amounts and carbohydrates such as pectin and up to 7.5% glucose and fructose [3]. The elderflowers contain high amounts of flavonoid glycosides and caffeic acid derivatives. Other constituents are triterpenes, sterols, free fatty acids and pectins [1]. A series of factors, such as habitat/location, harvest date, cultivar, fertilization, and maturation of the berries and the flowers can affect their content.

The antioxidant properties of elderberry extracts and its phenolic constituents have been reported in a series of papers, using different well established *in vitro* assays [2, 4, 5]. *In vitro* experiments also showed that elderberry alcohol extracts were effective against *Helicobacter pylori* and have anti-proliferative effects in cell cultures. Several *in vitro* studies have shown that elderberry extracts have immunomodulating activity and induce the cytokines IL-1, IL-6, IL-8 and TNF-α. Besides a standardized elderberry liquid alcohol extract showed inhibition of human pathogenic bacteria and human pathogenic influenza viruses *in vitro* [6]. Animal experiments (rats and chimpanzees) have shown reduction in flu and flu-like symptoms, anti-inflammatory and antioxidant effects [6-8]. Sambucol®, a standardized elderberry extract, has in a randomized, double blind, placebo-controlled study showed reduction of influenza symptoms [9]. Another clinical trial showed that low dose elderberry spray-dried extract had a minor effect on serum lipids and anti-oxidative capacity [10]. A recent clinical study

conducted by Tiralongo, et al. [11] showed that elderberry extracts reduced cold duration and influenza symptoms in air-travelers.

The elderflowers have *in vitro* showed potent anti-inflammatory activities, anti-bacterial activities and anti-viral activities [2]. In addition, Gray, et al. [12] and Bhattacharya, et al. [13] showed that elderflower alcohol extracts had an effect on blood glucose, insulin-like activity and increased the glucose uptake in porcine myotubes. In animal experiments elderflowers showed diuretic, diaphoretic and anti-inflammatory activities [1]. No human clinical studies on elderflowers alone have been found in the literature. However, combination products containing elderflowers have been studied. Elderflowers in combination with other herbs (Sinupret®) have been reported to have beneficial effects on bronchitis, headache and sinus infections in human studies [6]. Allergease®, an oral lozenge supplement which contains elderflowers and other herbs, has in a double-blind, placebo-controlled, crossover study showed decrease sneezing during acute antigen exposure and resulted in faster recovery [14].

In addition to 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 elderberries or elderflowers, juice or extracts. However, since the unripe berries contain cyanogenic glycosides, elderberries should be harvested when fully ripe and then heated to remove traces of these toxic substances [2].

Today, black elder are cultivated both for its ornamental value and berries and flowers can be ingredients for juices, wines, jams, beverages or food colorants, as well. Preparations of fruits and flowers are also used as nutritional supplements in the treatment of common cold and influenza symptoms and as an immunomodulator (Figure 2) [1, 2].

Figure 2. Nutritional supplements with elderberries which can be purchased in Norway. Photos from: https://sunkost.no/immunforsvar/svarthyll/

1.2 Chemistry of compounds from *S. nigra*

1.2.1 Pectic polysaccharides

Pectins are structurally the most complex family of polysaccharides in nature, and are thought to account for about one third of all primary cell wall macromolecules in higher plants. They are found in the primary cell wall, as an interpenetrating matrix supporting cellulose microfibrils, together with hemicellulose and proteins. Pectins are galacturonic acid-rich polysaccharides including homogalacturonan (HG), rhamnogalacturonan I (RG-I) and the substituted galacturonans; rhamnogalacturonan II (RG-II) and xylogalacturonan (XG). It has been estimated that approximately 90% of the uronic acids in the wall derive from the galacturonic acid (GalA) residues of pectic polysaccharides [15]. The precise chemical structure of pectin is under debate, although the structural elements of pectin are rather well described. There exists several models for describing the pectin structure, and currently there are two preferred proposed models, the “smooth and hairy region” (Figure 3A) and the “RG backbone” (Figure 3B) [16]. In the “smooth and hairy region” model, the backbone of the pectic polysaccharide consists of smooth regions made up of HG and interrupted by hairy regions consisting of RG-I regions with neutral sugar side chains. In the “RG backbone” model, the pectic polysaccharide consists of a RG-I backbone with both HG and neutral sugar chains as side chains [17].

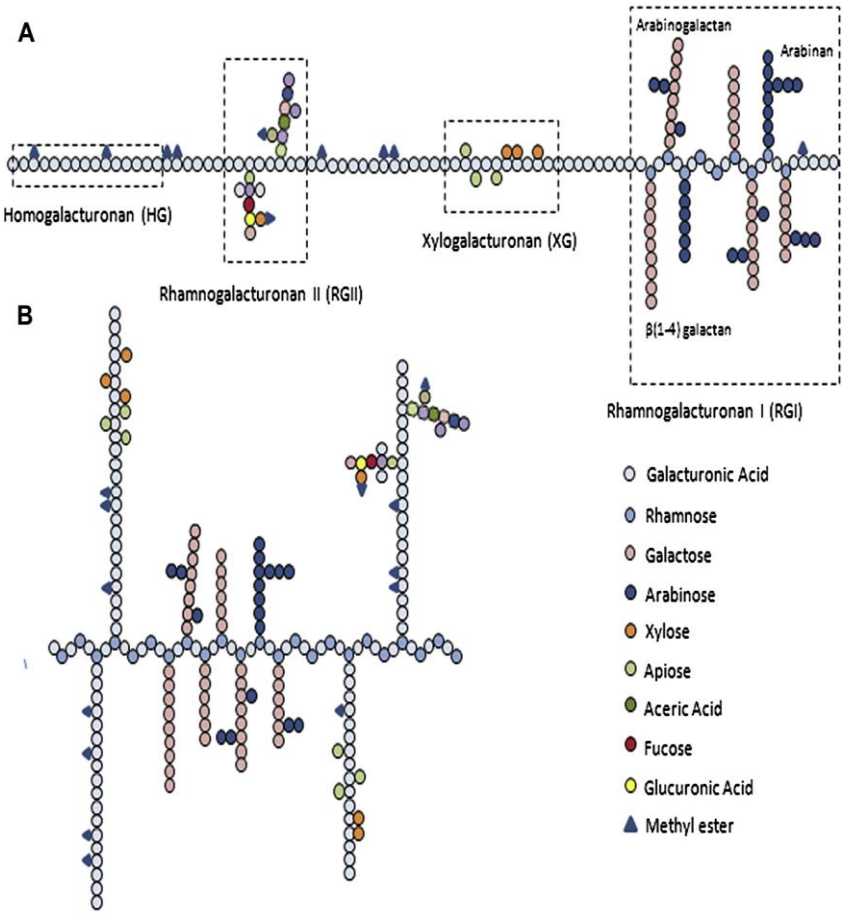


Figure 3. Schematic structure of pectin. **A)** The RG-I is considered attached to HG regions which are partially methyl-esterified and also contain RG-II and XG. Typical neutral side chains of RG-I are linear galactans and arabinans, branched galactans and arabinans, and arabinogalactans. **B)** An alternative structure whereby HG are side chains of RG-I. Figure from Maxwell, Belshaw, Waldron and Morris [16].

1.2.1.1 Homogalacturonan (HG)

The most abundant pectic polysaccharide is HG, a linear homopolysaccharide consisting of α-1,4-linked-*D*-GalA. Some of the GalA residues may be methyl-esterified at C6 carboxyl, and depending on the plant source, the GalA residues can also be *O*-acetylated in position 2 or 3 [18]. The methyl esterification might be present as blocks, or the substitution may be randomly distributed [19].

1.2.1.2 Rhamnogalacturonan-I (RG-I)

RG-I is a group of pectic polysaccharides that contains a backbone of alternating 1,2-linked α-L-rhamnose and 1,4-linked α-D-GalA residues. The RG-I are highly branched structures with neutral sugar side chains (arabinans, galactans and arabinogalactans) attached to position 4 of

rhamnose (Rha) [20]. The highly branched nature of RG-I is known as the “hairy region” of the pectin, in contrast to HG regions which are known as the “smooth” regions. The side chains of RG-I can be arabinans, arabinogalactans or galactans. Arabinans have a backbone of 1,5-linked arabinose (Ara), with branching points on position 2 or 3 of Ara [21]. Galactans consist of 1,4-linked galactose (Gal). Arabinogalactans can be divided into two subclasses, arabinogalactan-I (AG-I) or arabinogalactan-II (AG-II). AG-I is basically composed of 1,4-linked Gal units, normally with substitutions of one Ara unit or various sized arabinans on position 3 of some of the Gal units. AG-II is more complex compared to AG-I and can be highly branched with 1,3,6-linked Gal as branch points. AG-II consists of a galactan backbone with either 1,3-linked or 1,6-linked Gal as the main chain [22]. Ara or arabinans can be bound to O-3 or O-6 of Gal. The side chains may also contain terminal α-fucose, β-glucuronic acid (GlcA) and 4-O-Me-β-glucuronic acid residues [23]. In addition, the side chains can be esterified with phenolic acids [24].

1.2.1.3 Substituted galacturonans

Substituted galacturonans contain a backbone of linear 1,4-linked GalA, substituted with various sugars (Figure 3A). Rhamnogalacturonan-II (RG-II) is a low molecular mass (5-10 kDa) pectic polysaccharide. The characteristic part of RG-II is the presence of unusual sugars in the side chains, such as apiose, aceric acid, 2-O-Me-Xyl, 2-O-Me-Fuc, 3-deoxy-ᴅ-manno-2-octulosonic acid (KDO) and 3-deoxy-ᴅ-lyxo-2-heptulosaric acid [25]. The backbone of RG-II has at least seven 1,4-linked α-ᴅ-GalA residues, two structurally distinct disaccharides and two oligosaccharide chains attached to the backbone [26]. Other substituted galacturonans have also been identified. XG contains β-ᴅ-xylosyl (Xyl) residues attached to position 3 of the GalA backbone. The GalA residues of XG can be methyl-esterified as in HG [17].

1.2.2 Phenolic compounds

Plant-based foods and beverages such as fruits, berries, vegetables, chocolate, tea and wine are rich sources of phenolic compounds. These molecules are secondary metabolites in plants and are produced for protection against UV light, insects, viruses and bacteria [27]. Phenolic compounds are characterized by having at least one aromatic ring with one or more hydroxyl groups attached and can be classified as flavonoids and non-flavonoids [27] .

1.2.2.1 Flavonoids

The flavonoids are polyphenolic compounds comprising 15 carbons that share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C) (Figure 4). They can be divided into 6 major subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols, while those that are comparatively minor components of the diet are dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones and aurones [28]. The basic flavonoid skeleton can have numerous substituents. In plants, all flavonoids except flavanols are found in glycosylated forms, they can be either O- or C-linked [27].

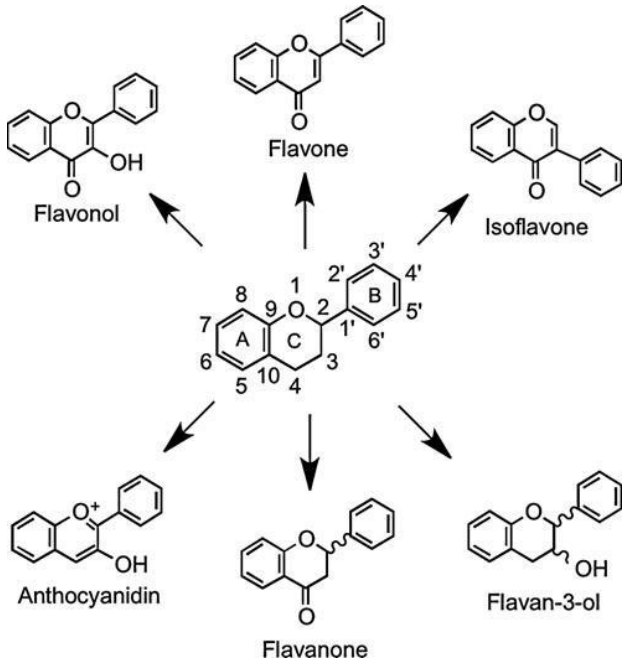


Figure 4. Structure of the flavonoid skeleton and some flavonoid subclasses. Figure from Del Rio et al. [27].

1.2.2.1.1 Flavanones

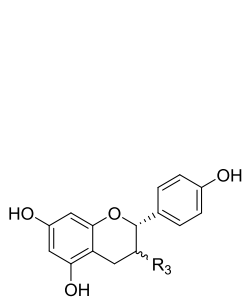
Flavanones such as naringenin are characterized by the absence of C2-C3 double bond and the presence of chiral centers at C-2 and C-3 [27]. In human foods, flavanones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit [16].

1.2.2.1.2 Flavonols

Chemically, flavonols differ from many other flavonoids since they have a double bond between C2-C3 and a hydroxyl group (OH) in position three of the C-ring (3-hydroxyflavones) [28]. The majority of flavonols are present as *O*-glycosides. In human diet, apples, plums, berries, grapes, onions, broccoli and tomatoes are major food sources of flavonols. Even cocoa, tea, both green and black ones, and red wine are good sources of flavonols [28]. The main flavonols in foods are quercetin-, kaempferol- and isorhamnetin glycosides.

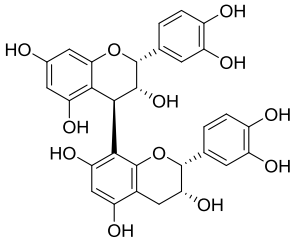
1.2.2.1.3 Flavan-3-ols and proanthocyanidins

Catechins and epicatechins are classified as flavan-3-ols monomers (Figure 5). Proanthocyanidins, which are also known as condensed tannins, are dimers, oligomers, and polymers of flavan-3-ols that are bound together by links between C4 and C8 (or C6) [28]. Procyanidins are a subclass of proanthocyanidins with (epi)catechin as the monomeric units (Figure 5). 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) [28]. The least frequent dimers are the A series, characterized by the presence of two linkages between the two flavanol units, one C4-C8 or C4-C6 and an additional one between C2 and C5 or C7. Proanthocyanidins are responsible for the astringent character of fruits and beverages (e.g. wine) and for the bitterness of chocolate [28].



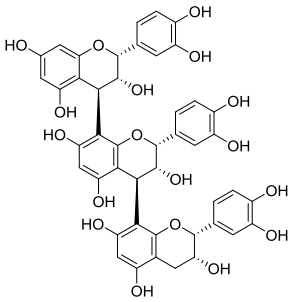
Catechin: R_3 = OH (β)
Epicatechin: R_3 = OH (α)

A



Procyanidin B2

B



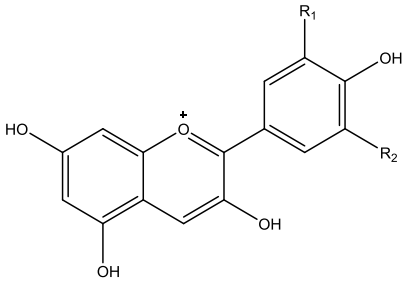
Procyanidin C1

C

Figure 5. Chemical structures of **A)** catechin and epicatechin, the monomeric units of procyanidins, **B)** procyanidin B2, an example of a procyanidin dimer and **C)** trimeric procyanidin C1.

1.2.2.1.4 Anthocyanins

Anthocyanins (glycosylated polyhydroxy derivatives of 2-phenylbenzopyrylium salts) are water-soluble pigments responsible for the blue, purple, red and orange colors of many fruits, vegetables and flowers [29]. Anthocyanins are mainly found in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries, bilberries, strawberries) [27]. Aglycones of anthocyanins are bound to sugar at the C3 and sometimes also at C5 and C7 positions and exist in the glycosylated form. Anthocyanins may also exist in the acylated form. At pH below 2 anthocyanins exist primarily in the form of red flavylium cations. The same anthocyanin may have different colours, depending on the pH of the solution. When the pH increases to 6, the flavylium cation converts into purple quinonoidal bases [27]. Several anthocyanidins have been reported with hydroxyl and methoxyl groups present at different positions in the B-ring on the basic structure. Six of them are commonly found in fruits and vegetables: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Figure 6) [27].

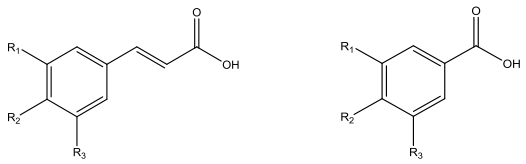


Anthocyanidin	**R_1**	**R_2**
Cyanidin	OH	H
Delphinidin	OH	OH
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 6. Structures of the most common anthocyanidins.

1.2.2.2 Non-flavonoids

Among the non-flavonoids of dietary significance are the phenolic acids. Phenolic acids are secondary metabolites from plants and fungi. They can be divided into two major groups, C6-C1 hydroxybenzoic acids and C6-C3 hydroxycinnamic acids, which are derived from molecules of benzoic and cinnamic acid (Figure 7), respectively [30]. Hydroxybenzoic acids are components of complex structures such as hydrolyzable tannins (gallotannins and ellagitannins). Among the hydroxybenzoic acids, gallic acid is the most common [31]. The hydroxycinnamic acids are more common than are the hydroxybenzoic acids and consist mainly of *p*-coumaric, caffeic and ferulic acids. Phenolic acids have been implicated as beneficial agents in a multitude of diseases, most commonly cardiovascular diseases. Phenolic acids have been shown to have antibiotic, antioxidant and anti-inflammatory properties and hence may inhibit or prevent the development of infectious and inflammatory diseases [32].



Cinamic acid derivates Benzoic acid derivates

Figure 7. Structures of cinamic acid derivates and benzoic acid derivates.

1.3 Bioavailability

The extent of the potential health benefits associated with the polyphenols studied *in vitro* are dependent *in vivo* on the absorption, metabolism, distribution, and excretion of these compounds within the body after ingestion. During the absorption, dietary polyphenols might be hydrolyzed and degraded by the intestinal enzymes or colonic microflora, and then conjugated in the intestinal cells or later in the liver [33]. Polyphenols consequently reach and accumulate in the target tissue and induce biological properties; the polyphenol derivatives mainly excrete through bile and urine. Most polyphenols are not subjected to phase I metabolism because the polyphenolic structures make them unfavorable substrates for the cytochrome P450s [33]. The polyphenolic compounds can directly undergo phase II metabolism, predominately methylation, glucuronidation and sulfation.

Most polyphenols pass to the colon where they are catabolized by the colonic microflora, yielding a diversity of phenolic acids such as homovanillic acid, caffeic acid and ferulic acid. Phenolic acids, when ingested in the free form, are rapidly absorbed from the small intestine. However, chlorogenic and neochlorogenic acids are naturally esterified and this impairs their absorption [34].

Human bioavailability studies have been employed for elderberry extracts and anthocyanins. The major finding in bioavailability studies in humans with elderberry anthocyanins was the determination of 0.1-0.4 μM in blood and urine after intake of elderberry extract (500-700 mg anthocyanins) [35-37]. Anthocyanins are some of the few polyphenols that can be detected unmetabolized (e.g. as glycosides) in plasma. The bioavailability of the anthocyanin metabolites was reported be 60- and 45-fold higher than their parent compounds in urine and plasma, respectively [35, 36]. There are big differences in the number of recovered metabolites in blood and urine and the amounts of recovered parent anthocyanins in the human studies.

The absorption of plant polysaccharides into the bloodstream after oral administration is not well understood and is a disputable issue. One possible mechanism for the uptake of polysaccharides in the lumen could be passage through microfold cells (M cells) in the Peyer's patches of the small intestinal lumen [38]. M cells are a unique subset of specialized epithelial cells for trans epithelial transport of macromolecules and particulate antigens [38].

1.4 Immunomodulating activity

Immunity is the body’s natural defense system against various infectious diseases. The factors that trigger immunity include previous infection, immunization, and various external stimuli. Based on the function, the immune system has been categorized into two broad categories, i.e., the innate immune system and the adaptive immune system [39]. The function and efficiency of the immune system are influenced by various exogenous and endogenous factors resulting in either immunosuppression or immunostimulation. Several agents possessing an activity to normalize or modulate pathophysiological processes are called immunomodulators. Natural products, plants, their extracts, and their active moieties such as polysaccharides, essential oils, steroids, terpenoids, phenolics, flavonoids and alkaloids have been reported with immunomodulatory potential [40-42].

1.4.1 The complement system

The complement system is one of the major effector pathways in the process of inflammation. The complement plays an important role in the first line defense against infections and it represents important effector functions of the innate and the adaptive immune system [43]. The complement is activated through the classical, the mannose binding (lectin) or the alternative pathway (Figure 8). The classical pathway is activated by antibodies and is initiated by IgM or IgG cluster with complement component 1 (C1) [43]. The alternative pathway is directly activated from C3 by microorganisms or some activators such as lipopolysaccharide through an "antibody-independent" mechanism [44]. In addition, another "antibody-independent" mannose-binding lectin (MBL) pathway has been established as the third activation pathway of complement system, and initiated from C4 [45].

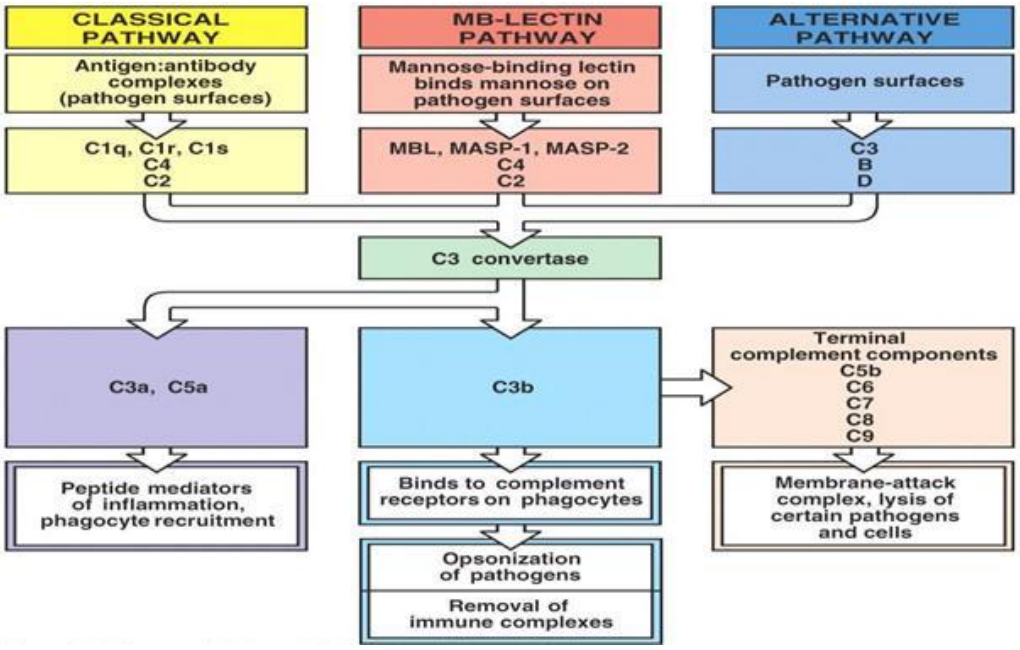


Figure 8. Activation steps and function of the complement system. Figure from Janeway, et al. [46]

Several medicinal plants such as *Plantago major*, *Echinacea purpurea*, *Panax ginseng*, *Angelica acutiloba* and *Biophytum umbraculum* (syn. *Biophytum petersianum*) have been reported with high complement fixating activities [47-49]. Structure-activity studies suggest that the hairy regions of RG-I, with complex galactan or AG-II side chains attached are important for the complement activity [21]. Also low molecular weight compounds such as flavonoids, anthocyanins and procyanidins have been reported complement fixating activity [50-52].

The complement fixating assay does not differentiate between activation and inhibition of the complement cascade because both result in inhibition of hemolysis [53]. Complement activators result in a decrease of hemolysis due to complement consumption, while complement inhibitors result in an inhibition of hemolysis by inhibiting a certain step in the complement cascade [54]. Another interesting feature of complement-activating plant polysaccharides is their possible use as an adjuvant. C3 activation products linked to an antigen can dramatically enhance the antibody response and possibly also have adjuvant effects to fight tumors and bacterial infections [44].

1.4.2 Activation and inhibition of macrophages and dendritic cells

Macrophages are important cells in the immune system that are formed in response to an infection or accumulating damaged or dead cells. Macrophages are formed through differentiation of monocytes, and take part in both innate and adaptive immunity [55]. Their role is to remove microorganisms during infections, in addition to interact with and stimulate lymphocytes. In addition, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines [55]. Macrophages that encourage inflammation are called M1 macrophages, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages [55].

Activated macrophages release many inflammatory mediators such as nitric oxide (NO). NO is synthesized from l-arginine by inducible NO synthase (iNOS) expression in numerous mammalian cells and tissues [56]. Under normal physiological conditions, NO plays an important role in the regulation of various pathophysiological processes such as neuronal communication, vasodilatation and neurotoxicity. High concentration of NO can lead to tissue damage and inflammatory diseases such as rheumatoid arthritis, cardiovascular diseases, chronic hepatitis and pulmonary fibrosis [56]. Thus, activation or inhibition of NO production in biological systems might play an important role in the treatment of infections and inflammatory diseases. Chronic inflammation is linked to cardiovascular diseases and diabetes [57].

Dendritic cells have many of the same properties as macrophages and are potent antigen representing cells that play a major role in the initiation and modulation of immune responses [58]. Dendritic cells present microbial antigens to T cells and provide inflammatory signals that modulate T cell differentiation. It is clear that dendritic cells contribute to T cell-independent immune responses. Dendritic cells are well equipped to detect microbial

pathogens by expressing distinct Toll-like receptor (TLR) combinations, enabling recognition of microbial molecules and consequent induction of cytokines [59].

1.5 Anti-diabetic activity

Type 2 diabetes (T2D) is a complex metabolic disorder associated with developing insulin resistance, impaired insulin signaling and β-cell dysfunction, abnormal glucose and lipid metabolism, inflammation and increased oxidative stress [60]. Among the multiple risk factors underlying the incidence and progression of T2D, diet is the main modifiable factor. In this area, recently the use of functional foods and their bioactive components have been considered as a new approach in the prevention and management of diabetes and its complications [60]. Due to their biological properties, polyphenols may be appropriate nutraceuticals and supplementary treatments for various aspects of diabetes mellitus.

1.5.1 Glucose- and oleic acid uptake in skeletal muscle cells and liver cells

The skeletal muscle and the liver play an important role in blood glucose control, storage and utilization of glucose [61]. Skeletal muscle, due to its large mass, is the principal organ for glucose disposal in the body and therefore effects on skeletal muscle cells can have profound effects on glucose homeostasis. It is of great importance to find and characterize insulin-independent pathways stimulating glucose uptake in skeletal muscle. Liver plays a major role in the regulation of blood glucose levels in tight cooperation with peripheral tissues. As estimated, liver is responsible of taking up one third of the postprandial glucose, and stores effectively glucose as glycogen via glycogenesis [60]. Polyunsaturated fatty acids and monounsaturated fatty acids have received a lot of attention due to their health benefits [62]. High consumption of oleic acid, which is a monounsaturated omega-9 fatty acid, reduced the risk of heart disease, diabetes and cardiovascular disease [62]. Oleic acid has also shown a beneficial effect on insulin sensitivity, adipocyte glucose transport and prevents T2D [62-65]. High concentrations of plasma free fatty acids are associated with increased risk for cardiovascular diseases.

1.5.2 Inhibition of α-amylase and α-glucosidase

Small intestinal α-glucosidase and pancreatic α-amylase are key enzymes of dietary carbohydrate digestion in humans. α-Amylase catalyzes the cleavage of α-D-(1-4)glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins into shorter oligosaccharides [66]. α-Glucosidase, which is also located in the brush-border surface

membrane of intestinal cells, activates the final step of the digestive process. These exo-type carbohydrase enzymes catalyzes the hydrolysis of complex carbohydrates and disaccharides to absorbable monosaccharides [66]. Inhibitors of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress postprandial hyperglycemia. The overall effect of inhibition is to reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, diminishing the postprandial effect of starch consumption on blood glucose levels. Currently there are some antidiabetic drugs which are associated with side effects, namely acarbose, miglitol and voglibose. These drugs act by inhibiting α-amylase and α-glucosidase activity [66]. Previously, several *in vitro* studies have been performed yielding potential α-glucosidase inhibitors from various food components and medicinal plants like cranberry, pepper, soy bean extracts, etc., and α-amylase inhibitors from oregano, cranberry extract etc. [4]. Therefore, natural α-glucosidase and α-amylase inhibitors from plant sources offer an attractive strategy for the control of postprandial hyperglycemia.

1.5.3 Free radicals and antioxidant activity

Free radicals are molecules with one or more unpaired electrons, often highly reactive. In our body and elsewhere in nature, O_2 can react with an electron to form the free radical superoxide ($O_2^{\cdot -}$) which can react and produce new reactive radicals [67]. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as reactive oxygen species (ROS), which have a tendency to donate oxygen to other substances and to make new radicals [67]. 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 ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals [68]. If free radicals overwhelm the body's ability to regulate them, a condition known as oxidative stress ensues. Increased oxidative stress appears to be a deleterious factor involved in insulin resistance, dyslipidemia, β-cell dysfunction and impaired glucose tolerance [69].

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 [67]. Testing of antioxidant activities of natural products and their potential beneficial effects on health has received much attention in recent decades. 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 [34].

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

A rapid, simple and inexpensive method to measure antioxidant capacity is the use of the free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH scavenging assay is based on the spectrophotometric measurement of the DPPH concentration change resulting from the reaction with a radical scavenger. On the basis of the chemical reactions involved, antioxidants can deactivate radicals by hydrogen atom transfer or by electron transfer reactions [34]. The DPPH assay was believed to involve hydrogen atom transfer reactions [70, 71], but other studies suggested that an electron transfer reaction is favored in the reaction between phenols having low pKa values and DPPH in strong hydrogen-bond-accepting solvents, such as methanol and ethanol [72, 73]. Radical scavenger effects may be involved in the reaction with receptors or enzymes that are regulated by antioxidant response elements (e.g. Nrf2) that regulate the redox status *in vivo*, and some of the anticipated beneficial health effects of polyphenols are today suggested to be mediated through these mechanisms [74].

1.5.3.2 Inhibition of 15-lipoxygenase (15-LO)

15-Lipoxygenase is an enzyme that catalyzes the stereospecific peroxidation of polyunsaturated fatty acids and esters, which leads to the formation of hydroxyperoxides as well as active radical intermediates that are involved in pathological processes in plants, animals and some bacteria [75, 76]. The primary lipid peroxidation products from arachidonic acid and linoleic acid are hydroperoxyeicosatetraenoic acid and hydroperoxyoctadecadienoic acid respectively [76]. These are readily converted to the hydroxy fatty acids, hydroxyeicosatetraenoic acid and hydroxyoctadecadienoic acid [77]. The 15-LO and their metabolites have been shown to be involved in a number of diseases such as cancer, cardiovascular diseases and diabetes (both type I and II) [75, 77].

1.5.3.3 Inhibition of xanthine oxidase (XO)

Xanthine oxidase (XO) is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and produces uric acid and superoxide [78]. XO is found mainly in the liver and gastrointestinal tract, but also in the kidney, brain and throughout the cardiovascular system [79]. The overproduction and/or underexcretion of uric acid lead to the incidence of hyperuricemia such as gout [80, 81]. An increasing number of researchers during the past

decade have also suggested that XO plays and important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases and cardiovascular diseases such as diabetes [80]. The action of XO enzymes also generates superoxide radicals and hydrogen peroxide, which can add or initiate oxidative stress [81]. Thus, inhibition of XO may therefore be beneficial to treat these aforementioned diseases.

2. AIMS OF THE STUDY

The overall aim of this thesis was to investigate potential health benefits of elderberries and elderflowers with main focus on the immunomodulatory effects and anti-diabetic potential of pectic polysaccharides and phenolic constituents.

Specific aims were as follows:

- To isolate and characterize pectic polysaccharides from elderberries and elderflowers based on bioassay guided fractionation (**Paper I-III**).
- To study the immunomodulating effects and the structure-activity relationship of the purified bioactive pectic polysaccharides from 50% EtOH, 50 °C and 100 °C water extracts from elderberries and elderflowers (**Paper I-III**).
- To study the effects of elderberry and elderflower crude extracts, phenolic constituents and metabolites on glucose- and fatty acid uptake in human skeletal muscle cells and human liver cells and the inhibition of the carbohydrate-hydrolyzing enzymes α-amylase and α-glucosidase in order to provide information on the anti-diabetic potential (**Paper IV-VI**).
- To evaluate *in vitro* antioxidant activity of elderberry and elderflower extracts, phenolic constituents and their metabolites (**Paper IV-VI**).
- To investigate the anti-inflammatory activity of elderberry and elderflower crude extracts, phenolic constituents and their metabolites (**Paper VII**).

3. SUMMARY OF PAPERS

3.1 Paper I

Structure-activity relationship of immunomodulating pectins from elderberries.
Giang Thanh Thi Ho, Abeeda Ahmed, Yuan-Feng Zou, Torun Helene Aslaksen, Helle Wangensteen & Hilde Barsett.

The aim of this work was to isolate pectic polysaccharides from the berries of *Sambucus nigra* and relate the structure to immunomodulating properties. Acidic fractions from 50% EtOH, 50° C and 100° C water extracts obtained after gelfiltration and anion-exchange chromatography showed potent dose-dependent complement-fixating activity and macrophage stimulating activity. The molecular weight of these acidic fractions was in the range of 410-933 kDa. The isolated fractions consisted of long HG regions and RG-I regions, in addition to AG-I and AG-II. Among the isolated fractions, the acidic fractions from 100 °C water extract showed the highest complement fixating activity with IC_{50} values of 19-27 µg/mL and the highest macrophage stimulating activity, detected by the release of nitric oxide. A decrease in Ara content and 1,3,6-linked Gal units resulted in reduced bioactivity after weak acid hydrolysis. The active acidic fractions from 50% EtOH extract, SnBe50-I-S3, was treated with endo-polygalacturonase and then fractionated by gelfiltration to give five sub-fractions. Sub-fraction-I and sub-fraction-II showed a higher complement fixating and macrophage stimulating activity compared to the native polymer. Structure elucidation indicated that RG-I regions with AG-I and AG-II side chains were the predominant structures in the sub-fraction-I, sub-fraction-II and sub-fraction-III. The sub-fraction-IV and sub-fraction-V, which contained high amounts of GalA and typical linkages for HG, showed a reduced bioactivity. These results indicated that branched moieties of arabinogalactans linked to rhamnogalacturonan regions are important for the immunomodulating activity observed for elderberry extracts.

3.2 Paper II

Structural characterization of bioactive pectic polysaccharides from elderflowers (*Sambuci flos*).
Giang Thanh Thi Ho, Yuan-Feng Zou, Torun Helene Aslaksen, Helle Wangensteen & Hilde Barsett.

The objective of this paper was to isolate, purify and characterize the structures of the pectic polysaccharides from elderflowers, and to determine their immunomodulating activity. All the purified acidic fractions from 50% EtOH, 50° C water and 100° C water extracts showed high

complement fixating activity (IC_{50} 2.0-7.5 µg/mL), all having higher activity in a dose-dependent manner compared to the positive control BPII. According to the linkage analysis, the acidic fractions were complex in structure, possibly with a rhamnogalacturonan backbone with side chains of arabinans, AG-I, AG-II and RG-II. An almost complete loss of Ara after treatment with oxalic acid indicated that the Ara was in furanose form. Lower amounts of 1,3,6 Gal, and 1,3 Gal compared to 1,6 Gal indicated that Ara was attached to position 3 of 1,6-linked Gal. After weak acid hydrolysis the complement fixating activity decreased considerably with IC_{50} values above 250 µg/mL. Stimulation of NO production in the macrophages was only observed at the highest concentration (100 µg/mL). The presence of ester groups in the polysaccharide fractions were reduced after treatment with NaOH. After de-esterification of the pectic polysaccharides the activity increased both in the complement fixating and macrophage stimulating tests. The presence of biologically active polysaccharides in elderflower increases the nutritional value of this plant.

3.3 Paper III

RG-I regions from elderflower pectins substituted on GalA are strong immunomodulators.
Giang Thanh Thi Ho, Yuan-Feng Zou, Helle Wangensteen & Hilde Barsett.

The aim of this paper was to further elucidate the structure and structure-activity relationship of the pectic polysaccharides isolated from elderflowers (Paper II). The purified fractions SnFl50-I-S2 from the 50% EtOH extract, 50WSnFl-I-S2 from the 50 °C water extract, and 100WSnFl-I-S2 and 100WSnFl-I-S3 from 100 °C water extract were subjected to enzymatic degradation with endo-polygalacturonase after de-esterification in order to isolate the hairy regions. Two sub-fractions from each fraction were isolated, one high molecular weight sub-fraction-I (25-29 kDa) and one medium molecular weight sub-fraction-II (6-17 kDa). The sub-fractions-I contained GalA:Rha ratio approximately to 1:1, which together with 1,2-linked Rha, 1,2,4-linked Rha and 1,4-linked GalA, indicated that RG-I was the predominant part of the fractions. Highly branched Gal in the sub-fractions-I indicated a complex structure. Sub-fractions-I contained almost equal amounts of 1,4 Gal and 1,3,6 Gal which indicated almost equal amounts of AG-I and AG-II. The sub-fractions-II contained structural features typical for both RG-I and RG-II. In the complement fixating and macrophage stimulating tests the sub-fractions-I showed higher bioactivity compared to both the native fractions and the sub-fractions-II. The important structural requirement for the observed immunomodulating activities seemed to be a rhamnogalacturonan backbone and the combination of both AG-I

and AG-II side chains and the molecular weight. In addition to this, two of three GalA units showed branch points either in C2 or C3 which might be important for the immunomodulating activity.

3.4 Paper IV

Effect of phenolic compounds from elderflowers on glucose- and fatty acid uptake in human myotubes and HepG2-Cells.
Giang Thanh Thi Ho, Eili Tranheim Kase, Helle Wangensteen & Hilde Barsett.

In this study, stimulation of glucose and oleic acid uptake by elderflower extracts, phenolic constituents and metabolites were tested *in vitro* using the human liver cells (HepG2) and human skeletal muscle cells. The 96% EtOH crude extract showed the highest increase of glucose and oleic acid uptake in the human skeletal muscle cells and HepG2-cells, followed by the 50% EtOH and the dichloromethane extract. Among the flavonoids, kaempferol and quercetin showed the highest stimulation of glucose uptake at 10 µM (39.1 ± 5.8% and 37.1 ± 4.6%, respectively), whereas kaempferol showed the highest stimulation of oleic acid uptake (25.0 ± 3.0%) in the human skeletal muscle cells. Rutin showed the highest increase of both glucose- and oleic acid uptake compared to the other glycosylated flavonoids in both cell lines. Chlorogenic acid showed an enhancement of oleic acid uptake at 10 µM of 17.3 ± 2.5% in the skeletal muscle cells and 25.6 ± 4.6% in the HepG2-cells. A small increase of glucose and oleic acid uptake was observed for the flavonoid metabolites 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-Me-catechol. The inhibition of the enzymes α-amylase, α-glucosidase, 15-LO and XO, and the scavenging of DPPH radical were also tested. The alcohol extracts and the phenolic constituents acted as strong antioxidants and showed a strong inhibition of both α-amylase and α-glucosidase. These results indicate that elderflower extracts have potential anti-diabetic properties, which could be explained by its high content of flavonoids and phenolic acids.

3.5 Paper V

Phenolic elderberry extracts, anthocyanins, procyanidins and metabolites influence glucose and fatty acid uptake in human skeletal muscle cells.
Giang Thanh Thi Ho, Eili Tranheim Kase, Helle Wangensteen & Hilde Barsett.

The aim of this work was to study elderberry extracts, pressed juice, anthocyanins, procyanidins, phenolic acids and their metabolites on glucose- and fatty acid uptake in human skeletal muscle cells, as well as their inhibition of α-amylase and α-glucosidase and

antioxidant effects. The alcohol extracts and the pressed juice showed higher stimulation of glucose- and oleic acid uptake compared to the water extracts. The main anthocyanins in elderberries, cyanidin-3-glucoside and cyanidin-3-sambubioside showed a high stimulation of glucose (38.0 ± 2.0% and 44.0 ± 3.7%, respectively) and oleic acid uptake (26.5 ± 2.2% and 29.3 ± 5.1%, respectively) at 10 µM. Among the metabolites, protocatechuic acid, phloroglucinol aldehyde, ferulic acid and caffeic acid showed the highest stimulation of both glucose and oleic acid uptake. The elderberry constituents and metabolites were also good antioxidants, and might play an important role in the controlling of postprandial hyperglycemia by their strong inhibition of the intestinal enzymes α-glucosidase and α-amylase. Metabolites are better absorbed, and might therefore have a more clinical relevance compared to the flavonoids. Based on these results elderberry might be valuable as functional food against diabetes.

3.6 Paper VI

Anti-diabetes and enzyme inhibitory effects of Norwegian berries – a comparison of 14 different berry extracts.

Giang Thanh Thi Ho, Thi Kim Yen Nguyen, Eili Tranheim Kase, Margey Tadesse, Hilde Barsett & Helle Wangensteen.

The aim of this study was to investigate 14 wild or cultivated Norwegian berries on the stimulation of glucose uptake in human liver cells (HepG2), and the inhibition of the enzymes α-amylase, α-glucosidase, 15-LO and XO. The berries were extracted with 80% EtOH by accelerated solvent extraction (ASE) and then purified by C-18 solid phase extraction (SPE). The SPE extract contained 5-25 times more phenolics compared to the ASE extract. Among the ASE extracts, the lingonberry, black chokeberry and elderberry extracts showed the highest glucose uptake in the HepG2-cells. The rowanberry SPE extract showed the highest maximal effect on glucose uptake, followed by cloudberry and crowberry SPE extracts. A high content of chlorogenic acids, ellagitannins, anthocyanins and flavonols might be important contributors to the increased glucose uptake observed for these berries. All the 14 Norwegian berry extracts showed a stronger inhibition against α-amylase and α-glucosidase compared to the anti-diabetic drug acarbose. The ASE extracts were inactive as 15-LO and XO inhibitors. After SPE purification the enzyme inhibitory activities increased, with crowberry and cloudberry extracts as the most active 15-LO inhibitors and bog whortleberry and lingonberry extracts as the most active XO-inhibitors. Removal of free sugars and type of phenolics might be important for the mode of action. In conclusion, a high consumption of

phenolic rich berries might have a potential to reduce the risk for cardiovascular diseases such as type 2 diabetes.

3.7 Paper VII

Elderberry and elderflower extracts, phenolic compounds, and metabolites and their effect on complement, RAW 264.7 macrophages and dendritic cells.
Giang Thanh Thi Ho, Helle Wangensteen & Hilde Barsett.

The main objective of this study was to investigate the effects of the elderberry and elderflower extracts, constituents and their metabolites on the complement fixating activity and on the NO production in LPS stimulated RAW 264.7 macrophages and murine dendritic D2SC/I cells. The 96% EtOH and the acidic MeOH berry extracts showed higher complement fixating activity than any of the berry compounds tested. Also the 96% EtOH flower extract showed higher complement fixating activity compared to the flower components. This could be due to unidentified compounds or to synergistic effects. Anthocyanins and procyanidins showed high complement fixating activity, and high NO-inhibitory activity in the macrophages but were in general less active in the dendritic cells. Flavonol glycosides showed high complement fixating activity, but their aglycones were almost inactive. Most of the flavonoids were very effective as NO inhibitors, but there were great variations in activity between the compounds. Rutin was a stronger NO inhibitor in the dendritic cells than in the macrophages, and also more active than the other quercetin-glycosides. The phenolic acids (chlorogenic acid and neochlorogenic acid) were also somewhat more active as NO inhibitors in the dendritic cells than in the macrophages. The metabolites did not possess any particular activity in the complement system, but some of the metabolites possessed rather strong NO inhibitory activity both in the macrophages and the dendritic cells. Caffeic acid and 3-hydroxyphenylacetic acid were those with the highest NO inhibitory activity. None of the tested compounds showed any cytotoxicity against the macrophages and dendritic cells, except from 4-Me-catechol, which was cytotoxic at 100 µM. These results showed that elderberries and elderflowers might have a potential to regulate inflammatory diseases.

4. RESULTS AND DISCUSSION

4.1 Isolation of pectic polysaccharides

Berries (Be) **(paper I)** and flowers (Fl) **(paper II** and **III)** from *S. nigra* (Sn) were extracted and purified according to the fractionation scheme (Figure 9) in order to isolate pectic polysaccharides. In general, dried pulverized berries or flowers were extracted with 96% EtOH using a Soxhlet apparatus to remove plant compounds of lipid nature as well as low molecular weight compounds. The residue after this treatment was then sequentially extracted with boiling 50% EtOH, 50 °C water and 100 °C water. The obtained crude extracts were then fractionated by gel filtration to give the high molecular weight fractions SnBe50-I or SnFl50-I from 50% EtOH, 50WSnBe-I or 50WSnFl-I from 50 °C water and 100WSnBe-I or 100WSnFl-I from 100 °C water. The high molecular weight fractions were further fractionated by anion exchange chromatography. This led to the isolation of one neutral and four acidic fractions. The neutral fractions (N) were obtained by elution with distilled water and acidic fractions (S1–S4) with 0-1.5 M NaCl gradient. The carbohydrate elution profiles were determined by the phenol–sulfuric acid method [82]. Ten acidic fractions from elderberries (SnBe50-I-S1, SnBe50-I-S2, SnBe50-I-S3 and SnBe50-I-S4 from 50% EtOH, 50WSnBe-I-S2 and 50WSnBe-I-S3 from 50 °C water, and 100WSnBe-I-S1, 100WSnBe-I-S2, 100WSnBe-I-S3, 100WSnBe-I-S4 from 100 °C water) were isolated and subjected to carbohydrate analysis and biological activity tests, and five of these were chosen for further studies. Seven acidic fractions from elderflowers were chosen for further investigation: SnFl50-I-S2 and SnFl50-I-S3 from 50% ethanol, 50WSnFl-I-S2, 50WSnFl-I-S3 and 50WSnFl-I-S4 from 50 °C water and 100WSnFl-I-S2 and 100WSnFl-I-S3 from 100 °C water. The pectic polysaccharide fractions were selected based on their effects in the complement fixating test and macrophage activation, in addition to the amount of material available and content of LPS contamination. The neutral fractions showed no activity in the bioassays, and were not included in further studies. The content of total phenolics was determined by using the Folin-Ciocalteu method [83], and was in the range of 0.1-1.2% for the acidic fraction in elderberries and 0.01-0.7% for the elderflowers. The biorad protein assay [84] showed negligible content of protein for the elderberries (0.1-0.4%) and the elderflowers (0.03-0.5%).

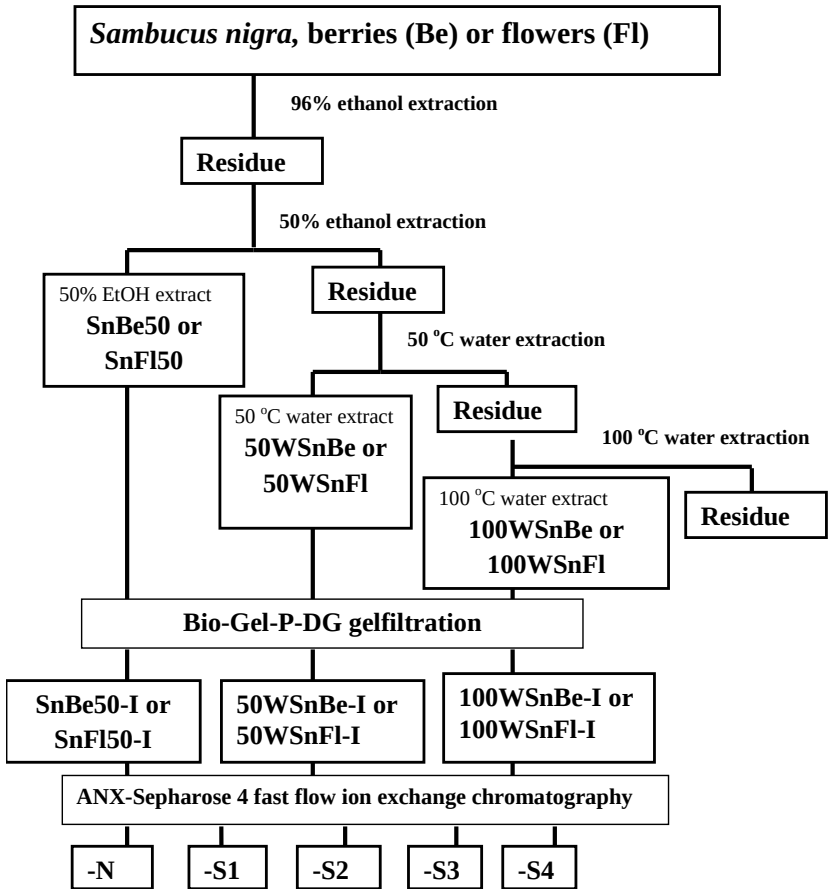


Figure 9. Fractionation scheme of berries (Be) and flowers (Fl) from *S. nigra* (Sn).

4.2 Structure elucidation

4.2.1 Structures of pectins from elderberries

Structural characteristics were determined by methylation and GC-MS of five acidic fractions from elderberries: SnBe50-I-S2, SnBe50-I-S3, 50WSnBe-I-S2, 100WSnBe-I-S1and 100WSnBe-I-S2 (**Paper I**). These five acidic polysaccharide fractions had slightly different monosaccharide compositions, but all contained monosaccharides typical for pectic polysaccharides (Table 1). The approximate molecular weights of the polysaccharide fractions were determined by size exclusion chromatography, comparing the elution profile of the polysaccharide fractions with the dextran standards. The molecular weights for these acidic fractions were determined to be in the range of 618-813 kDa. The fractions SnBe50-I-S2, SnBe50-I-S3, 50WSnBe-I-S2, 100WSnBe-I-S1and 100WSnBe-I-S2 contained glycosidic linkages characteristic for HG and RG-I (Table 1). The main structural feature of the acidic fractions was 1,4-linked GalA, with a few branching points in position 3 of GalA [21]. The

Rha units were 1,2-linked with branch points on position 4 which is a structural feature of RG-I. The low ratio of Rha (1,2 Rha and 1,2,4 Rha) to GalA (1,4 GalA and 1,3,4 GalA) indicated that these fractions consisted of pectic polymers with long HG backbone and shorter areas of RG-I. The presence of t-Gal, 1,3 Gal, 1,4 Gal, 1,6 Gal with branching points at 1,3,6 Gal, in addition to Ara, indicated the presence of AG-I and AG-II. Ara units might be linked to O-3 or O-6 of Gal [85]. The presence of AG-II was also confirmed by the strong positive reaction in the Yariv test. High amount of 1,3,6 Gal and strong positive reaction in the Yariv test observed for SnBe50-I-S2 and SnBe50-I-S3, indicated that these fractions consisted of more AG-II structural units. AG-II has similar sugar moieties as arabinogalactan proteins (AGP), and possible bindings of AGPs with pectins have previously been reported [86-88]. Pectins with AG-II side chains are reported with very small amounts of protein [89]. Since the protein content detected in the isolated acidic fractions was under 0.4%, it is reasonable to anticipate that an AG-II polymer is most likely. The phenolic compounds (<1.2%) which are present in the fractions might give an indication of side chains being esterified with phenolic acids such as ferulic acids [90]. Arabinan might be present in all the fractions as well, indicated by the presence of 1,5 Ara, t-Ara, 1,3,5 Ara, 1,2,5 Ara and 1,2,3 Ara. Arabinan might be linked to Rha in the rhamnogalacturonan backbone through 1,4-linked Gal. This has been found for several other RG-Is isolated from plant cell walls [85]. Terminally GlcA was identified in all the fractions and this might be directly linked to position 3 of 1,4-linked GalA in the RG-I backbone, or may also be a part of the AG-II side chains [91, 92]. High amount of 1,4 Xyl, 1,4 Glc and 1,4,6 Glc in the fractions 50WSnBe-I-S2, 100WSnBe-I-S1and 100WSnBe-I-S2 might indicate the presence of hemicellulose such as xyloglucans. Xyloglucans is a structural feature reported to be present in bilberries [93]. Xylogalacturonan (t-Xyl and 1,3,4 GalA) and arabinoxylan (1,4 Xyl, 1,2 Ara, 1,3 Ara or 1,2,3 Ara) might be another structural features present in these acidic elderberry fractions [94].

Table 1. The glycosidic linkages (mol%), AG-II content, molecular weight, LPS content and KDO present in the fractions obtained by anion exchange chromatography from 50% EtOH (SnBe-I-S2, SnBe-I-S3), 50 °C water (50WSnBe-I-S2) and 100 °C water (100WSnBe-I-S1, 100WSnBe-I-S2) extracts from berries of *Sambucus nigra* before and after weak acid hydrolysis (AH).

	SnBe50-I-S2	SnBe50-I-S2-AH	SnBe50-I-S3	SnBE50-I-S3AH	50WSnBe-I-S2	50WSnBe-I-S2-AH	100WSnBe-I-S1	100WSnBe-I-S1-AH	100WSnBe-I-S2	100WSnBe-I-S2-AH
t-Ara*f*	3.3	-	2.2	0.3	2.1	0.1	5.8	0.3	1.3	0.4
1,2 Ara	1.7	-	1.7	Trace	2.3	-	1.7	Trace	0.8	0.2
1,3 Ara	0.8	-	0.5	0.2	0.8	-	5.5	0.5	1.3	0.1
1,4*p*/ 1,5*f* Ara	3.1	0.6	4.6	0.1	1.2	0.1	8.0	0.5	1.7	1.9
1,3,5 Ara	2.3	-	0.4	-	0.4	-	3.2	0.9	2.0	0.4
1,2,5 Ara	1.2	-	0.8	0.2	3.5	0.3	1.6	-	7.0	Trace
1,2,3 Ara	1.5	-	0.7	Trace	0.4	0.3	2.8	-	1.6	0.6
t-Rha	0.5	0.8	0.1	0.6	0.9	1.7	0.2	0.8	1.8	1.4
1,2 Rha	2.5	2,0	1.7	0.5	1.3	1.5	1.0	3.2	2.6	1.8
1,3 Rha	0.5	0.4	0.3	0.2	0.1	0.6	Trace	-	-	-
1,3,4 Rha	-	-	0.7	0.3	-	-	0.1	-	0.1	0.1
1,2,4 Rha	0.8	-	-	0.1	2.5	-	0.3	0.3	1.4	Trace
t-Xyl	0.4	0.9	0.2	0.7	1.8	1.8	3.3	3.6	5.3	4.8
1, 4 Xyl	0.7	1.9	0.2	0.2	4.0	4.9	11.0	13.3	27.1	30.3
t-Man	3.7	3.0	4.9	3.7	4.4	-	20.1	19.1	6.5	14.1
t-Gal	2.4	2.7	-	6.1	2.1	3.7	1.9	3.3	0.9	1.3
1,3 Gal	7.3	6.9	5.7	5.6	2.0	1.8	5.2	5.0	4.7	4.2
1,4 Gal	1.4	4.5	5.7	2.4	1.9	2.1	0.5	2.6	1.5	2.2
1,6 Gal	1.6	2.6	3.5	1.0	1.3	2.2	0.5	2.9	1.1	1.3
1,2,4 Gal	-	-	3.0	3.9	0.8	3.2	0.4	2.7	Trace	2.7
1,3,6 Gal	3.5	-	2.8	0.2	1.9	0.1	2.0	0.9	2.1	0.1
1,4,6 Gal	-	-	3.9	-	1.1	Trace	1.0	2.3	0.4	-
t-Glc	0.5	-	-	1.5	1.1	1.2	2.0	2.2	1.9	1.8
1,4 Glc	1.5	2.0	1.9	Trace	3.9	3.3	6.8	5.2	4.3	3.5
1,3 Glc	-	-	1.6	5.5	-	0.5	4.8	0.3	Trace	-
1,4,6 Glc	0.3	0.8	-	-	0.3	-	0.5	-	-	-
t-GlcA	0.5	-	0.3	-	1.9	-	0.6	-	1.8	-
1,4 GlcA	0.4	-	0.1	0.9	-	-	-	-	-	-
t-GalA	3.9	4.0	2.1	0.9	1.6	3.4	0.3	2.3	2.7	1.5
1,4 GalA	49.3	61.9	49.1	51.4	53.5	67.2	8.0	26.6	17.7	24.3
1,3,4 GalA	4.2	5.0	1.3	13.5	0.5	-	0.7	1.2	0.2	1.0
t-4-O-Me-GlcA	0.2	-	-	-	0.4	0.2	0.2	-	0.3	-
AGII[a]	++	-	++	-	+	-	+	-	+	-
Mw (kDa)[b]	813	nt	709	nt	618	nt	813	nt	618	nt
% LPS[c]	0.3	0.1	0.1	0.01	1.1	0.1	0.1	0.01	0.2	0.1
KDO[d]	-	-	-	-	-	-	-	-	-	-

[a] The presence of AG-II was determined by precipitation with the β-glycosyl Yariv reagent.
[b] The molecular weight (Mw) was determined by size exclusion chromatography.
[c] The LPS content (%,w/w) was determined by GC-MS.
[d] The presence of KDO was determined by TBA assay.
nt: Not tested

In order to further elucidate the structure and structure-activity relationships, weak acid hydrolysis and enzymatic degradation of the elderberry pectic polymers were performed **(paper I)**. The use of weak acid hydrolysis (AH) was undertaken on the fractions SnBe50-I-S2, SnBe50-I-S3, 50WSnBe-I-S2, 100WSnBe-I-S1and 100WSnBe-I-S2, yielding fractions SnBe50-I-S2-AH, SnBe50-I-S3-AH, 50WSnBe-I-S2-AH, 100WSnBe-I-S1-AH and 100WSnBe-I-S2-AH. Glycosidic linkages of Ara in furanose form are generally more easily hydrolyzed under mild acidic conditions compared to residues in pyranose form. The Ara content and some branch points of the Rha and Gal were decreased after the treatment with oxalic acid. According to the linkage analysis (Table 1) there has been a relative decrease of 1,3 and 1,3,6-linked Gal, and a relative increase of 1,6-linked Gal and terminal Gal after acid hydrolysis. This could be due to arabinosyl units attached to position 3 of Gal and to 1,6-linked Gal. The negative Yariv test also indicated the absence of AG-II after weak acid hydrolysis.

One acidic fraction from 50% EtOH, SnBe50-I-S3, was treated with endo-polygalacturonase resulting in five new sub-fractions (in addition to oligosaccharides): SnBe50-I-S3-I (54 kDa), SnBe50-I-S3-II (44 kDa), SnBe50-I-S3-III (36 kDA), SnBe50-I-S3-IV (19 kDa) and SnBe50-I-S3-V (11 kDA) (Table 2). Endo-polygalacturonase hydrolyses HG regions of de-esterified GalA 1,4-linkages [25]. Linkages normally found in RG-I backbone such as 1,4-linked GalA, 1,3,4-linked GalA and 1,2-linked Rha, and side chains on position 4 of Rha were identified in the sub-fractions. SnBe50-I-S3-I, SnBe50-I-S3-II and SnBe50-I-S3-III consisted of more or larger RG-I regions compared to sub-fractions -IV and -V. The presence of 1,3-linked, 1,6-linked and 1,3,6-linked Gal indicated the presence of AG-II structures in SnBe50-I-S3-I, SnBe50-I-S3-II and SnBe50-I-S3-III. This was also confirmed by a positive reaction with the Yariv reagent. All the sub-fractions contained 1,4-linked Gal which indicated that also AG-I was present. These results suggested that SnBe50-I-S3-I, SnBe50-I-S3-II and SnBe50-I-S3-III are associated with RG-I which consisted of a backbone of rhamnogalacturonan with attached side chains including AG-I and AG-II side chains. The fractions SnBe50-I-S3-IV and SnBe50-I-S3-V showed high content of GalA suggesting areas with HG structure. None of the fractions showed RG-II structural elements. This assumption was based on a negative result in the TBA assay, indicating that KDO was not present. KDO is a relatively rare monosaccharide in pectins, but is an important monomer in RG-II [25]. Neither of the other typical RG-II structures such as 1,3 apiose nor 1,3 fucose were identified.

Table 2. The glycosidic linkages (mol%), AG-II content, molecular weight, LPS content and KDO of the sub-fractions I-V obtained after enzymatic degradation from SnBe50-I-S3 from 50% EtOH extract from berries of *Sambucus nigra*.

	SnBe50-I-S3- I	SnBe50-I-S3-II	SnBe50-I-S3-III	SnBe50-I-S3-IV	SnBe50-I-S3-V
t-Ara*f*	4.5	4.4	1.9	3.7	3.9
1,2 Ara	3.8	5.8	2.9	-	-
1,3 Ara	3.3	2.9	0.9	-	-
1,4*p*/1,5*f* Ara	4.5	2.6	3.1	1.5	1.6
1,3,5 Ara	1.2	1.6	3.7	2.3	1.9
1,2,5 Ara	1.5	1.6	1.3	0.6	-
1,2,3 Ara	0.1	1.1	1.1	0.2	0.1
t-Rha	1.0	1.4	1.6	1.0	0.9
1,2 Rha	3.0	4.8	8.3	7.5	3.3
1,3 Rha	1.7	2.3	1.7	1.0	0.4
1,2,4 Rha	0.6	1.1	1.0	1.1	0.9
1,3,4 Rha	0.9	2.2	2.9	0.2	-
t-Xyl	1.3	1.1	1.0	2.1	3.9
1,4 Xyl	0.6	0.8	0.9	0.6	-
t-Man	2.8	3.8	0.4	0.4	0.5
1,3,6 Man	1.0	0.9	4.3	2.5	1.0
t-Gal	15.0	8.8	8.0	7.5	3.4
1,3 Gal	11.1	8.0	7.9	6.9	4.1
1,4 Gal	5.1	5.1	6.2	4.7	1.1
1,6 Gal	6.1	4.2	1.2	-	-
1,2,4 Gal	3.1	3.1	2.3	3.1	1.1
1,3,6 Gal	5.7	4.9	1.3	-	-
1,4,6 Gal	0.6	0.9	1.0	0.1	0.2
t-Glc	1.2	1.6	2.1	-	-
1,4 Glc	0.9	1.3	1.1	5.4	2.7
1,4,6 Glc	1.3	2.1	1.0	-	-
t-GlcA	-	0.5	1.3	1.2	0.5
1,4 GlcA	2.8	3.8	4.5	2.0	0.6
t-GalA	1.0	1.8	2.3	9.9	19.3
1,4 GalA	6.5	10.0	21.4	19.6	38.0
1,3,4 GalA	7.8	5.5	1.4	14.9	10.6
t-4-O-Me-GlcA	-	-	-	-	-
AGII[a]	++	++	+	-	-
Mw (kDa)[b]	54.1	43.9	35.7	19.2	11.4
% LPS[c]	0.05	0.04	0.02	0.01	0.01
KDO[d]	-	-	-	-	-

[a] The presence of AG-II was determined by precipitation with the β-glycosyl Yariv reagent.
[b] The molecular weight (Mw) was determined by size exclusion chromatography.
[c] The LPS content (%,w/w) was determined by GC-MS.
[d] The presence of KDO wad determined by TBA assay.

4.2.2 Structures of pectins from elderflowers

Pectic polysaccharides from elderflowers were also isolated and purified according to the fractionation scheme (Figure 9). Structural elucidation of seven acidic fractions (SnFl50-I-S2 and SnFl50-I-S3 from 50% ethanol, 50WSnFl-I-S2, 50WSnFl-I-S3 and 50WSnFl-I-S4 from 50 °C water and 100WSnFl-I-S2 and 100WSnFl-I-S3 from 100 °C water) was performed (**paper II**). The molecular weight of these fractions was in the range of 470-813 kDa. Linkages normally found in HG and RG-I backbone were identified in all the fractions (Table 3 and Table 4) [95]. The most acidic fraction from the 50 °C water extract, 50WSnFl-I-S4, consisted mainly of HG, since a higher amount of 1,4-GalA was found compared to the other purified fractions. The relative amounts of Rha versus GalA in SnFl50-I-S3 (1:2.7), 100WSnFl-I-S2 (1:2.6), 100WSnFl-I-S3 (1:2.3) indicated a backbone-chain consisting of either a larger sequence or more RG-I regions compared to other polysaccharide fractions [95]. The acidic polymers might contain RG-II regions due to the presence of the monosaccharide KDO. The variation of linkages to Ara and Gal residues in the different fractions can give large numbers of alternative side chain structures that might be arranged in many different ways along the backbone. The presence of both 1,3-, 1,4- ,1,6-linked Gal units and t-Ara indicates that both AG-I and AG-II are present in the side chains [40]. A positive reaction with the Yariv reagent indicated that these fractions contained AG-II structures. 50WSnFl-I-S4 and 100WSnFl-I-S3 contained lower amounts of 1,3,6 Gal compared to the other fractions and a weaker precipitation in the Yariv. The presence of 1,2,5-linked Ara in addition to terminal Ara, 1,5-Ara and 1,3,5-Ara might indicate arabinan side chains [96]. Small amounts of 1,4-Xyl, 1,4-Glc, t-Xyl and 1,3,4-GalA could be due to xyloglucans and xylogalacturonans [94, 97]. High content of xylose, mannose and glucose in 50WSnFl-I-S3 could be due to hemicellulose associated with pectin. In SnFl50-I-S2, 50WSnFl-I-S2 and 50WSnFl-I-S3 linkages such as 1,4-Xyl substituted with 2-,3- or 2,3-linked Ara residues were detected, which is typical linkages for arabinoxylan [97]. These units may be located in the hairy regions of the acidic fractions [97].

Table 3. The glycosidic linkages (mol%), AG-II content, molecular weight, LPS content and KDO present in the fractions obtained by anion exchange chromatography from 50% EtOH (SnFl50-I-S2, SnFl50-I-S3) and 50 °C water extracts (50WSnFl-I-S2) from flowers of *Sambucus nigra* before and after weak acid hydrolysis (AH).

	SnFl50-I-S2	SnFl50-I-S2-AH	SnFl50-I-S3	SnFl50-I-S3-AH	50WSnFl-I-S2	50WSnFl-I-S2-AH
t-Ara*f*	5.2	-	12.3	-	8.6	-
1,2 Ara	0.8	-	3.5	-	2.5	-
1,3 Ara	0.8	-	0.8	-	2.9	-
1,4*p*/1,5*f* Ara	6.7	0.3	0.9	0.2	5.8	-
1,3,5 Ara	2.3	-	5.2	-	6.0	-
1,2,5 Ara	3.6	-	2.0	-	2.2	-
t-Rha	0.8	0.9	1.6	4.5	1.0	2.0
1,2 Rha	2.4	2.4	6.5	8.9	1.8	2.2
1,3 Rha	0.1	0.1	2.9	5.1	-	-
1,2,3 Rha	0.4	-	-	-	0.7	Trace
1,3,4 Rha	-	-	0.1	2.2	-	-
1,2,4 Rha	1.6	0.9	2.3	-	0.9	Trace
t-Xyl	0.3	Trace	0.4	0.1	0.8	0.2
1,4-Xyl	0.4	0.5	-	-	0.5	-
Man	1.1	Trace	1.3	0.2	1.0	Trace
t-Gal	2.4	5.1	2.5	4.1	2.2	3.2
1,3 Gal	2.3	1.0	3.2	2.5	4.8	1.8
1,4 Gal	7.9	6.6	1.6	1.1	4.7	3.2
1,6 Gal	2.4	3.7	4.3	6.7	2.5	2.9
1,2,4 Gal	1.0	Trace	2.6	5.9	-	-
1,3,6 Gal	6.9	Trace	5.7	Trace	5.0	Trace
t-Glc	1.4	-	0.4	-	1.0	-
1,4 Glc	1.4	2.0	-	1.3	1.0	4.4
t-GlcA	1.2	-	0.2	0.8	0.2	-
1,4 GlcA	0.9	-	1.4	-	0.2	-
t-GalA	2.3	3.4	0.8	3.1	2.2	4.6
1,4 GalA	40.4	69.4	34.2	50.8	35.9	67.3
1,3,4 GalA	1.0	-	1.1	-	3.1	7.0
1,2,4 GalA	1.0	3.3	Trace	-	1.2	-
t-4-O-Me-GlcA	1.0	-	2.1	2.5	1.4	1.2
AGII [a]	++	-	++	-	++	-
Mw (kDa) [b]	813	nt	789	nt	709	nt
% LPS[c]	0.07	0.01	0.12	0.01	0.04	0.01
KDO[d]	+	nt	+	nt	+	nt

[a] The presence of AG-II was determined by precipitation with the β-glycosyl Yariv reagent.
[b] The molecular weight (Mw) was determined by size exclusion chromatography.
[c] The LPS content (%,w/w) was determined by GC-MS.
[d] The presence of KDO was determined by TBA assay.
nt: Not tested

Table 4. The glycosidic linkages (mol%), AG-II content, molecular weight, LPS content and KDO present in the fractions obtained by anion exchange chromatography from 50 °C water (50WSnFl50-I-S3 and 50WSnFl-I-S4) and 100 °C water extracts (100WSnFl-I-S2 and 100WSnFl-I-S3) after weak acid hydrolysis (AH) from flowers of *Sambucus nigra.*

	50WSnFl-I-S3	50WSnFl-I-S3-AH	50WSnFl-I-S4	50WSnFl-I-S4-AH	100WSnFl-I-S2	100WSnFl-I-S2-AH	100WSnFl-I-S3	100WSnFl-I-S3-AH
t-Ara*f*	2.2	-	2.4	-	7.1	-	1.7	-
1,2 Ara	2.2	-	1.0	-	2.0	-	0.4	-
1,3 Ara	0.3	-	1.5	-	2.1	-	0.4	-
1,4*p*/1,5*f* Ara	5.5	-	1.0	-	3.7	-	3.2	-
1,3,5 Ara	4.2	-	1.9	-	1.2	-	1.0	-
1,2,5 Ara	0.1	-	0.8	-	2.1	-	1.1	-
t-Rha	1.2	1.8	4.2	4.3	3.1	4.4	3.8	4.8
1,2 Rha	2.1	2.2	4.6	3.8	6.2	5.4	10.3	10.2
1,3 Rha	-	-	-	-	0.1	0.2	-	-
1,2,3 Rha	Trace	0.4	1.9	2.2	0.4	0.6	2.7	0.1
1,3,4 Rha	0.2	1.2	1.9	2.1	1.2	1.9	2.6	Trace
1,2,4 Rha	1.0	-	4.2	2.2	4.7	3.0	3.9	2.9
t-Xyl	2.5	2.1	1.3	1.9	3.4	2.3	2.4	0.5
1,4 Xyl	5.4	4.9	-	-	-	-	-	0.6
Man	2.5	1.0	0.3	-	0.5	-	0.5	Trace
t-Gal	2.4	1.6	2.1	3.5	3.9	5.8	1.0	5.2
1,3 Gal	1.5	1.0	4.2	3.2	0.3	-	1.2	0.3
1,4 Gal	5.4	5.1	Trace	1.6	4.5	3.9	2.9	6.3
1,6 Gal	1.2	1.4	1.0	1.1	1.9	2.1	Trace	0.6
1,2,4 Gal	3.6	1.9	1.6	-	-	-	0.8	-
1,3,6 Gal	2.6	Trace	1.2	Trace	5.1	Trace	1.5	Trace
t-Glc	2.9	1.1	-	-	0.3	0.3	1.2	-
1,4 Glc	2.8	3.9	2.9	5.4	2.5	3.5	2.1	-
t-GlcA	1.8	-	0.4	-	0.1	-	0.1	-
1,4 GlcA	-	-	-	1.9	0.2	-	-	-
t-GalA	1.1	1.2	3.6	2.8	2.0	3.4	2.7	4.1
1,4 GalA	43.1	67.0	54.1	64.0	36.5	60.1	45.9	58.0
1,3,4 GalA	1.0	2.0	0.5	-	1.2	Trace	5.7	4.2
1,2,4 GalA	-	-	-	-	0.8	1.9	-	2.2
t-4-O-Me-GlcA	1.2	-	1.3	-	0.9	1.2	0.9	-
AGII[a]	+	-	+	-	++	-	++	-
Mw (kDa)[b]	618	nt	470	nt	813	nt	709	nt
% LPS[c]	0.04	0.01	0.03	0.01	0.02	0.01	0.03	-
KDO[d]	+	nt	+	nt	++	nt	++	nt

[a] The presence of AG-II was determined by precipitation with the β-glycosyl Yariv reagent.
[b] The molecular weight (Mw) was determined by size exclusion chromatography.
[c] The LPS content (%,w/w) was determined by GC-MS.
[d] The presence of KDO was determined by TBA assay.
nt: Not tested

The acidic polysaccharide fractions from elderflowers were treated with a weak acid (oxalic acid) which resulted in a loss of Ara and 1,3,6-linked Gal (Table 3 and Table 4). The amount of 1,3-linked Gal was somewhat reduced and 1,6-linked and t-Gal were elevated after weak acid hydrolysis. In sum this indicates that Ara was linked to position 3 of Gal and of 1,6-linked Gal.

The presence of ester in the elderflower acidic fractions was determined by Fourier transform infrared spectroscopy (FT-IR). Ester groups on galacturonic acid have a characteristic absorption at 1735 cm^{-1}, and a clear peak led to the assumption that a certain amount of uronic acid in the fractions was esterified. The ester groups were de-esterified and linkage analysis showed almost the same distribution of linkages as their respective native polysaccharides.

SnFl50-I-S2 from 50% EtOH extract, 50WSnFl-I-S2 from 50 °C water extract, 100WSnFl-I-S2 and 100WSnFl-I-S3 from 100 °C water extract were subjected to enzymatic degradation with endo-polygalacturonase after de-esterification (**paper III)**. This led to the isolation of two pectic sub-units from each mother fraction, one high molecular weight sub-fraction-I (SnFl50-I-S2-I, 50WSnFl-I-S2-I, 100WSnFl-I-S2-I and 100WSnFl-I-S3-I) and one medium molecular weight sub-fraction-II (SnFl50-I-S2-II, 50WSnFl-I-S2-II, 100WSnFl-I-S2-II and 100WSnFl-I-S3-II), in addition to oligogalacturonides. Linkage analysis suggested RG-I like features being the predominant part of the high molecular weight sub-fractions-I with a GalA:Rha ratio of approximately 1:1 (Table 5). Rhamnose was linked 1,2 and 1,2,4 and GalA linked 1,4. In addition two of three 1,4-linked GalA in the rhamnogalacturonan backbone were branched in either position 2 or 3. Highly branched Gal in sub-fractions-I indicated a complex structure, where 31.9-40.8% of the Gal residues existed in 1,2,4-, 1,3,4- or 1,3,6-linkages. The sub-fractions-I with a molecular weight between 25-29 kDa contained almost equal amounts of 1,4 Gal and 1,3,6 Gal, which may indicate equal amounts of AG-I and AG-II. Linkages typical for arabinan were detected in all the fractions. More than 40% of the Ara were 1,5-linked in 100WSnFl-I-S2 indicating a linear arabinan. The arabinans could be attached directly to the rhamnogalacturonan backbone or attached to the arabinogalactan units [21]. T-GlcA or 4-O-Me-GlcA present in the fractions might be linked to AG-II side chains or to the position 2- or 3- of 1,4-linked GalA in the RG-I backbone [91, 92]. It has been reported that a single GlcA residue was attached to O-3 of a 1,4-linked GalA in sugar beet RG-I [92]. Austarheim et al. [98] suggested that a single 4-O-Me-GlcA residue was linked to GalA in the rhamnogalacturonan backbone in *Cola cardifolia*. The amount of 1,3- and 1,3,6-Gal were somewhat higher in sub-fractions-I than in sub-fractions-II suggesting that AG-II is more

prevalent in sub-fractions-I. This was also confirmed by a stronger reaction in the Yariv test. The AG-II moieties present might consist of inner chains composed of 1,3-linked Gal units to which Gal residues are substituted in position 6 by shorter outer side chains consisting of 1,3 or 1,6-linked Gal residues. A proposed structure of RG-I backbone based on glycosidic linkages analysis are shown in Figure 10.

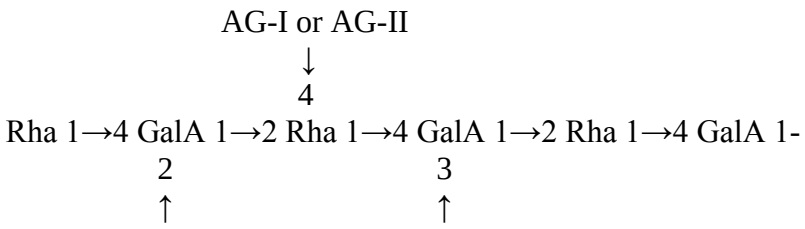


Figure 10. Proposed structure of the backbone of RG-I regions isolated from elderflower pectins.

The medium molecular weight sub-fractions-II with a molecular weight in the range of 6-17 kDa contained many of the same elements as sub-fractions-I. The medium molecular weight sub-fractions-II consisted of more 1,4-linked GalA and linkages characteristic for RG-II type structures (Table 5). T-Gal and 1,2,4 Gal are typical residues of RG-II and also typical in AG-I, and represent more than 50% of the Gal in the sub-fractions-II. The content of 1,4-linked GalA and 1,3,4 GalA could also be due to backbones of both RG-I and RG-II moieties [99, 100]. KDO was detected in all the sub-fractions, but showed a much stronger positive reaction in the sub-fractions-II in the TBA assay. Small amounts of glycosidic linkages characteristic for RG-II type structures, such as t-Ara, t-Rha, 1,2 Rha, 1,3 Rha, 1,2,4 Gal, t-Gal, 1,3 apiose, and 1,3 fucose were detected in sub-fractions-II [101]. In addition, monometylated pentose and monometylated hexose were seen as very small peaks before Ara in the GC chromatogram. Small amounts of RG-II seem to be present in the elderflower fractions. RG-I is more abundant than RG-II in most cell walls, where RG-I represent 10-15% and RG-II 2-5% of pectic polysaccharides [25]. RG-II is a complex structure in the plant cell wall and has been found in several medicinal plants such as *Panax ginseng* [99, 100].

Table 5. The glycosidic linkages (mol%), AG-II content, molecular weight, LPS content and KDO present in the sub-fractions-I (SnFl50-I-S2-I, 50WSnFl-I-S2-I, 100WSnFl-I-S2-I, 100WSnFl-I-S3-I) and sub-fractions-II (SnFl50-I-S2-II, 50WSnFl-I-S2-II, 100WSnFl-I-S2-II, 100WSnFl-I-S3-II) obtained after enzymatic degradation with endo-α-D-(1–4)-polygalacturonase from flowers of *Sambucus nigra.*

	SnFl50-I-S2-I	SnFl50-I-S2-II	50WSnFl-I-S2-I	50WSnFl-I-S2-II	100WSnFl-I-S2-I	100WSnFl-I-S2-II	100WSnFl-I-S3-I	100WSnFl-I-S3-II
t-Ara*f*	9.0	7.2	11.4	7.3	5.7	4.8	3.7	1.6
1,2 Ara	3.3	-	4.4	3.5	2.0	2.0	2.6	1.0
1,3 Ara	1.0	-	1.3	3.1	0.6	3.0	2.4	2.0
1,4*p*/1,5 Ara*f*	5.1	7.5	4.6	2.7	8.8	6.4	4.6	5.2
1,3,5 Ara	1.7	3.3	7.1	5.5	1.4	4.4	2.2	0.5
1,2,5 Ara	2.6	4.5	0.7	2.1	1.1	2.1	0.4	0.8
t-Rha	2.8	5.1	4.7	3.6	4.7	4.3	4.5	2.7
1,2 Rha	3.6	6.5	3.2	4.8	4.4	6.3	6.7	4.2
1,2,3 Rha	-	-	0.7	-	0.9	2.0	4.5	4.2
1,3,4 Rha	-	2.6	2.4	2.0	1.9	3.0	3.6	4.2
1,2,4 Rha	4.1	1.9	3.3	-	4.3	3.0	4.8	-
t-Xyl	0.1	1.2	-	0.4	-	0.6	0.1	1.0
1,4 Xyl	0.8	0.9	1.8	0.2	4.6	Trace	1.5	1.1
t-Man	3.0	1.3	2.0	0.5	1.5	1.4	0.8	1.2
t-Gal	7.1	3.4	3.6	7.1	2.9	5.8	5.1	6.1
1,3 Gal	4.3	3.0	3.5	2.7	3.7	1.1	5.4	2.8
1,4 Gal	8.6	5.5	5.2	4.9	9.9	1.5	5.2	2.9
1,6 Gal	3.7	3.3	2.8	2.5	4.7	1.8	1.4	1.2
1,2,4 Gal	2.4	8.3	3.0	8.8	1.6	4.1	1.1	5.1
1,3,4 Gal	2.8	1.4	2.7	1.8	2.2	1.9	1.7	0.9
1,3,6 Gal	10.1	3.7	4.7	2.6	7.8	2.7	5.2	1.1
t-Glc	-	-	-	-	2.3	1.0	1.1	2.2
1,4 Glc	0.9	0.2	3.6	0.4	4.0	2.0	1.2	1.0
t-GlcA	6.4	2.0	1.3	1.9	0.7	1.7	2.2	3.3
1,4 GlcA	3.1	3.0	1.0	0.5	1.3	2.2	0.8	0.9
t-GalA	1.2	3.2	7.1	1.4	1.5	4.0	5.3	3.0
1,4 GalA	3.8	13.5	4.3	20.6	4.7	19.3	8.1	28.1
1,3,4 GalA	3.2	4.6	3.5	5.9	3.6	3.7	5.2	8.0
1,2,4 GalA	3.4	-	3.0	-	3.8	-	4.4	-
t-4-O-Me-GlcA	1.6	1.3	2.7	1.5	2.9	1.6	4.1	2.1
1,3 Apiose	-	+	-	+	-	+	-	+
1,3 Rha/ 1,3 Fuc	-	+	-	+	-	+	-	+
AGII[a]	++	+	++	+	++	+	++	+
Mw (kDa)[b]	29.0	7.5	29.0	7.5	29.0	17.0	25.0	6.0
% LPS[c]	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.01
KDO[d]	+	++	+	++	+	++	+	++

[a] The presence of AG-II was determined by precipitation with the β-glycosyl Yariv reagent.
[b] The molecular weight (Mw) was determined by size exclusion chromatography.
[c] The LPS content (%,w/w) was determined by GC-MS.
[d] The presence of KDO wad determined by TBA assay.

4.3 Immunomodulating properties and pectin structure requirements

Compounds that are capable of interacting with the immune system to up-regulate or down-regulate specific parts of the host response can be classified as immune modulators. A wide range of plant-derived polysaccharides, such as pectins and β-glucans have been reported to exhibit a variety of immunological activities [21, 40]. The effects of the polysaccharide fractions isolated from elderberries and elderflowers on complement system and macrophages were analyzed *in vitro* and described in **paper I, II** and **III**. In order to study structure–activity relationship, a comparison of the complement fixing activity and macrophage stimulating activity of the original polymer and the fractions obtained after weak acid hydrolysis, ester hydrolysis and enzymatic degradation were performed.

4.3.1 Complement fixating activity

The complement system is essential for the operation of the innate as well as the adaptive immune system. By the activation of the complement system with pectic polysaccharides, the fragments such as C3a and C5a are produced from C3 and C5, and these fragments are believed to modulate the immune system [53]. Due to the important role of the complement system in the immune system, complement modulation is involved in various diseases and considered as an interesting target for inflammatory diseases [53]. Plant-derived polysaccharides, such as pectins, β-glucan, heteroglycans, e.g. glucuronarabinoxylan have shown to exhibit effects on the complement system [53, 54].

The acidic polysaccharide fractions obtained from the elderberries and the elderflowers after anion exchange chromatography showed potent human complement fixation activity *in vitro* (Figure 11) **(papers I-III)**. It is evident from the figures that the acidic pectic polysaccharides isolated from elderflowers showed the highest complement fixating activity compared to elderberries and to the positive control BPII from *Biophytum umbraculum* (syn. *Biophytum petersianum*) [102] with IC_{50} values in the range of 2.0-7.5 µg/mL **(paper II)**. Acidic polysaccharide fractions isolated from the 100 °C water extract of elderberries, showed high complement fixating activity with IC_{50} values in the range of 18.9-27.2 µg/mL **(paper I)**. The high amount of Xyl and Glc in 100WSnBe-I-S1 and 100WSnBe-I-S2, in addition to low amount of the uronic acids could be an explanation of the increased activity. These fractions were somewhat less active than the positive control BPII, but still quite active compared with other polysaccharides previously studied, for example *Plantago major* (Figure 11A) [103].

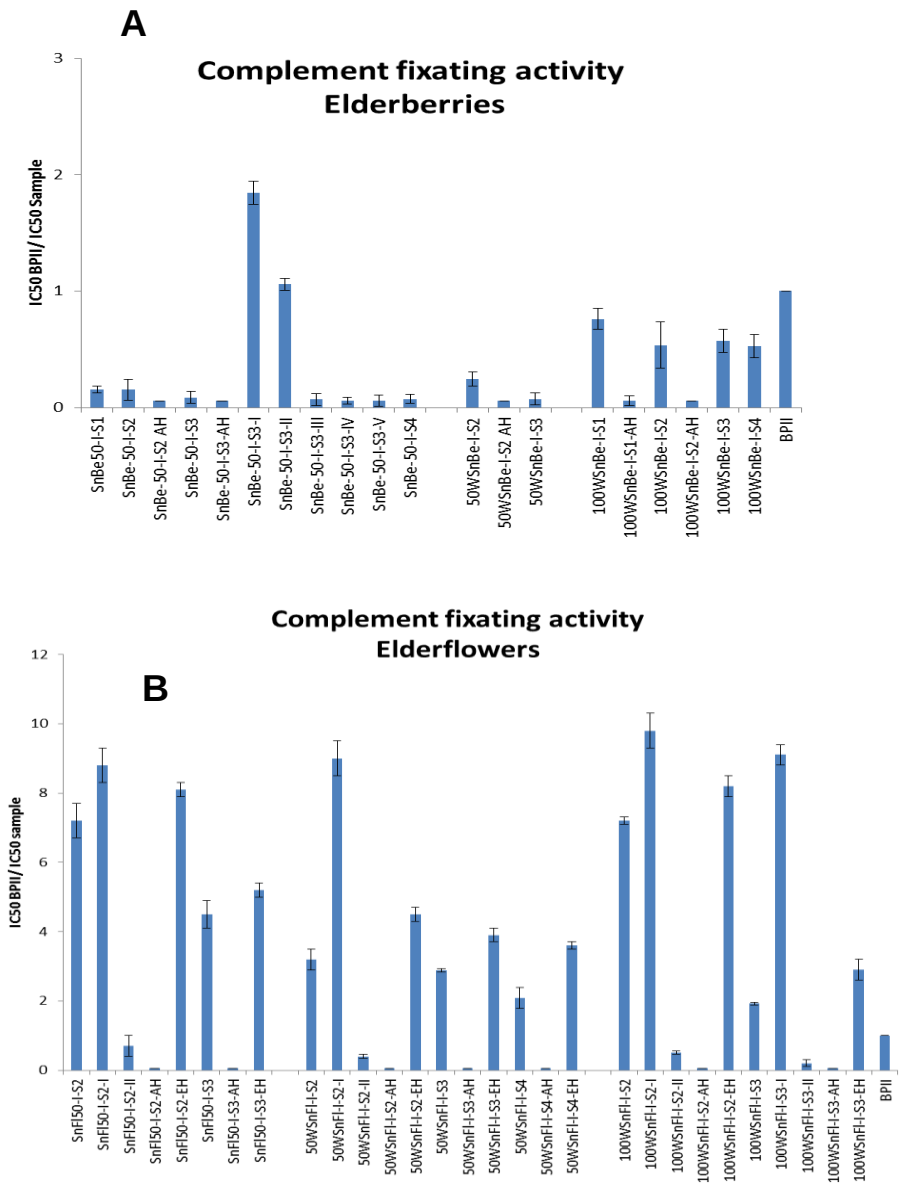


Figure 11. Complement fixation activity, expressed as the ratio IC_{50} BPII/IC_{50} Test sample and shows how active each individual test sample is compared to the positive control BPII from *Biophytum umbraculum* (syn. *Biophytum petersianum*). **A)** Complement fixation activity of the acidic fractions from 50% EtOH (SnBe50-I-S1, SnBe50-I-S2, SnBe50-I-S3, SnBe50-I-S4), 50 °C water (50WSnBe-I-S2, 50WSnBe-I-S3) and 100 °C water (100WSnBe-I-S1, 100WSnBe-I-S2, 100WSnBe-I-S3, 100WSnBe-I-S4) extracts from elderberries, both before and after weak acid hydrolysis (AH) and enzymatic degradation (sub-fraction I-IV from SnBe50-I-S3). **B)** Complement fixation activity of the acidic fractions from 50% EtOH (SnFl50-I-S2, SnFl50-I-S3), 50 °C water (50WSnFl-I-S2, 50WSnFl-I-S3, 50WSnFl-I-S4) and 100 °C water (100WSnBFl-I-S2, 100WSnFl-I-S3) extracts from elderflowers, both before and after weak acid hydrolysis (AH), after ester hydrolysis (EH) and after enzymatic degradation giving two sub-fractions (I-II). The results represent the mean of three independent experiments.

Certain structural features may be important for the complement fixating activity. The activity of the sub-fraction-I and sub-fraction-II from elderberries (SnBe50-I-S3-I and SnBe50-I-S3-II) and sub-fractions-I from elderflowers (SnFl50-I-S2-I, 50WSnFl-I-S2-I, 100WSnFl-I-S2-I and 100WSnFl-I-S3-I) after enzymatic degradation showed higher complement fixation activity than their respective native fractions. These fractions consist mainly of RG-I with AG-I and AG-II side chains, which may explain the high activity observed. Substantial amounts of RG-I regions with neutral side chains composed of Gal and/ or Ara units seemed to be important for the activity. Yamada and Kiyohara [40, 54] have also reported that the hairy RG-I regions have higher activity in the complement fixating assay compared to the native fraction, while oligo-galacturonans have a weak or negligible activity. Neutral side chains consisting of 6-linked galactosyl chains containing t-GlcA or 4-O-Me-GlcA attached to a 3-linked Gal backbone in the ramified region have been proposed to be involved in activation of complement [104].

The sub-fractions-I from the elderflowers consisting of a GalA:Rha ratio of approximately 1:1 showed the highest complement fixating activity. Two of three 1,4-linked GalA units in the rhamnogalacturonan backbone of the high molecular weight sub-fractions-I in elderflowers had branch points either in C2 or C3. This could be important for the immunomodulating activity. The high molecular weight sub-fractions-I contained almost equal amounts of AG-I and AG-II, which may be important for the high complement fixing activity. The combination of AG-I and AG-II side chains have been reported to exhibit higher complement fixating activities than with only AG-II side chains alone [47, 105]. Arabinan are present in a high amount in 100WSnFl-I-S2-I, and might also influence the activity. The sub-fractions-II from the elderflowers, containing some RG-II structural elements, showed lower complement fixation activity than the respective native fraction and sub-fractions-I.

Sub-fractions-III, -IV and -V from elderberries after enzymatic treatment exhibited low complement fixating activity. High amount of GalA, which indicates HG structures, is often considered inactive, and can explain why these fractions have low or no activities in the complement fixing assay [106, 107].

The removal of arabinosyl residues in furanose form from the pectic polymers from elderberries and elderflowers after weak acid hydrolysis, led to a complete reduction in the complement fixing activity with IC_{50} value above 250 µg/mL. Weak acid hydrolysis also led to a decrease of 1,3,6-linked Gal and 1,3-linked Gal, and thereby reduced the side chain

complexity which may have contributed to the reduced activity. This indicates the importance of the side chains containing Ara and Gal for the complement fixating activity of the pectic polymers. It has been reported that removal of Ara in furanose form in an arabinogalactan (type II)-protein from *Echinacea purpurea* resulted in a considerably lower activity in complement-induced hemolysis [53]. The importance of Ara to activate the complement system has also been reported by Inngjerdingen et al. [108]. On the other hand, Yamada and Kiyohara [54] reported that the activation of complement by various pectins is dependent on the presence of the galactosyl chains attached to the rhamnogalacturonan backbone, and that Ara residues are not required. Therefore a certain three-dimensional structure of the exposed side chains may be important for the complement activation. After ester hydrolysis of the pectic polymers from elderflowers the complement fixating activity increased. This leads to the assumption that the amount of ester groups affects the complement fixating activity.

Polysaccharides with high molecular weight are reported to be more active compared to those with lower molecular weights [47, 106]. Sub-fraction-IV and sub-fraction-V from elderberries (11-19 kDa) and sub-fractions-II from elderflowers (6-17 kDa) possessed low complement fixating activity and have the lowest molecular weight. However, the molecular weight of the sub-fractions-I from elderberries and elderflowers was lower than the native acidic fractions, but did still exhibit higher complement fixating activity. The same findings have also been reported previously [104, 109]. Molecular weight is an important factor, but the bioactivity is truly also dependent on chemical compositions and structural differences.

4.3.2 Macrophage activation

Macrophages are specialized cells that neutralize foreign substances, infectious microbes and cancer cells through phagocytosis [22]. Macrophages defend against external pathogens by releasing cytotoxic and inflammatory molecules such as nitric oxide (NO), reactive oxygen species (ROS) and secreting pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1[22]. Polysaccharides can activate macrophages via complement receptor (CR3), mannose receptor (MR), scavenger receptor (SR), dectin-I and toll-like receptor (TLR4) [110]. TLR4 has been identified as a receptor for plant-derived polysaccharides [111]. Binding to TLR4 leads to the production of pro-inflammatory cytokines and iNOS [110]. The ability of the pectic polysaccharides isolated from elderberries and elderflowers to stimulate the mouse macrophages to produce NO is shown in Figure 12 and Figure 13.

The acidic fractions from elderflowers obtained after anion exchange chromatography showed stronger stimulating effects on NO release compared to the similar fractions isolated from elderberries (Figure 12A and Figure 13A). High amounts of Xyl and Glc present in 100WSnBe-I-S1 and 100WSnBe-I-S2 from elderberries could be important for the strong stimulating effect on NO release.

After enzymatic degradation, the sub-fractions-I from elderflowers with amount of Rha:GalA in 1:1 ratio indicating RG-I, showed the high release of NO. The sub-fractions-I, 50WSnFl-I-S2-I and 100WSnFl-I-S2-I, showed a NO release of 16.8 ± 0.9 µM and 16.6 ± 0.9 µM, respectively (Figure 13B). The sub-fraction-I and sub-fraction-II from the elderberries, SnBe50-I-S3-I and SnBe50-I-S3-II, showed a NO release of 10.0 ± 0.03 µM and 9.5 ± 0.03 µM at 100 µg/mL, respectively (Figure 12B). RG-I like features, highly branched Gal and the arabinogalactan side chains in these sub-fractions seemed to be a part of the structural requirements for the induction of macrophage response which also are in agreement with previous report [54]. A minimum amount of AG-II and 6-linked galactosyl side chains has been reported to be important for the polysaccharide fractions for the induction of the macrophage response. However, there is no correlation between increased amount of AG-II and macrophage activation [40, 49].

Sub-fractions-II from the elderflowers which were shown to contain RG-II regions were found to be less active than the native fractions and the sub-fractions-I. RG-II or high relative amount of GalA could be the reasons for the reduced bioactivity. Inngjerdingen et al. [112] isolated a pectic polysaccharide fraction containing RG-II structures from *B. petersianum,* which was found to be less active than RG-I containing fractions. However, in another study RG-II polymer isolated from the leaves of *P. ginseng* and showed IL-6 enhancing properties as well as a potent secretion enhancing activity of the nerve growth factor (NGF) [99]. Different amounts of linkages may lead to different length variation, and these variations may cause activity differences.

The production of NO from the macrophages was observed only in the highest concentration (100 µg/mL) for all the weak acid hydrolyzed fractions from elderberries and elderflowers. This indicates that side chains containing Ara and Gal are important for the activation of the macrophages. An increase of NO production was observed after ester hydrolysis in the fractions obtained from the elderflowers. The presence of ester groups seems to affect the macrophage stimulating activities. This is in agreement to results from the complement test

system. The results from MTT assay did not show any cytotoxic effects against RAW 264.7 macrophages at all the concentration tested (91.9-102.1% cell viability), indicating that the observed effects were not due to cell cytotoxicity. Among the immunomodulating pectins there are differences in the potencies expressed by the individual pectin. Chain length, types of linkages, molecular weight, conformation and availability of active sites may affect the biological activity.

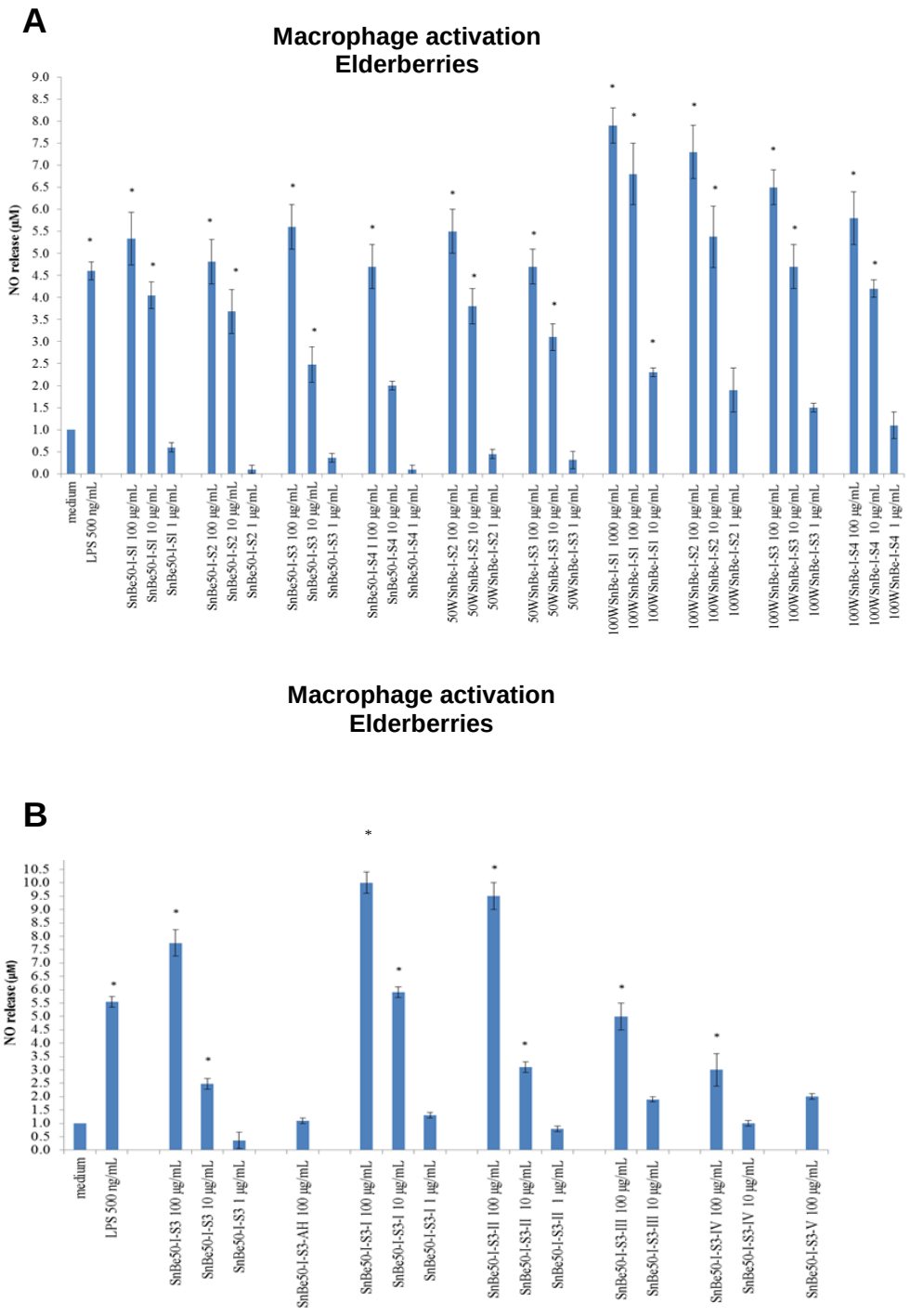


Figure 12. Activation of RAW 264.7 macrophages with pectic polysaccharides isolated from elderberries. **A)** Measurement of nitric oxide (NO) release from RAW 264.7 cells in the acidic fractions obtained by anion exchange chromatography from 50% EtOH (SnBe50-I-S1, SnBe50-I-S2, SnBe50-I-S3, SnBe50-I-S4), 50 °C water (50WSnBe-I-S2, 50WSnBe-I-S3) and 100 °C water (100WSnBe-I-S1, 100WSnBe-I-S2, 100WSnBe-I-S3, 100WSnBe-I-S4) extracts. **B)** NO measurement of the native polymer in SnBe50-I-S3, the fraction obtained after weak acid hydrolysis SnBe50-I-S3-AH and the fractions SnBe50-I-S3-I, SnBe50-I-S3-II, SnBe50-I-S3-III, SnBe50-I-S3-IV and SnBe50-I-S3-V obtained after enzymatic degradation. The figures show NO release (µM) given as means ± SEM (n=3) from separate experiments. *$p < 0.05$ vs. medium.

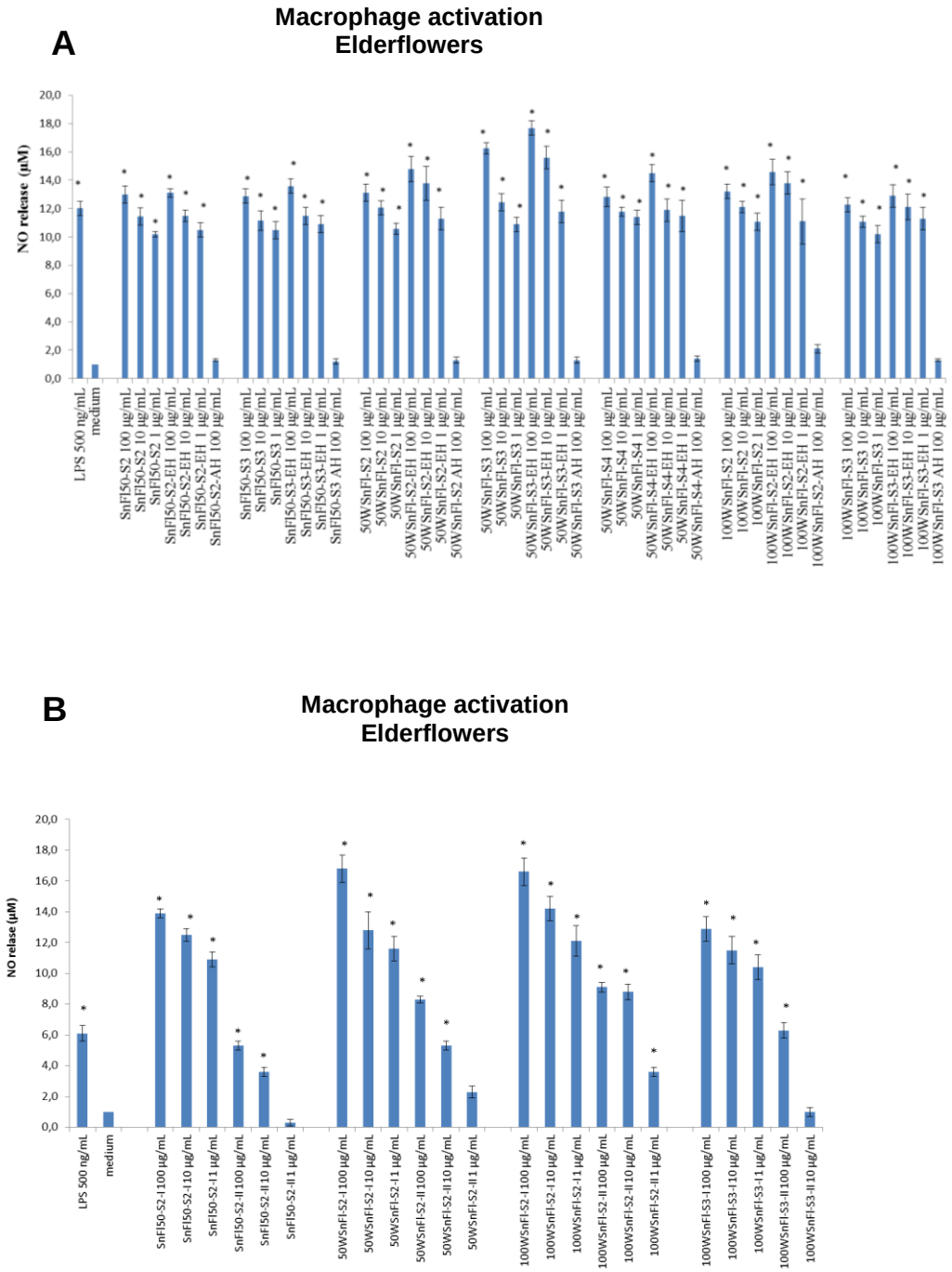


Figure 13. Activation of RAW 264.7 macrophages with pectic polysaccharides isolated from elderflowers. **A)** Measurement of nitric oxide (NO) release from RAW 264.7 cells in the acidic fractions obtained by anion exchange chromatography from 50% EtOH (SnFl50-I-S2, SnFl50-I-S3), 50 °C water (50WSnFl-I-S2, 50WSnFl-I-S3, 50WSnFl-I-S4) and 100 °C water (100WSnBFl-I-S2, 100WSnFl-I-S3) extracts, and the fraction obtained after weak acid hydrolysis (AH) and ester hydrolysis (EH). **B)** NO measurement of the sub-fraction-I (SnFl50-S2-I, 50WSnFl-S2-I, 100WSnFl-S2-I, 100WSnFl-S3-I) and sub-fraction-II (SnFl50-S2-II, 50WSnFl-S2-II, 100WSnFl-S2-II, 100WSnFl-S3-II) obtained after enzymatic degradation. LPS was used as positive control, and medium as negative control. The figures show NO release (µM) given as means ± SEM (n=3) from separate experiments. *$p < 0.05$ vs. medium.

4.4 Consideration regarding potential lipopolysaccharide (LPS) contamination

Contamination of LPS, also known as endotoxin, is an important issue affecting potential biological effects of a test substance. LPS is the major component of the outer membrane of Gram-negative bacteria and one of the most potent immune stimulators. It consists of an O-antigen (or O polysaccharide), a core oligosaccharide and lipid A, and binds to the CD14/TLR4/MD2 receptor complex on many cell types, especially on macrophages and dendritic cells. LPS protects the bacterial outer membrane from chemical attack and contributes to the structural integrity of the bacteria.

The LPS content in the polysaccharide samples was analyzed by GC-MS. The content of LPS in all the fractions tested was estimated to be between 0.01-2.8% **(paper I-III)**. The elderflower fractions at the highest concentration (100 µg/mL) tested in the macrophage test contained LPS contamination between 10-120 ng/mL. All these fractions managed to release more NO compared to the LPS control (500 ng/mL) (Figure 13). The same finding was also observed for several of the elderberry fractions. SnBe50-I-S3-I and SnBe50-I-S3-II (100 µg/mL) which was the most active sub-fractions isolated from elderberries, contained low amount of LPS contamination (50 ng/mL and 40 ng/mL, respectively), showed much higher NO release than the LPS control (500 ng/mL) (Figure 12B). High release of NO in combination with low LPS contamination in the fractions compared to LPS control, anticipates that LPS was not responsible for the total production of NO seen in the active fractions. Interestingly, the LPS content decreased after weak acid hydrolysis, ester hydrolysis and enzymatic degradation possibly due to some degradation of LPS and purification process of polysaccharide sub-fractions. The influence of LPS contamination on the complement fixing activity was disregarded as previous studies reported that the amount of LPS in the polymer did not influence the activity observed [106, 113].

In order to remove the LPS from the fractions various methods were tried. Some of the fractions were passed through a Detoxy-Gel ™ column (polymyxin B), solid phase extraction (SPE) column or EndoTrap ® column. All these columns showed insufficient LPS removal.

4.5 Phenolic constituents and metabolites from *S. nigra*

Freeze-dried and pulverized elderberries (**paper V** and **paper VII**) and pulverized elderflowers (**paper IV** and **paper VII**) were extracted with dichloromethane (DCM) followed by 96% EtOH in an accelerated solvent extraction instrument (ASE). The plant residues were further extracted with 50% EtOH at 70 °C, followed by extraction with 50 °C and 100 °C water (Figure 14). Fresh elderberries were cold-pressed, filtered to remove the peels and seeds and evaporated to obtain the pressed juice. The pressed juice was then heated up to 68 °C to remove the toxic cyanogenic glycosides (**paper V** and **paper VII**). The phenol-sulfuric acid method [82] and ^{1}H NMR analyses revealed that carbohydrates were present in the 50% EtOH, 50 °C and 100 °C water elderberry and elderflower extracts. Based on the ^{1}H and ^{13}C NMR analyses the 96% EtOH elderberry and elderflower extracts showed signals from aromatic compounds, organic acids and carbohydrates. Signals from rutin [114] and caffeoyl moieties [115] were detected in the 96% EtOH elderflower extract. The content of total phenols in the elderberry extracts were determined by using the Folin-Ciocateu method. The highest content of phenolics was found in the pressed juice (69.1 ± 1.1 gallic acid equivalents (GAE)/g extract), the 96% EtOH (55.3 ± 3.0 GAE/g extract) and the acidic MeOH (49.4 ± 2.0 GAE/g extract) extracts.

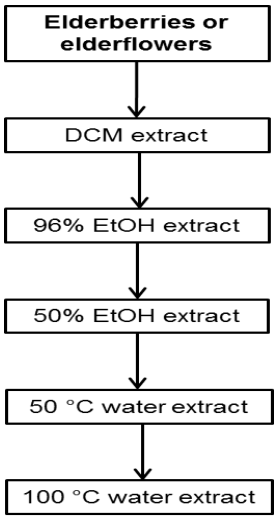
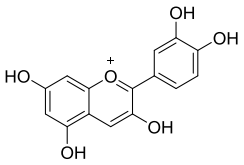


Figure 14. Illustration of the extraction procedure for the elderberries and the elderflowers.

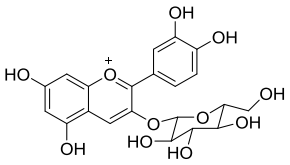
Elderberries in addition to 13 other berries were defatted with hexane prior to extraction with 80% MeOH on an ASE (**paper VI**). The obtained elderberry ASE extract was then purified using a solid phase extraction C18 column (SPE) giving the elderberry SPE extract. The content of total phenols in 80% MeOH elderberry ASE and SPE extracts were determined by using the Folin-Ciocalteu method [83]. The elderberry SPE extract (262.4 mg GAE/g extract) contained more of total phenolis compared to the elderberry ASE extract (53.2 mg GAE/g extract). Based on ^{1}H NMR analysis the methanol ASE extract was dominated by signals from free sugars and organic acids. However, the ^{1}H NMR spectra of the methanol SPE extract clearly showed that the free sugars had disappeared and characteristic signals from phenolic constituents were identified.

In order to isolate anthocyanins, the elderberries were extracted with 0.5% TFA in MeOH at room temperature and partitioned between ethyl acetate and water (**paper V** and **paper VII**). The aqueous phase containing the anthocyanins was purified by Amberlite XAD-7HP and Sephadex LH-20 with eluents of different polarity as described in **paper V** and **VII**. The anthocyanins were eluted with 50% EtOH, and this fraction was purified by preparative HPLC equipped with a Microsorb 60-8 C18 column. HPLC analysis of the obtained anthocyanins were performed on a LaChrom Elite HPLC system equipped with an L-2455 diode array detector. A Chromolith Performance C18 column was used for separation. Cyanidin (270 mg), cyanidin-3-glucoside (340 mg), cyanidin-3-sambubioside (250 mg) from elderberries were isolated and identified (Figure 15) (**paper V**). The anthocyanins are found in glycosylated forms in the elderberries, and the presence of the cyanidin aglycone might be due to degradation from cyanidin-3-glucoside and cyanidin-3-sambubioside. The anthocyanins were identified by NMR spectroscopy (^{1}H, ^{13}C, COSY, APT, HSQC and HMBC) and the spectroscopic data were in accordance with those reported in the literature [116, 117].

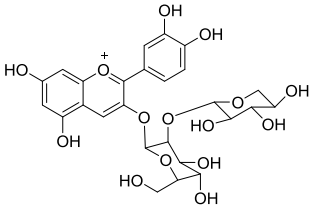
S.nigra contains components with high biological activity, such as flavonols, phenolic acids, anthocyanins and proanthocyanidins [2]. Due to the complex bioavailability of polyphenols, it is difficult to know what the most relevant substance is after intake of elderberries and elderflowers in humans. Hence, the most common constituents from elderberries and elderflowers and their metabolites were included in this study to investigate whether these metabolites could contribute to an effect [2, 35, 36]. Chemical structures of the phenolic constituents from elderberries and elderflowers employed in this thesis are shown in Figure 15 and Figure 16.



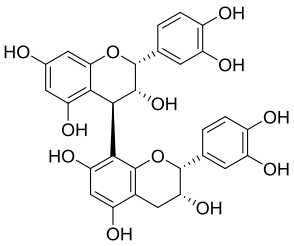
Cyanidin



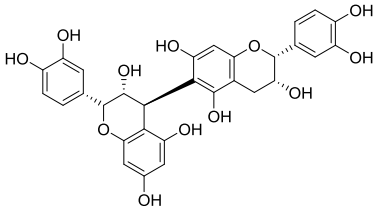
Cyanidin-3-glucoside



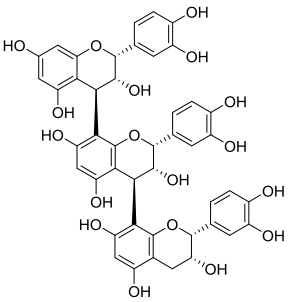
Cyanidin-3-sambubioside



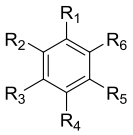
Procyanidin B2



Procyanidin B5

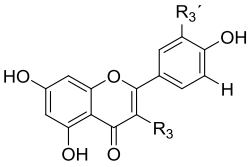


Procyanidin C1

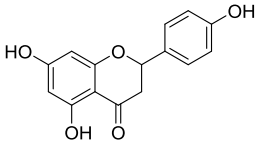


Chemical compound	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**
p-Coumaric acid	CH=CH-COOH	H	H	OH	H	H
Homovanillic acid	CH_2-COOH	H	H	OH	OCH_3	H
Phloroglucinol aldehyde	CHO	OH	H	OH	H	OH
4-Hydroxybenzoic acid	COOH	H	H	OH	H	H
Hippuric acid	CO-NH-CH_2-COOH	H	H	H	H	H
Ferulic acid	CH=CH-COOH	H	OCH_3	OH	H	H
4-Hydroxybenzaldehyde	CHO	H	H	OH	H	H
Protocatechuic acid	COOH	H	H	OH	OH	H
Caffeic acid	CH=CH-COOH	H	H	OH	OH	H
Vanillic acid	COOH	H	H	OH	OCH_3	H

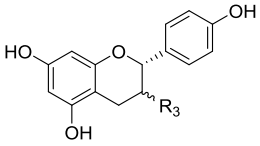
Figure 15. Structures of elderberry constituents and their metabolites.



A

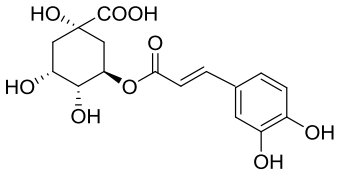


B

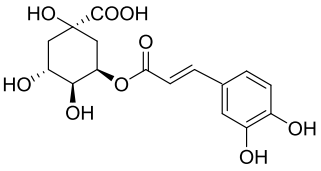


C

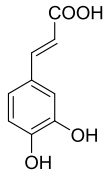
Flavonoid	**Structure**	**R_3**	**R_3´**
Quercetin	A	OH	OH
Quercetin-3-glucoside	A	*O*-glucose	OH
Quercetin-3-rhamnoside	A	*O*-rhamnose	OH
Rutin (quercetin-3-rutinoside)	A	*O*-rutinose	OH
Kaempferol	A	OH	H
Kaempferol-3-rutinoside	A	*O*-rutinose	H
Isorhamnetin	A	OH	OCH_3
Isorhamnetin-3-rutinoside	A	*O*-rutinose	OCH_3
Naringenin	B	-	-
Catechin	C	OH (β)	-
Epicatechin	C	OH (α)	-



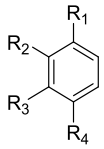
Chlorogenic acid



Neochlorogenic acid



Caffeic acid



D

Metabolites	**Structure**	**R_1**	**R_2**	**R_3**	**R_4**
Benzoic acid	D	COOH	H	H	H
3-Hydroxyphenylacetic acid	D	CH_2-COOH	H	OH	H
3,4-Dihydroxyphenylacetic acid	D	CH_2-COOH	H	OH	OH
4-Me-catechol	D	OH	OH	H	CH_3

Figure 16. Structures of elderflower constituents and their metabolites.

4.6 Biological activity of flavonoids, phenolic acids and metabolites from *S. nigra*

4.6.1 Glucose- and oleic acid uptake in human skeletal muscle cells and human liver cells

Due to the increasing number of people with type 2 diabetes (T2D) (estimated to be 285 million people, corresponding to 6.4% of the world's adult population in 2010), there is considerable interest in identifying compounds that can improve glucose- and fatty acid uptake [118]. Plant based diets have been recommended to reduce the risk of T2D [119]. Glucose uptake into muscle fibers and liver provides the cells with important energy substrate and has major impact on whole body glucose homeostasis [61]. There is a positive correlation between the intake of unsaturated fatty acids and the prevention of T2D [120]. Oleic acid is a common monounsaturated fatty acid in human diet and has a beneficial effect on adipocyte glucose transport and prevents T2D [62]. Thus, substances that stimulate glucose and unsaturated free fatty acid uptake in the skeletal muscles and liver might play an important role in the glucose homeostasis, and are of therapeutic interest in the treatment of T2D.

Among 14 Norwegian berry extracts tested, the extract of elderberries showed high stimulation of glucose uptake in the hepatocellular liver carcinoma cell line (HepG2) (**paper VI**). A closer look on elderberry extracts, constituents and metabolites on their stimulation of glucose- and oleic acid uptake in the human skeletal muscle cells (Figure 17) is further investigated and described in **paper V**. The 96% EtOH and the acidic MeOH extracts from elderberries showed the highest increase of glucose- and oleic acid uptake in the range of 34.0-37.4% compared to DMSO control (Figure 17A-B). The effects of the water extracts were less prominent than the alcohol extracts. The high glucose- and oleic acid uptake of the acidic MeOH and EtOH extracts may be ascribed to their high content of polyphenols. The isolated anthocyanins and proanthocyanidins from elderberries exhibited significant stimulation of glucose- and oleic acid uptake at all concentrations tested (0.1-10 µM) in human skeletal muscle cells (Figure 17 C-D). Cyanidin-3-glucoside and cyanidin-3-sambubioside showed the highest stimulation of glucose uptake at 10 µM. Cyanidin-3-glucoside isolated from Chinese bayberry has been reported to increase glucose uptake in a dose-dependent manner in HepG2-cells [121]. Only small differences were observed between the effects of anthocyanins and the procyanidins on the uptake of oleic acid. Since proanthocyanidin B2 (dimer) and C1 (trimer) appear to be almost equally active at the same

molar concentration, there is no clear correlation between degree of polymerization and glucose- and oleic acid uptake. All the tested metabolites which are detected in urine, plasma or/and feces after elderberries intake showed a dose-dependent increase of glucose uptake. Among the metabolites, protocatechuic acid and pholoroglucinol aldehyde, which are the degradation products of the anthocyanins [35, 36], showed the highest increase of glucose (26.7-28.6%) and oleic acid (30.8-31.3%) uptake at 10 µM (Figure 17 E-F). Caffeic acid, *p*-coumaric acid and ferulic acid are previously found to increase the glucose uptake in porcine myotubes [13]. Homovanillic acid, vanillic acid and 4-hydroxybenzoic acid, among the most abundant metabolites in urine, showed a significant increase of glucose and oleic acid uptake at the highest tested concentration (10 µM). The abundant plasma metabolite, 4-hydroxybenzaldehyde, showed an increase in glucose- and oleic acid uptake at all tested concentrations.

Stimulation of glucose- and oleic acid uptake by elderflower extracts, constituents and metabolites were tested *in vitro* using HepG2-cells and human skeletal muscle cells (Figure 18) (**paper IV**). Among the crude extracts, the 96% EtOH extract showed the highest increase in glucose uptake and oleic acid uptake in human skeletal muscle cells and HepG2-cells followed by the 50% EtOH and the DCM extracts (Figure 18 A-B). Elderflower flavonoids and phenolic acids increased the glucose- and oleic acid uptake in skeletal muscle cells (Figure 18 C-D) and HepG2-cells in a concentration dependent way. Kaempferol, quercetin and naringenin showed the highest glucose- and oleic acid increase at 10 µM in the human skeletal muscles. The same effects were also observed for the HepG2-cells. Kaempferol has been previously reported to increase glucose uptake in porcine myotubes, 3T3-L1 adipocytes and HepG2-cells [13, 122, 123]. Quercetin has been found to increase the glucose uptake in 3T3-L1 adipocytes, but was ineffective in the HepG2-cell lines [123]. The different cell types and experimental setup might be the reasons for the inconsistency in the effects of quercetin found in these two studies. The flavanone naringenin (0.1-10 µM) has been found to increase the glucose uptake in porcine myotubes and also to activate PPARɣ [13, 124]. The glycosides of quercetin, kaempferol and isorhamnetin showed all a high stimulation of glucose and oleic acid uptake. However, glycosylated forms seemed to have a lower increase of glucose uptake compared to the corresponding aglycone. The sugar moiety attached to flavonoid aglycone seems therefore to influence the glucose- and oleic acid uptake in both skeletal muscle cells and HepG2-cells. Among the flavonoid glycosides, rutin showed the highest increase of glucose- and oleic acid uptake at the highest tested concentration. At 10 µM chlorogenic acid

showed an enhancement of oleic acid uptake of 17.3 ± 2.5% in the skeletal muscle cells and 25.6 ± 4.6% in the HepG2-cells. Rutin and chlorogenic acid that are present in high amounts in elderflower [125] might be the most important contributors to the enhanced uptake of glucose- and oleic acid by elderflowers. Epicatechin and its isomer catechin appeared almost equally active at the same molar concentration in the skeletal muscle cells. However, in the HepG2-cells epicatechin showed a significant higher increase of oleic acid compared to catechin at 1 and 10 µM. This indicates that minor structural differences might influence their ability to increase glucose or oleic acid uptake in the HepG2-cells. The degradation products of the flavonoids and phenolic acids, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-Me-catechol, showed a small increase of glucose- and oleic acid uptake at the highest concentration tested (10 µM) in both the skeletal muscle cells (Figure 18 E-F) and HepG2-cells. The metabolites in physiological concentrations increased the glucose- and fatty acid uptake in human skeletal muscle cells and human liver cells. Since the metabolites are better absorbed and will easier reach the systemic tissue, the activity of the metabolites might be more physiologic relevant than their parent compounds (flavonols, anthocyanins). It has been reported that polyphenols and plant extracts increase the glucose uptake in the muscle cell by a mechanism that involves the AMP-activated protein kinase (AMPK) pathway, which might lead to increased plasma glucose transporter (GLUT) 1 and GLUT 4 activity [126, 127]. Phosphatidylinositide 3-kinase (PI3k) might also be involved as a key signaling pathway for up-regulation of glucose- and fatty acid uptake [128, 129]. Different plant extracts such as elderflower, anise and thyme have been reported to have an effect on the PPARs. The PPARs are transcription factors within the large family of nuclear receptors that are crucially involved in the regulation of carbohydrate and lipid metabolism [130].

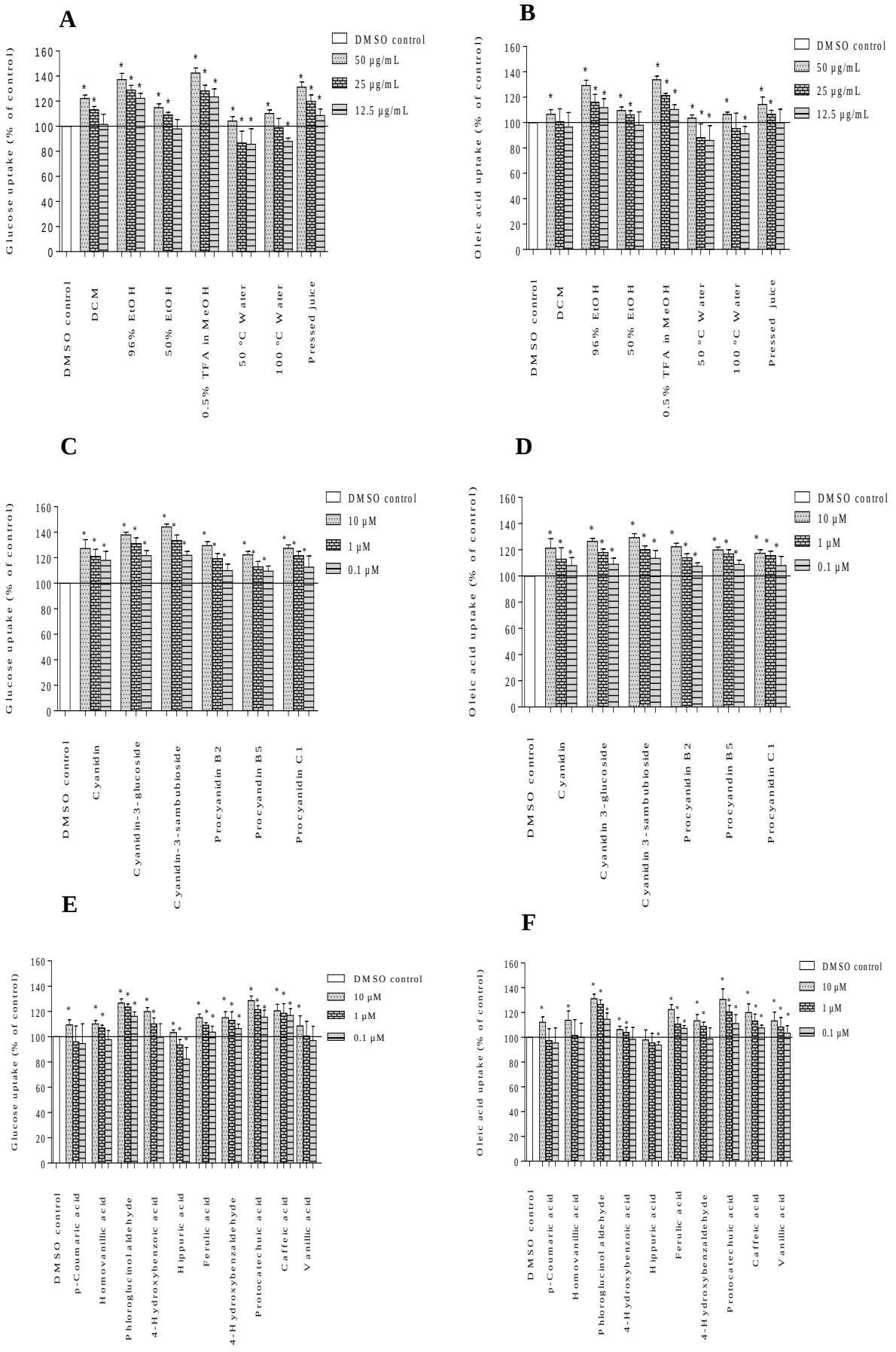


Figure 17. Effects of elderberry extracts, constituents and their metabolites on glucose- (**A,C, E**) and oleic acid (**B, D, F**) uptake in human skeletal muscle cells.

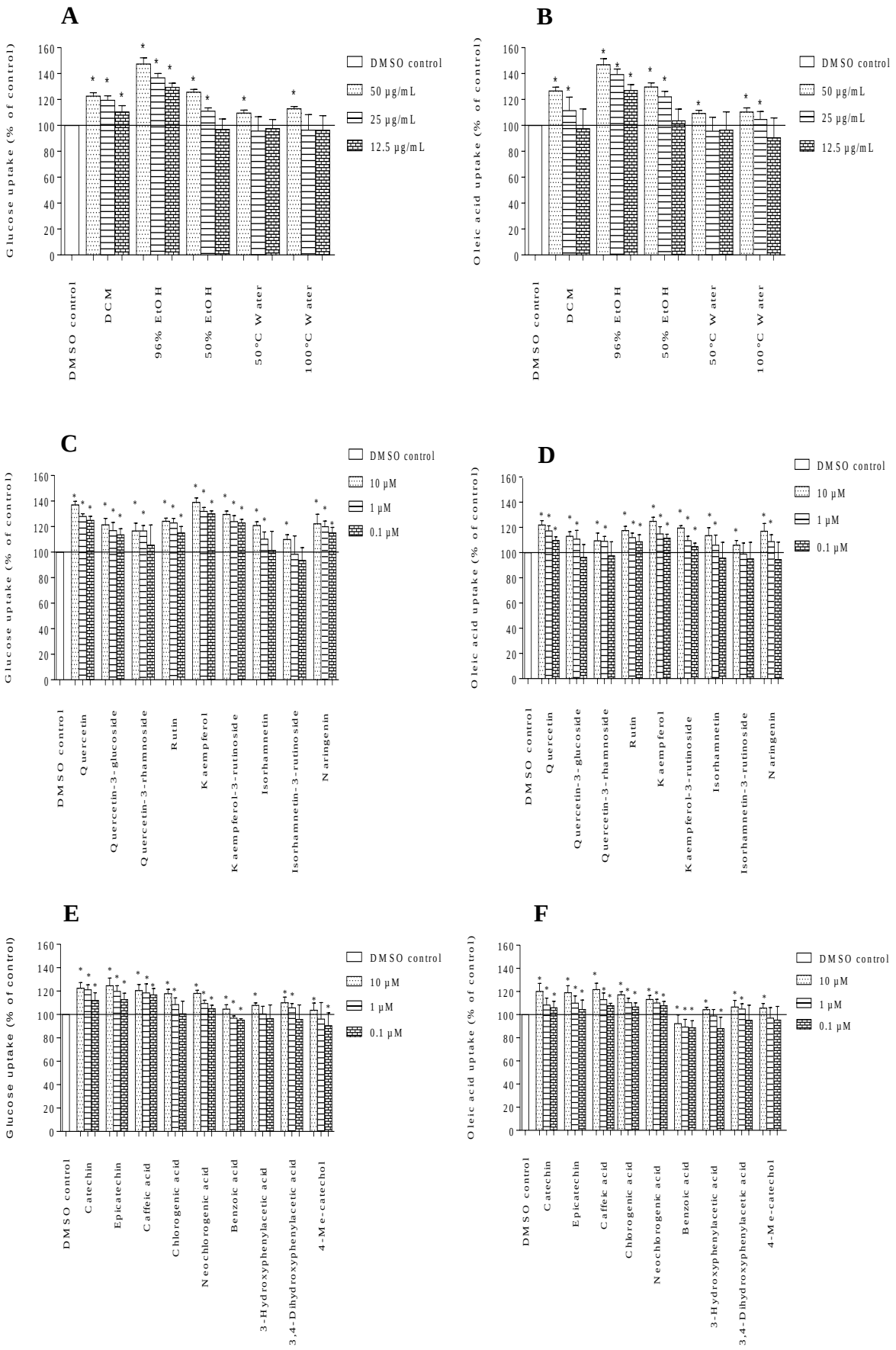


Figure 18. Effects of elderflower extracts, constituents and their metabolites on glucose- (**A,C, E**) and oleic acid (**B, D, F**) uptake in human skeletal muscle cells.

4.6.2 α-Amylase and α-glucosidase inhibitory activity

One important therapeutic approach for treating diabetes is to control postprandial hyperglycemia. The control of postprandial hyperglycemia is critical in the early therapy for diabetes. This can be done through inhibition of the carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase which will delay the digestion of carbohydrates and the absorption of glucose from the intestine [131].

Elderberry and elderflower extracts, constituents and metabolites were investigated for their abilities to inhibit porcine pancreatic α-amylase and yeast α-glucosidase (**paper IV-VI)**. In general, all the elderberry and elderflower crude extracts (except from DCM extracts) possessed stronger inhibition of α-amylase and α-glucosidase compared to the positive control acarbose, which is a widely used antidiabetic drug (Table 6). Among the crude extracts, the 96% EtOH elderflower extract showed strongest inhibition of both α-amylase (IC_{50} 2.8 ± 1.1 µg/mL) and α-glucosidase (IC_{50} 4.8 ± 0.5 µg/mL), followed by the acidic MeOH elderberry extract (IC_{50} 4.6 ± 1.9 µg/mL for α-glucosidase, 3.9 ± 1.3 µg/mL for α-amylase) and the 96% EtOH elderberry extract (IC_{50} 7.2 ± 1.3 µg/mL for α-glucosidase, 6.8 ± 2.1 µg/mL for α-amylase). This might be ascribed to their high content of polyphenols. The 50 °C water and 100 °C water extracts from elderberries and elderflowers showed higher α-glucosidase and α-amylase inhibition compared to acarbose which could be due to the polysaccharides present in these extracts. Polysaccharides have been shown to inhibit α-glucosidase and α-amylase, decreasing blood glucose levels and also act as active hypoglycemic agents [132, 133]. However, the mechanism of action is unclear. In comparison with other Norwegian berries the elderberries showed strong α-amylase and α-glucosidase inhibitory activity (**paper VI**). Interestingly, the elderberry SPE extract was a stronger α-amylase and α-glucosidase inhibitors compared to the elderberry ASE extract (**paper VI)**. Showing that up-concentration of polyphenols and removal of free sugars enhanced the α-amylase and α-glucosidase inhibition. It is known that some anthocyanin extracts from plant exert a potent *in vitro* α-glucosidase inhibitory activity effect [134]. Also, McDougall et al. [135] found that the extent of inhibition of α-glucosidase is related to the anthocyanin content in different berries. Of the isolated anthocyanins in elderberries, cyanidin-3-glucoside and cyanidin-3-sambubioside possessed the highest inhibitory activity against α-glucosidase and α-amylase, much higher inhibition compared to cyanidin and to acarbose (Table 7) (**paper V**). These results indicated that the presences of 3-*O*-glucoside and 3-*O*-sambubioside substituents are important for inducing inhibition of α-amylase and α-glucosidase. The activity of the anthocyanins is

consistent with the literature [136]. The inhibitory activity against α-glucosidase and α-amylase, reduction in blood glucose concentrations and enhanced insulin sensitivity in T2D rats have been previously reported for these anthocyanins [136-138]. Trimeric procyanidin C1 was also found to possess stronger inhibitory activity compared to the dimeric procyandins B2 and B5. This is in agreement with a previous report [136]. The most active flavonoid aglycones from elderflowers were quercetin, kaempferol, naringenin and isorhamnetin (**paper IV**) (Table 7). The inhibitory activity of the flavonoids and the anthocyanins might depend on hydrogen bonds between the hydroxyl groups of the polyphenol ligands and the conjugation position, and the class of sugar moiety that serve as the region for binding to the active site of α-amylase and α-glucosidase [138, 139]. Glycosylation of flavonols decrease the inhibitory effect against α-amylase and α-glucosidase which are observed for quercetin and its glycosides, kaempferol and its glycosides and isorhamnetin and its glycosides. This is in accordance with previous reports [139, 140]. It was suggested that glycoside substitution might lead to steric hindrance which weakens the binding interaction between flavonoids and the α-amylase and α-glucosidase enzymes [139]. However, these results are in contrast to the activity of anthocyanins compared to their aglycone. A study performed by Manaharan et al. [141] reported that myricetin 3-*O*-rhamnoside showed stronger inhibition of α-amylase than of myricetin. Overall, it is difficult to draw general or universally applicable comments regarding the impact of glycosylation on flavonoids' and anthocyanins' biological benefits. High α-amylase and α-glucosidase inhibitory activities were observed for chlorogenic acid and neochlorogenic acids, as well, in accordance with a previous study [139]. Minor structural differences might influence their ability to inhibit α-amylase and α-glucosidase which were observed for catechin and its isomer epicatehin, and chlorogenic acid and its isomer neochlorogenic acid. The inhibitory activity of epicatechin has been reported to be higher than catechin [142]. However, in a separate study catechin showed higher inhibitory activity than epicatechin against α-glucosidase [143].

The metabolites and degradation products were all much more active than the positive control acarbose, where protocatechuic acid, pholoroglucinol aldehyde and caffeic acid were the most active one. In our experiments, protocatechuic acid showed higher inhibitory activity compared to other reports [144, 145]. Interestingly, protocatechuic acid was highly potent as α-glucosidase inhibitor, as opposed to the results by Schäfer and Högger [146], who reported protocatechuic acid to be inactive. It should be noted that inhibitory activity may vary between α-glucosidase or α-amylase from different sources [139].

4.6.3 Antioxidant activity

The antioxidant activities of the elderberry and elderflower crude extracts, constituents and metabolites were evaluated in **paper IV, V** and **VI**. Accumulated evidence has suggested that diabetic patients are under oxidative stress, with an imbalance between the free radical generating and radical scavenging capacities [147, 148]. 15-LO and XO are peroxidative and prooxidative enzymes, respectively, and sources of ROS [148]. Substances that both prevent free radical formation and inhibit the production of ROS might play an important role in the diabetes therapy. 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 elderberry and elderflower substances. The results are summarized in Table 6 and Table 7.

The DCM extract and the water extracts from elderberries and elderflowers were inactive in all the assays. Among all the extracts, the EtOH extracts from elderflower possessed the strongest DPPH-radical scavenging activity. The EtOH and the acidic MeOH extract from elderberries possessed stronger DPPH-radical scavenging activity compared to the water extracts. Alcohol extracts from the elderberries and the elderflowers have been reported high antioxidant activity in DPPH assay, ABTS scavenging assay, FRAP assay and ORAC assay [5, 125, 149-151]. In **paper V**, isolated anthocyanins and procyanidins from elderberries were shown to have a strong radical scavenging activity. The radical scavenging activity and the antioxidant effect of cyanidin and cyanidin-3-glucoside are previously reported [136, 152]. The radical scavenging ability of the procyanidins is in good accordance with the results reported by Bräunlich et al. [153]. Procyanidins have high hydrogen donating capacity, and the number of OH groups is correlated to the molecular size of procyanidins, which is reported to be important for scavenging of free radicals in the reaction with DPPH [154]. Among the constituents in elderflower (**paper IV**), quercetin and kaempferol showed the strongest scavenging of DPPH. These flavonoids are known to possess strong antioxidant activity and are involved in the Nrf2-ARE signaling pathway [140, 155]. Our results are consistent with previous reports on antioxidant activity of flavonoids being dependent on the number and position of substituted OH groups and with the presence of sugar residues [140, 156]. Chlorogenic acid (IC_{50} 17.5 ± 3.9 µM) which is present in large amounts in both the elderberries and elderflowers [157] showed strong DPPH-radical scavenging activity. Chlorogenic acid has shown both *in vitro* and *in vivo* anti-oxidant effect and is correlated with many health-promoting properties [158-160]. The metabolites and degradation products from elderberries and elderflowers possessed moderate radical scavenging activity compared to the

positive control quercetin, with protocatechuic acid, pholoroglucinol aldehyde and 4-Me-catechol as the most active ones.

The inhibitory potency of the extracts and constituents from elderberries and elderflowers towards peroxidation of linoleic acid catalyzed by soybean 15-LO was studied. Among the elderberry crude extracts, the SPE extract and the acidic MeOH possessed moderate 15-LO inhibition (**paper V** and **paper VI**). The 96% and 50% EtOH elderflower extracts possessed much stronger inhibitory activity towards 15-LO compared to the elderberry extracts. The high 15-LO inhibitory activity observed for the flower extracts could not be ascribed to any of the tested constituents, suggesting that the flower extracts may contain additional unidentified 15-LO inhibitors or the effect are caused by synergistic effects (**paper IV**). Procyanidin C1 was the strongest inhibitor among the procyanidins, cyanidin was the strongest among the anthocyanins and quercetin was the strongest among the other flavonoids. The 15-LO inhibitory activity of anthocyanins and procyanidins are in fair accordance with previous investigations [136]. Rutin has previously been reported to be a good 15-LO inhibitor, in good accordance with our findings [161]. Also in this test system the presence of glycosyl groups attached to the flavonoid aglycone decreased the inhibitory activity, which is in agreement with previous report [156]. The metabolites and degradation products from elderberries and elderflowers possessed moderate 15-LO inhibitory activities with protocatechuic acid and phloroglucinol aldehyde as the most active metabolites (IC_{50} 120.8 ± 6.7 µM and 124.2 ± 2.2 µM, respectively).

The inhibitory effect of elderberry and elderflower crude extracts, constituents and metabolites towards the superoxide-producing enzyme XO from cow's milk was studied. The acidic MeOH elderberry extract and the 96% EtOH elderflower extract possessed modest activity in the XO inhibition assay. Anthocyanins and procyanidins possessed weak XO inhibitory ability, while the flavonols (kaempferol, quercetin and isorhamnetin) possessed strong XO-inhibition. The XO-inhibition of kaempferol and isorhamnetin is consistent with previous investigations [162, 163]. Lin et al. [164] has reported that flavonoid aglycones are relatively strong inhibitors of XO, as the presence of a glycosyl group would decrease the inhibitory activity. This seems to be in agreement with our study, where flavonoid glycosides and cyanidin glycosides showed a weaker inhibition against XO compared to its aglycone. Chlorogenic acid (IC_{50} 24.2 ± 5.3 µM) and neochlorogenic acid (IC_{50} 26.2 ± 3.1 µM) were both strong inhibitors of XO, which are in fair accordance with previous results [165, 166].

The metabolites were inactive as XO inhibitors, except for protocatechuic acid, pholoroglucinol aldehyde and caffeic acid which showed moderate activity.

Table 6. Scavenging of DPPH radical, 15-LO, XO, α-glucosidase and α-amylase inhibitory activity of elderberry and elderflower crude extracts. IC_{50} values ± SD are shown.

Test compound	DPPH (µg/mL)	15-LO (µg/mL)	XO (µg/mL)	α-glucosidase (µg/mL)	α-amylase (µg/mL)
Elderberry extracts					
DCM	>167	>167	>167	123 ± 5.3	115 ± 4.9
96% EtOH	138.8 ± 5.1	153.9 ± 3.6	>167	7.2 ± 1.3	6.8 ± 2.1
50% EtOH	158.9 ± 5.2	>167	135.9 ± 4.2	13.9 ± 2.1	15.6 ± 2.3
50°C Water	>167	>167	>167	63.1 ± 3.9	56.8 ± 4.1
100°C Water	>167	>167	>167	45.8 ± 4.6	32.2 ± 3.6
0.5% TFA in MeOH	95.9 ± 3.9	108.3 ± 4.5	89.8 ± 3.9	4.6 ± 1.9	3.9 ± 1.3
Pressed juice	128.3 ± 4.3	143.5 ± 3.9	145.9 ± 4.4	10.8 ± 3.1	8.9 ± 2.6
ASE extract	nt	>167	>167	13.5 ± 2.0	10.7 ± 0.8
SPE extract	nt	100.3 ± 5.4	>167	8.2 ± 0.9	7.1 ± 0.8
Elderflower extracts					
DCM	>167	125.9 ± 3.9	>167	105 ± 5.6	103 ± 5.9
96% EtOH	9.2 ± 0.9	17.9 ± 3.6	59.3 ± 6.3	4.8 ± 0.5	2.8 ± 1.1
50% EtOH	20.2 ± 3.9	24.4 ± 3.1	79.5 ± 4.1	8.9 ± 1.1	3.1 ± 1.3
50 °C Water 100 °C Water	32.0 ± 2.9	75.9 ± 6.5	135.6 ± 7.8	65.3 ± 4.6	66.2 ± 5.6
Quercetin (control)	2.8 ± 0.3	29.3 ± 1.9	0.7 ± 0.2	nt	nt
Acarbose (control)	nt	nt	nt	84.7 ± 3.8	73.3 ± 4.3

nt: Not tested

Table 7. Scavenging of DPPH radical, 15-LO, XO, α-glucosidase and α-amylase inhibitory activity of phenolic compounds from elderberries and elderflowers. IC_{50} values ± SD are shown.

Test compound	DPPH [1] (µM)	15-LO [1] (µM)	XO [1] (µM)	α-glucosidase [2] (µM)	α-amylase [3] (µM)
Phenolic compounds					
Cyanidin	22.1 ± 1.1	102.6 ± 4.4	109.8 ± 2.9	18.4 ± 1.3	16.2 ± 2.7
Cyanidin-3-glucoside	33.6 ± 1.9	132.8 ± 4.8	129.0 ± 2.9	5.0 ± 0.2	3.7 ± 0.8
Cyandin-3-sambubioside	29.2 ± 1.4	123.4 ± 3.2	126.6 ± 2.8	2.8 ± 0.9	2.3 ± 0.5
Procyanidin B2	7.4 ± 0.3	113.6 ± 5.3	110.4 ± 4.7	9.0 ± 0.9	6.6 ± 2.7
Procyanidin B5	8.5 ± 1.1	120.7 ± 3.8	115.6 ± 3.9	11.9 ± 4.7	7.0 ± 5.5
Procyanidin C1	3.2 ± 0.4	104.3 ± 4.5	108.7 ± 5.8	5.2 ± 0.5	2.6 ± 0.9
Quercetin [1]	9.3 ± 1.5	95.9 ± 1.3	2.3 ± 0.3	2.6 ± 0.9	2.1 ± 0.5
Quercetin-3-glucoside	17.6 ± 3.2	102.3 ± 5.3	105.9 ± 5.3	4.1 ± 1.9	3.0 ± 1.2
Quercetin-3-rhamnoside	19.1 ± 2.1	108.4 ± 4.6	104.6 ± 4.6	3.9 ± 1.4	3.5 ± 0.9
Rutin (quercetin-3-rutinoside)	22.5 ± 1.6	99.3 ± 1.1	42.9 ± 2.9	4.6 ± 2.3	4.1 ± 0.8
Kaempferol	10.6 ± 3.9	93.7 ± 3.7	1.8 ± 0.3	4.5 ± 1.2	3.6 ± 1.1
Kaempferol-3-rutinoside	30.6 ± 3.9	108.7 ± 5.6	63.8 ± 2.1	23.9 ± 1.1	19.1 ± 0.5
Isorhamnetin	63.3 ± 2.3	103.1 ± 2.4	2.8 ± 0.7	8.1 ± 3.1	7.5 ± 0.9
Isorhamnetin-3-rutinoside	85.0 ± 2.1	115.3 ± 6.2	125.0 ± 3.9	25.2 ± 2.9	26.2 ± 0.7
Naringenin	23.3 ± 1.4	124.1 ± 3.5	95.1 ± 4.5	7.5 ± 1.1	6.2 ± 1.1
Catechin	19.0 ± 1.1	128.1 ± 5.9	>167	18.5 ± 2.2	14.1 ± 0.8
Epicatechin	15.6 ± 2.3	115.6 ± 7.9	>167	12.1 ± 2.3	9.7 ± 2.1
Chlorogenic acid	17.5 ± 3.9	106.2 ± 2.3	24.2 ± 5.3	10.5 ± 2.1	9.1 ± 1.1
Neochlorogenic acid	19.6 ± 1.6	115.1 ± 5.8	26.2 ± 3.1	13.1 ± 1.3	15.4 ± 3.2
Metabolites					
p-Coumaric acid	>167	129.9 ± 3.2	>167	29.3 ± 4.4	25.9 ± 4.4
Homovanillic acid	>167	153.9 ± 4.2	>167	35.7 ± 3.1	26.3 ± 2.5
Phloroglucinol aldehyde	75.9 ± 3.5	124.2 ± 2.2	114.4 ± 4.5	16.0 ± 1.7	12.5 ± 0.9
4-Hydroxybenzoic acid	>167	155.4 ± 5.8	>167	43.5 ± 2.8	35.4 ± 2.7
Hippuric acid	>167	128.6 ± 4.8	>167	71.2± 3.6	62.8 ± 4.6
Ferulic acid	125.8 ± 5.7	135.4 ± 4.2	>167	19.3 ± 3.4	15.9 ± 4.1
4-Hydroxybenzaldehyde	>167	145.1 ± 5.3	>167	21.3 ± 3.8	14.8 ± 2.8
Protocatechuic acid	66.9 ± 3.2	120.8 ± 6.7	103.3 ± 2.8	13.3 ± 0.8	10.4 ± 0.6
Caffeic acid	90.3 ± 4.3	125.9 ± 4.7	107.3 ± 3.2	18.5 ± 0.9	13.9 ± 0.7
Vanillic acid	>167	129.7 ± 3.3	>167	25.6 ± 3.2	19.5 ± 4.2
Benzoic acid	145.3± 5.8	137.6 ± 6.5	>167	128.9 ± 3.8	124.1 ±5.3
3-Hydroxyphenylacetic acid	125.3± 4.8	133.9 ± 5.8	>167	68.9 ± 3.8	44.8 ± 5.3
3,4-Dihydroxyphenylacetic acid	115.9 ±1.4	135.5 ± 7.3	>167	78.5 ± 1.6	74.9 ± 1.7
4-Me-catechol	40.5 ± 3.6	129.0 ± 5.2	>167	98.9 ± 3.5	94.8 ± 6.8

[1] Quercetin was used as positive control in DPPH, 15-LO and XO assays. [2] Acarbose was used as a positive control (IC_{50} 131.2 ± 9.3 µM). [3] Acarbose was used as a positive control (IC_{50} 113.5 ± 4.6 µM).

4.6.4 Complement fixating activity

Modulation of complement activity may have therapeutic value when related to inflammatory diseases. Elderberry and elderflower extracts, constituents and metabolites were investigated for their effects on the complement system (**Paper VII**). All the crude extracts showed relatively high and dose dependent complement fixating activity, except from the DCM crude extracts. The 96% EtOH elderberry and elderflower extracts were the most active among the crude extracts showing higher activity compared to the highly active positive control BPII (Table 8).

Table 8. Complement fixating activity of crude extracts from elderberries and elderflowers

Test compounds	IC_{50} (µg/mL)[a]
Elderberry extracts	
DCM	>200
96% EtOH	7.8 ± 2.3
50% EtOH	13.4 ± 2.9
0.5% TFA in MeOH	12.3 ± 1.9
50 °C water	44.9 ± 5.3
100 °C water	23.3 ± 3.5
Pressed juice	142.4 ± 13.1
Elderflower extracts	
DCM	>200
96% EtOH	6.5 ± 1.5
50% EtOH	8.9 ± 2.2
50 °C water	32.3 ± 5.2
100°C water	14.4 ± 3.1
BPII (positive control)	17.7 ± 1.5

[a]IC_{50}: Concentration to give 50% inhibition of hemolysis.

Cyanidin, cyanidin-3-glucoside and cyanidin-3-sambubioside, present in high amounts in elderberries showed strong complement fixating activity (Table 9). These results are in good agreement with those reported previously [50, 51]. Anthocyanins seemed to have a lower inhibition of hemolysis compared to the corresponding aglycone. These results may indicate that the effect is influenced by the sugar units linked to the anthocyanidin. Trimeric procyanidin C1 possessed stronger complement fixating activity compared to the dimeric procyanidins B2 and B5 and the monomer epicatechin. Among the flavonoids, rutin, kaempferol-3-rutinoside and quercetin-3-glucoside showed the strongest complement fixating activity. It appeared that the flavonoid glycosides were more active compared to their aglycones in this system, which is also reported previously [50, 167]. The flavonols quercetin, kaempferol, isorhamentin showed low complement fixating activity with IC_{50}> 193.8 µM. In a study performed by Min et al. [168] quercetin was reported to be inactive in the complement

system. An earlier study showed that kaempferol and quercetin-3-rhamnoside had weak complement fixating activity with IC_{50} values of 730 µM and 440 µM, respectively [168, 169]. These results are in contrast to the complement fixating activity observed for anthocyanins and their aglycone in this study. The metabolites from the elderberries and elderflowers showed no particular complement fixating activity at the highest concentration tested (IC_{50} >200 µM).

Table 9. Complement fixating activity of phenolic constituents and metabolites from elderberries and elderflowers.

Test compounds	IC_{50} (µM)[a]
Flavonoids	
Cyanidin	74.2 ± 3.4
Cyanidin-3-glucoside	87.1 ± 5.6
Cyanidin-3-sambubioside	82.8 ± 3.9
Procyanidin B2	70.6 ± 4.5
Procyanidin B5	65.0 ± 3.1
Procyanidin C1	19.4 ± 2.1
Quercetin	193.8 ± 5.6
Quercetin-3-glucoside	76.5 ± 2.6
Quercetin-3-rhamnoside	95.1 ± 3.1
Rutin	40.0 ± 2.6
Kaempferol	>200
Kaempferol-3-rutinoside	70.6 ± 3.8
Isorhamnetin	>200
Isorhamnetin-3-rutinoside	127.8 ± 4.8
Naringenin	>200
Catechin	>200
Epicatechin	>200
Chlorogeninc acid	>200
Neochlorogenic acid	>200
Metabolites	
p-Coumaric acid	>200
Homovanillic acid	>200
Phoroglucinol aldehyde	>200
4-Hydroxybenzoic acid	>200
Hippuric acid	>200
Ferulic acid	>200
4-Hydroxybenzaldehyde	>200
Protocatechuic acid	>200
Caffeic acid	>200
Vanillic acid	>200
Benzoic acid	>200
3-Hydroxybenzoic acid	>200
3,4-Dihydroxyphenylacetic acid	>200
4-Me-catechol	>200

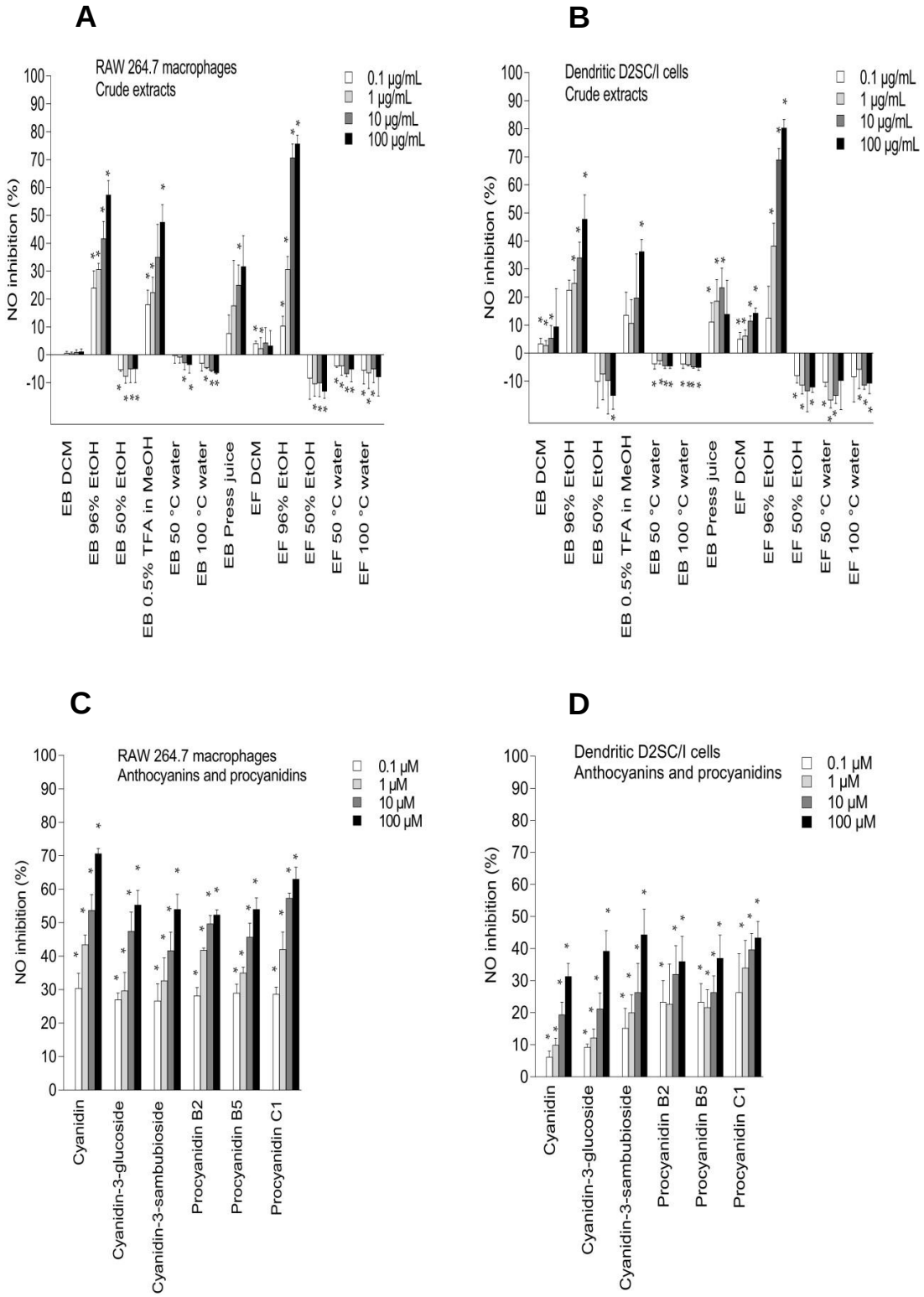
[a]IC_{50}: Concentration to give 50% inhibition of hemolysis.

4.6.5 Inhibition of NO in LPS activated RAW 264.7 macrophages and dendritic D2SC/I cells

Macrophages and dendritic cells play a critical role in the regulation of the immune response [57]. Exposure of macrophages or dendritic cells to external bacterial toxins like lipopolysaccharides (LPS) has extensively been shown to stimulate the secretion of nitric oxide (NO) [170]. The main function of the dendritic cells is to act as antigen presenting cells for T cell activation. Increased levels of NO may inhibit T cell proliferation and apoptosis in dendritic cells [58]. Overproduction of NO induces tissue damage associated with acute and chronic inflammations. Therefore, inhibition of the production of pro-inflammatory mediators such as NO is an important goal in the treatment of various inflammatory diseases such as rheumatoid arthritis, cardiovascular diseases and T2D [56].

Elderberry and elderflower extracts, constituents and metabolites were tested for inhibition of NO production in LPS activated RAW 264.7 macrophages and murine dendritic D2SC/I cells (Figure 19) (**paper VII**). The 96% EtOH elderberry and elderflower extracts showed the highest inhibition on NO production in LPS activated RAW 264.7 macrophages and dendritic D2SC/I cells. High inhibitory activity on NO observed in these extracts might be related to the high content of phenolic compounds or to synergistic effect. The isolated anthocyanins and procyanidins showed all a dose dependent inhibition on NO production in both cell lines. Cyanidin showed the strongest inhibitory effect compared to cyanidin-3-glucoside and cyanidin-3-sambubioside in the RAW 264.7 cells. Cyanidin has been reported previously to be a stronger NO inhibitor than cyanidin-3-glucoside in RAW 264.7 macrophages [56]. Interestingly, cyandin-3-glucoside and cyanidin-3-sambubioside, showed a higher inhibitory effect on NO production compared to its aglycone cyanidin in the dendritic cells at the highest concentration tested. The potency of the procyanidins is somewhat the same as in the complement assay, where procyanidin C1 was the most active. Only minor differences were found between procyanidin B2 and its isomer procyanidin B5. This is in accordance with previous study [51]. The glycosylated forms of quercetin, kaempferol and isorhamnetin showed a lower NO inhibition compared to their aglycones in both the cell lines. Glycosylation of the flavonoids might lead to increased hydrophilicity and steric hindrance, which again results in reduced absorption or penetration into the cells. Similar results have also been reported by Hämäläinen, et al. [171] and Wang and Mazza [56]. The phenolic constituents showed somewhat similar effects of NO-inhibition in macrophages and dendritic cells. However, there were some variations in activity from one compound to the other. Rutin

gave higher NO inhibition in dendritic cells than in the macrophages, and was also more active compared to the other glycosides. Kaempferol on the other side possessed strong NO inhibition in the RAW 264.7 macrophages and weaker in the dendritic D2SC/I cells. The polyphenols seemed therefore to act on different cellular targets in the RAW 264.7 cells and dendritic D2SC/I cells. Chlorogenic acid possessed strong NO inhibition in both cell lines. However, chlorogenic acid was reported to be inactive as NO-inhibitor in RAW 264.7 cells and in J774 macrophages in a previous study [56, 171]. Some of the metabolites showed stronger NO-inhibition compared to its parent compounds. Caffeic acid and 3,4-dihydroxyphenylacetic acid showed a stronger NO inhibition in dendritic cells at 100 µM compared to any of the parent flavonoids. Protocatechuic acid and pholoroglucinol aldehyde, which are the major metabolites from anthocyanins, showed almost the same inhibitory effect on NO production compared to their parent compounds in RAW 264.7 cells at 100 µM. 4-Me-catechol was toxic at 100 µM against RAW 264.7 cells and dendritic D2SC/I cells (45.3-51.2% cell viability) and the strong NO inhibition observed for 4-Me-catechol at 100 µM could be caused by cell cytotoxicity. None of the other tested compounds showed any cytotoxicity. The strong inhibitory activity against NO in both macrophages and dendritic cells suggests that elderberries and elderflowers might have inflammatory modulating properties. Different targets are involved in the anti-inflammatory activities of polyphenols: these can be subdivided into targets related to the arachidonic acid-dependent pathways such as cyclooxygenase (COX) inhibition, lipoxygenase inhibition, and phospholipase A2 inhibition. Within the arachidonic acid-independent pathways, nitrous oxide synthase (NOS), nuclear factor kB (NFkB) and NSAID activated gene-1 (NAG-1) are targets of polyphenols [172].


A
RAW 264.7 macrophages
Crude extracts
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
NO inhibition (%)
EB DCM
EB 96% EtOH
EB 50% EtOH
EB 0.5% TFA in MeOH
EB 50 °C water
EB 100 °C water
EB Press juice
EF DCM
EF 96% EtOH
EF 50% EtOH
EF 50 °C water
EF 100 °C water
B
Dendritic D2SC/I cells
Crude extracts
C
RAW 264.7 macrophages
Anthocyanins and procyanidins
0.1 µM
1 µM
10 µM
100 µM
Cyanidin
Cyanidin-3-glucoside
Cyanidin-3-sambubioside
Procyanidin B2
Procyanidin B5
Procyanidin C1
D
Dendritic D2SC/I cells
Anthocyanins and procyanidins

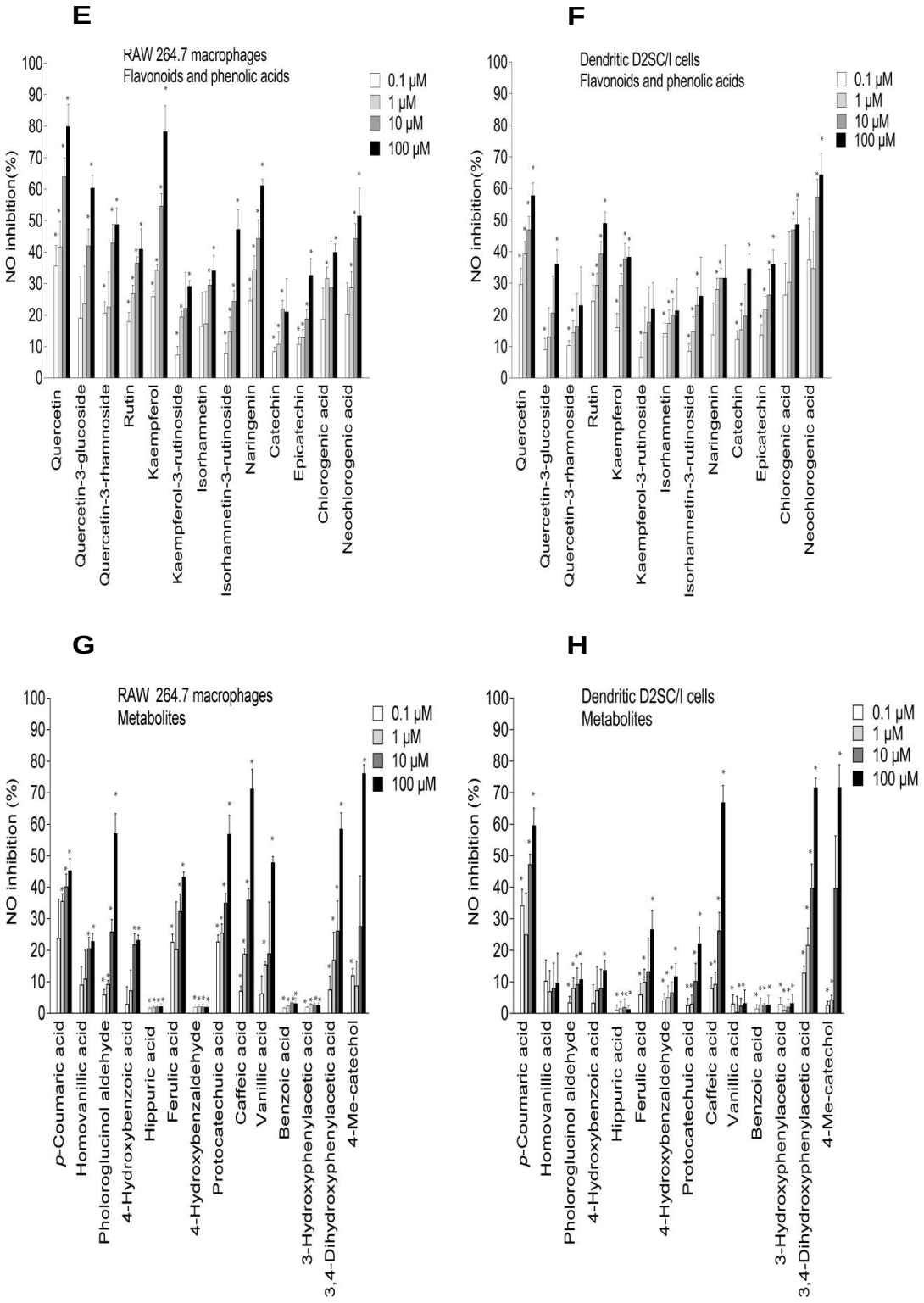


Figure 19. Inhibitory effects of elderberry (EB) and elderflower (EF) crude extracts **(A and B)**, anthocyanins and proanthocyanidins **(C and D)**, flavonoids and phenolic acids **(E and F)** and metabolites **(G and H)** on NO producion in LPS activated RAW 264.7 macrophages **(A, C, E, G)** and dendritic D2SC/I cells **(B, D, F, H)**. The experiments were repeated independently three times, and results shown are expressed as the average ± SEM. * $p < 0.05$ as compared to response of LPS (0.1% DMSO) alone.

5. CONCLUSIONS

The relationship between plant food intake and health has been the focal point of much scientific investigation in recent years to try to identify the specific plant components that convey health benefits. Berries and flowers of *Sambucus nigra* have been used in the traditional medicine, and are today widely used across Europe as food supplements. The present study provides a better understanding of the potential health benefits of pectic polysaccharides and phenolic compounds from elderberries and elderflowers.

Several elderberry and elderflower constituents such as anthocyanins, procyanidins, flavonols, phenolic acids and their metabolites showed strong stimulation of glucose- and fatty acid uptake in human skeletal muscle cells and human liver cells, high antioxidant activity, strong inhibition against the carbohydrate-hydrolyzing enzyme α-amylase and α-glucosidase and immunomodulating activity. The observed biological effects might have a clinical relevance, since the lowest concentrations of some of the phenolics used in this study have been reported *in vivo* as well. The activity of the metabolites might be physiologically more relevant compared to the flavonoids as they are better absorbed and will therefore easier reach the target *in vivo*. The pectic polysaccharides from the elderberries and elderflowers showed strong immunomodulating activity. It is clear that rhamnogalacturonan backbone to which side chains of arabinogalactans were attached was important for both the complement fixating activity and macrophage stimulating activity. The molecular weight, the ramified regions and the degree of branch points are important factors for the immunomodulating activity. The presence of these highly active polysaccharides and polyphenols in elderberries and elderflowers might explain the traditional use of *S. nigra* against diabetes, infections, cold and flu. This could be attributed to the presence of polysaccharides and polyphenols alone or caused by synergistic effects.

Although *in vitro* data on the anti-oxidative, anti-diabetic and anti-inflammatory effects are very promising, current clinical evidence of effectiveness is poor to moderate. Further studies are needed to demonstrate clinical effects.

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PAPERS I-VII

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