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Comparison of Carbohydrate Structures and Immunomodulating Properties of Extracts from Berries and Flowers of *Sambucus nigra* L.

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Authors' Contributions

Author H.B. designed the study, wrote the protocol, supervised, managed the literature searches and wrote the manuscript. Author T.H.A. managed most of the extractions and several analyses of the study. Author P.G. managed the NO-test. Author T.E.M. was responsible for the complement fixing assay. Author B.S.P. organized polysaccharide structural analysis. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To investigate if the immunomodulating activity of compounds present in berries and flowers of *S. nigra* were of the same order, or different, and also if the most active components were of high or low molecular weight nature.
Methodology: Defatted material of berries and flowers of *S. nigra* were extracted with 50% ethanol and with water of 50ºC and 100ºC. High molecular weight fractions were obtained after gel filtration on BioGelP6DG. The different fractions were investigated for their monosaccharide contents and carbohydrate structures. The immunomodulating effects were investigated using a complement fixing assay as well as a system for measuring the production of NO after stimulation of macrophages with the different fractions.
Results: All fractions contained substantial amounts of carbohydrates. Removal of low molecular weight material revealed polysaccharide fractions containing monosaccharides typical for pectins and showed enhanced bioactivity.
Conclusion: High molecular weight fractions from elderflowers showed higher bioactivity

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than the equally extracted fractions from elderberries. The 100ºC water flower fraction gave after gel filtration the fraction with the highest activity and with the longest backbone of rhamnogalacturonan I. Based on this; one should encourage the drinking of juice made form flowers in the same way as has been done for the juice made from berries, since both may have a beneficial heath effect.

Keywords: Sambucus nigra; berries; flowers; complement; macrophage stimulation; carbohydrates.

1. INTRODUCTION

Black Elder, *S. nigra* L. Fam. Adoxaceae (formerly Fam. Caprifoliaceae), is a common shrub in Europe with sweet-smelling flowers and shiny black berries. *S. nigra*, flos and *S. nigra*, fructus, have both a long history of medicinal use for a wide variety of conditions, mainly as a diaphoretic and for treatment of common cold and other feverish conditions. The flowers have also been used as a diuretic remedy and the berries as a laxative (Blumenthal et al., 2000; Weiss and Fintelmann, 2000; WHO, 2002; Fossum et al., 2008). Today, preparations of fruits and flowers are used in the treatment of common cold and influenza symptoms and as an immunostimulant (Fossum et al., 2008).

A clinical trial in 1995 showed reduction of symptoms by consuming a standardized elderberry extract (Sambucol (R)) during an outbreak of influenza B/Panama in Southern Israel (Zakay-Rones et al., 1995). Another clinical trial in 2004 using elderberry extract (Sambucol (R)) showed reduction of symptoms after treatment of influenza A and B virus infection in Norway (Zakay-Rones et al., 2004). Elderberry spray-dried extract at low dose showed a minor effect on serum lipids and antioxidative capacity in a randomized, double-blind, placebo-controlled study (Murkovic et al., 2004). No human clinical studies have been described in the literature on elderflower as a single plant.

In vitro experiments show that fruits of black elder are effective against oxidative stress, viruses and possibly *Helicobacter pylori* and have antiproliferative effects in cell cultures. Animal experiments have shown anti-inflammatory and antioxidant effects and reductions of influenza-like symptoms (Vlachojannis et al., 2010). Several studies have shown that elderberry extracts have immunomodulating activity and induce the cytokines IL-1, IL-6, IL-8 and TNFα (Barak et al., 2002; Waknine-Grinberg et al., 2009). A standardized elderberry liquid extract was also shown to inhibit human pathogenic bacteria as well as human pathogenic influenza viruses (Krawitz et al., 2011).

Flavonoids from elderberry extract were shown to bind to H1N1 virions and, when bound, block the ability of the viruses to infect host cells. Two compounds (5,7,3´4´-tetra-O-methylquercetin and 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl) chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate) were identified as H1N1-binding molecules (Roschek et al., 2009). Other compounds identified in extracts of elderberries are phenolic acids and flavonoids (Christensen et al., 2008), volatile substances (Kaack, 2008a) and pentacyclic triterpenes (Hearst et al., 2010). Several different anthocyanins have been isolated from elderberries and identified (Rieger et al., 2008; Veberic et al., 2009). Anthocyanins were detected in human plasma after oral administration of an elderberry extract, first time in 1999 (Cao and Prior, 1999).

In vitro data show that flowers of black elder have antioxidant, anti-inflammatory, antiviral and insulin-like activity, and animal experiments show anti-inflammatory, diuretic and secretolytic activity (Fossum et al., 2008; Barros et al., 2011). Phytochemicals identified in elder flower or berry were toxic to nosocomial pathogens, particularly *S. aureus* (MRSA) (Hearst et al., 2010).

Compounds identified in extracts of elderflowers are phenolic acids and flavonoids (Christensen et al., 2008; Barros et al., 2011), volatile substances (Kaack, 2008b) and pentacyclic triterpenes (Hearst et al., 2010). There is no single identified compound in *S. nigra* preparations that can be associated with the medicinal effects.

Several of the medicinal plants used in traditional medicine contain polysaccharides showing biological effects related to different part of the immune system (Yamada and Kiyohara, 1999; Yamada and Kiyohara, 2007; Yamada et al., 2009). Polysaccharides with biological activity often contain uronic acids, as in pectins. Among the structural moiety of pectins, the ramified region often contains the active sites for the complement fixing activity, mitogenic activity and enhancement of immune complex clearance (Paulsen and Barsett, 2005). There are no studies on polysaccharides with immunomodulating activity from *S. nigra* reported in the literature.

In the present study, fractions rich in pectic polysaccharide were isolated by the same procedure from fruits and flowers of *S. nigra* and compared with respect to carbohydrate composition, structural patterns, complement fixing activity and macrophage activation.

2. MATERIALS AND METHODS

2.1 Plant Material

Dried berries (prod. no: 100308) and flowers (prod. no: 100309) of *S. nigra* L, were purchased from Odin´s Marked, Norway (org.no: 876905892). Certified ecologically cultivated flowers and berries were imported from Hungary. The plant material was pulverized (0, 4 mm) by a mechanical grinder and stored dry in closed vessels below 5ºC.

2.2 Extraction

The extraction and fractionation procedure is outlined in Fig. 1. Low molecular weight compounds and lipophilic constituents, were removed from the plant material of *S. nigra* by exhaustive Soxhlet extraction with 96% ethanol (Nergard et al., 2004), using plant material/solvent ratios of 1:4 (w/v). The Soxhlet extraction was performed until the extract was colorless. The residual plant material of the berries and flowers were further extracted with boiling 50% aqueous ethanol for 2 hour and repeated until the extract was colorless. The extracts were filtered through Whatman GF/A glass fiber filter. The ethanol in the combined extracts for each plant part were evaporated by rotary evaporation under reduced pressure and 40ºC, and the fractions were dialyzed against distilled water in a Spectra/Por Membrane dialysis tube (Spectrum) with molecular weight cut off of 3.5 kDa, then freeze dried and called SnBe50 and SnFl50 respectively.

The procedure followed by extraction of the plant material of berries and flowers with 50ºC H_2O, pH 5, two times for 2 hours and by extraction of the residual plant material with 100ºC two times for 2 hours. All extractions were performed under reflux. The extracts were filtered,

and the combined water extracts for each extraction and plant material were concentrated by rotary evaporation under reduced pressure and 40ºC, dialyzed and freeze dried. The fractions given by 50ºC water extraction were called 50WSnBe and 50WSnFl respectively and the fractions given by 100ºC water extraction were called 100WSnBe and 100WSnFl.

2.3 Gel Filtration on Bio-Gel P-6 DG

The dialyzed and freeze dried fractions (3 g) were dissolved in degassed distilled water (100 ml), filtered through Millex®-HA 0.45 µm filter (Millipore) and applied onto a Bio-Gel P-6 DG (Bio-Rad) column (Ø: 5,0 cm, L: 26 cm). The samples were eluted with degassed distilled water at 1,0 ml/min, and 10 ml fractions were collected. The carbohydrate profile was monitored using the phenol-sulphuric acid method (Dubois et al., 1956). The absorption at 490 nm was plotted as a function of elution volume, and the relevant high molecular weight fractions from each crude extract were pooled. Dextran standard T500 (500kDa) and raffinose were used for calibration of the column. The high molecular weight fractions obtained from the original fruit/berry fractions were called SnBe50-I, 50WSnBe-I and 100WSnBe-I and the high molecular weight fractions obtained from the original flower fractions were called SnFl50-I, 50WSnFl-I and 100WSnFl-I.

2.4 Carbohydrate Composition

The samples (1 mg) were subjected to methanolysis with 4 M HCl in anhydrous MeOH for 24 hours at 80ºC (Chambers and Clamp, 1971; Barsett and Smestad Paulsen, 1991). Mannitol was used as an internal standard. After methanolysis the reagents were removed under a stream of N_2 and the methyl-glycosides dried in vacuum over P_2O_5 prior to conversion into the corresponding TMS-derivatives. The samples were subjected to capillary gas chromatography (DB-5, Carlo Erba 6000 Vegas Series 2) as described by Barsett and Smestad Paulsen (1991).

2.5 Quantitative Determination of Phenolic Content

The total phenolic compounds were determined according to the Folin-Ciocalteu assay (Swain and Hillis, 1959), and modified as described by Rombouts et al. (1986). The absorbance was measured at 750 nm in a 4049 Novaspec single ray spectrophotometer (LKB, Biochrom) using ferulic acid as standard. The total phenolic content was determined as ferulic acid equivalents (FA/sample), 100%.

2.6 Determination of Protein Content

The protein content of the samples was detected using the protein assay of Lowry et al. (1951) modified by Peterson (1979).

2.7 Linkages Analysis of the Polysaccharides

Prior to methylation of the polymers, the uronic acids were reduced to their corresponding neutral sugars as described by Sims and Bacic (1995). The reduced polymers were methylated using the method of Ciucanu and Kerek (1984), modified by McConville et. al (1990). The fully methylated polysaccharides were hydrolysed with TFA, the monomers reduced with 1M $NaBD_4$ in 2M NH_4OH and the resulting partly methylated alditols were acetylated to partially methylated alditol acetates, PMAA, by adding 200 µl 1-

methylimidazole and 2 ml acetic acid anhydride. The PMAA were dissolved in 100 µl MeOH and analyzed by GC-MS on Fisons GC 8065 using split injection and a Fisons fused silica column (30 m x 0.2 mm i.d.) with a film thickness of 0.20 mm coupled with a Hewlett-Packard Mass Selective Detector 5970. The compounds present were identified by retention time and interpretation of the mass spectra. The relative amounts of each linkage type were estimated from the total amount of each monosaccharide obtained from the methanolysis analysis and the relative amount of each PMAA present

2.8 The Yariv- β-glucosyl Test

The presence of arabinogalactan type II (AGII) structures was detected by the single radial diffusion in an agarose gel containing the β-glucosyl Yariv reagent as described by van Holst and Clarke, (1985). A positive reaction was identified by a reddish circle (halo) around the well into which the samples had been applied. Gum Arabic was used as a positive control.

2.9 Complement Fixing Assay

The complement fixing test is based on inhibition of haemolysis of antibody sensitized sheep red blood cells, SRBC, by human sera as described by Michaelsen et al. (Method A) (2000). PMII, a pectin fraction from the leaves of Plantago major, was used as a positive control (Samuelsen et al., 1996). Inhibition of lysis induced by the test samples were calculated by the formula $((A_{control} - A_{test})/ A_{control}) \times 100\%$. From these data a dose-response curve was constructed and the concentration of test sample giving 50% inhibition of lysis (ICH_{50}) was calculated. A low ICH_{50} value means a high complement fixing activity. This biological test system can have some day to day variation and thus the ratio ICH_{50} PMII / ICH_{50} sample was calculated. A high ratio means high complement fixing activity.

2.10 Measurement of Nitric Oxide Release

The RAW 264.7 mouse macrophage cell line were plated at a density of $5x10^5$ cells/mL in 96-well flat bottomed plates ($5x10^4$ cells/well), and cultured with the indicated concentrations of polysaccharides, 500ng/ml LPS (from *P. aeruginosa* 10, Sigma-Aldrich) or medium alone. Cells were incubated for 24 hrs and then centrifuged at 1300 rpm for 2 min. Cell-free supernatants were harvested, and the amount of nitrite (a stable breakdown product of nitric oxide) was determined using a colorimetric method with $NaNO_2$ as a standard. The method was adopted from Promegas Technical bulletin 229. The culture supernatant (50 µl) was mixed with an equal volume of Griess reagent A (1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid) and incubated at room temperature in the dark for 10 min. After addition of 50 µl 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in water (Griess reagent B) the absorbance was measured at 540 nm.

2.11 Statistical Analysis

Experimental values were expressed as mean ± SEM, and represent the means from four independent experiments with duplicates unless stated otherwise. The statistical significance of differences between two mean values was evaluated by the two tailed unpaired t-test, where values of $p < 0.05$ were considered to be statistically significant.

3. RESULTS AND DISCUSSION

The plant material was extracted with 96% ethanol of appr. 70ºC in order to remove plant compounds of lipid nature as well as low molecular weight material of intermediate lipophilic nature. The residue after this treatment was then sequentially extracted with boiling 50% ethanol, 50ºC water and 100ºC water as described in Fig. 1.

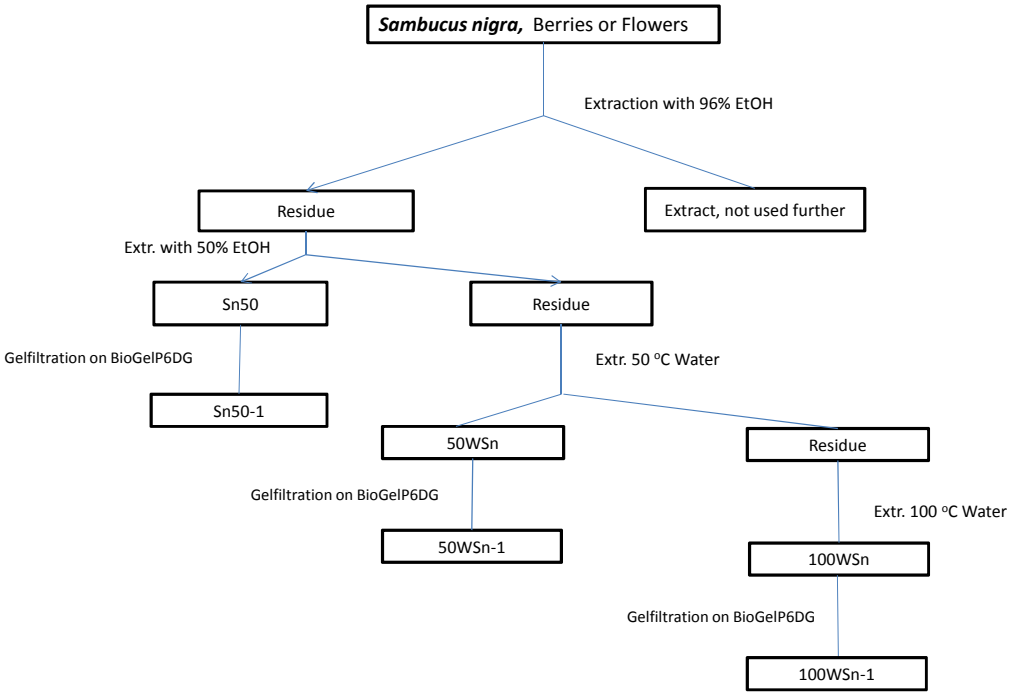

Fig. 1. Extraction scheme used both for berries and flowers of *S. nigra*.

The crude extracts, denominated Sn50, 50WSn and 100WSn from both berries (-Be) and flowers (-Fl) were analyzed for their content of carbohydrates and monomer composition, the effect on the complement system and the presence of Arabinogalactan II structures using the Yariv reagent.

Table 1 gives the monosaccharide composition as well as total carbohydrate of all crude extracts. The fractions from the flowers contain higher amount of the monosaccharides arabinose, galactose, rhamnose and galacturonic acid than the same type of fractions from the berries, but presence of these monosaccharides in all the fractions strongly indicate that pectins may be part of the crude extracts. The fractions from the berries contain relatively high amount of xylose and glucose, much higher than the flower fractions. It is well known that fruits/berries and vegetables contain xyloglucans (Hilz et al., 2005; Hoffman et al., 2005). This could also be the case for the berries of *S. nigra,* which could be explained by the content of xylose and glucose in these fractions. Starch may be present due to the amounts of glucose found, and the presence of low molecular weight carbohydrate cannot be ruled out, as especially the 50% ethanol extracts would contain low molecular weight compounds. The fraction SnBe50 from the berries and the fractions SnFl50, 50WSnFl and 100WSnFl from the flowers gave positive response with the Yariv reagent, which indicates

the presence of arabinogalactan type II (AGII) structures in the polysaccharides from these crude fractions.

One of the objects for the present study was to investigate if there were compounds present in the extracts of berries and flowers of *S. nigra* that would have an immunomodulating effect. We therefore tested the effect in the complement assay, a test system that indicates immunomodulating activity. The complement fixing test is based on inhibition of hemolysis of antibody sensitized sheep erythrocytes (SRBC) (Michaelsen et al., 2000). The activity for all fractions was concentration dependent at the range of concentrations studied and the activity is presented as ICH_{50} (the lowest concentration of sample needed to give 50% inhibition of hemolysis of SRBC). The relative ICH_{50} values compared to that of a positive control are given in Fig. 2.

Table 1. Monosaccharide compositions of the crude extracts isolated from berries and flowers of *S. nigra* given as mol% of carbohydrate present

	SnBe50	50WSnBe	100WSnBe	SnFl50	50WSnFl	100WSnFl
Arabinose	7,7	9,0	16,8	13,4	11,0	15,4
Rhamnose	2,7	5,6	4,8	9,4	8,0	9,6
Xylose	18,8	16,3	11,3	1,8	4,3	3,4
Mannose	8,2	8,3	10,0	4,4	3,5	6,6
Galactose	8,0	11,6	9,9	18,3	11,3	13,9
Glucose	46,6	24,2	23,3	18,0	11,0	12,8
Glucuronic acid	n.d.	n.d.	1,5	3,8	1,6	2,4
Galacturonic acid	8,0	25,0	22,4	30,9	49,3	35,9
% carbohydrate in the fraction	25,6	15,1	39,2	25,8	28,1	45,6

n.d. = not detected

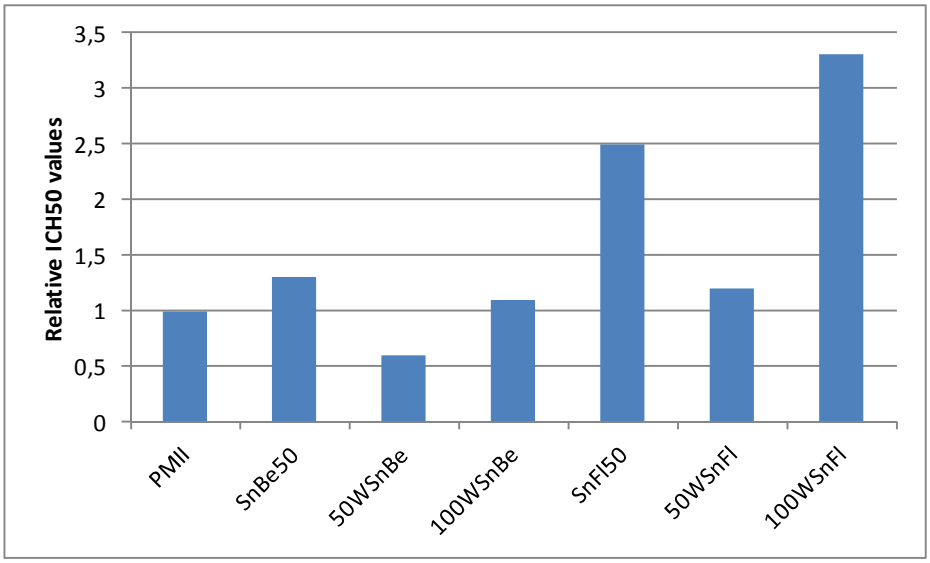

Fig. 2. Relative ICH_{50} values of the extracts from berries and flowers of *S. nigra*, presented as the ICH_{50} of the standard polysaccharide PMII divided by the ICH_{50} of the fractions.

The results presented are from a typical set of the experiments performed

Interestingly, all the crude extracts apart from 50WSnBe, had an effect in the complement assay equal to or higher than that of the very active polysaccharide standard compound PMII, isolated form *Plantago major* (Samuelsen et al., 1996). Furthermore, the extracts from the flowers all had higher activities than the paralell extracts from the berries. Due to this, further purification of the extracts was performed, and low molecular weight material was removed by gel filtration in aqueous medium on a BioGel P6DG column. The monosaccharide composition of the high molecular weight materials is shown in Table 2. The results of the linkage analysis of the same fractions are shown in Table 3.

The most striking difference from monosaccharide compositions of the crude extract is the reduction of the amount of glucose present in all fractions. The relatively high amounts of arabinose, galactose and galacturonic acid is also interesting from a structural point of view when taking into account that pectins containing these sugars are quite common in all plant parts in general. The fractions SnB50-I, SnFl50-I and 50WSnFl-I in particular, have much higher content of arabinose and galactose than their parent crude extracts. The high molecular weight fractions from flowers contain higher amount of pectic polysaccharides than the equal fractions from berries due to higher content of galacturonic acid. The berry fractions still contain more xylose and glucose than the fractions from flowers.

Table 2. Monosaccharide composition of the high molecular weight fractions isolated after gel filtration on BioGel P6DG of the crude extracts from berries and flowers of *S. nigra* given as mol% of carbohydrate present

	SnBe50-I	50WSnBe-I	100WSnBe-I	SnFl50-I	50WSnFl-I	100WSnFl-I
Arabinose	25,0	12,1	20,6	19,9	14,9	13,4
Rhamnose	4,8	10,0	5,9	6,1	9,1	16,8
Xylose	5,4	17,2	13,2	2,5	2,8	3,1
Mannose	9,2	8,9	9,2	4,1	3,4	2,9
Galactose	24,6	14,6	12,5	26,7	20,2	15,9
Glucose	12,3	13,2	12,7	4,5	5,1	5,8
Glucuronic acid	2,2	n.d.	1,4	3,7	2,7	n.d.
Galacturonic acid	16,5	24,0	24,5	32,5	41,8	42,1

n.d. = not detected

The linkage analysis (Table 3) show a low content of terminal xylose in all the fractions, and the 50ºC and 100ºC water fractions of the berries have relatively high amount of 1-4 linked xylose. The same two fractions also have a relatively high amount of 1-4 linked glucose with glucose 1-4,6 branch points. This could be due to xyloglucans with side chains consisting of more than one xylose unit, which is in accordance with xyloglucans found in bilberries (Hilz et al., 2007). The linkage analysis indicates that some xylose could also be terminally linked to C3 in the galacturonic acid backbone, which is a structure well known in the literature (Schols et al., 1995). The methylation analysis also showed that the main part of the galacturonic acid is 1-4 linked. This is typical in pectic polysaccharides such as homogalacturonan and rhamnogalacturonan I (RG-I), the latter which consist of a polymer that have a core of alternating α-1-4 linked D-galacturonic acid and α-1-2-L-rhamnose units (McNeil et al., 1982; Ishii et al., 1989; Paulsen and Barsett, 2005; Waldron and Faulds, 2007). The linkage analysis showed 1-2 linked rhamnose and rhamnose 1-2,4 branchpoints, which is consistent with RG-I. High amount of arabinose and galactose could indicate sidechains of arabinogalactans. The arabinogalactans attached to the rhamnose units are frequently found to be of the arabinogalactan type I (AG-I) and/or arabinogalactan type II

(AG-II) (precipitates with the Yariv-reagent) (van Holst and Clarke, 1985; Paulsen and Barsett, 2005). AG-II has as its main core a galactan that can have either 3 or 6 linkages in the main chain and is highly branched with the 1-3, 6 linked galactose units at the branching points. The methylation analysis showed rather high amount of terminally linked arabinose in all the high molecular weight fractions, and together with 1-3 linked, 1-6 linked galactose and high amount of galactose 1-3, 6 branch points, this could indicate AG-II. Concomitant with the linkage analysis, all the fractions gave concentration dependent precipitation with the Yariv-reagent.

Table 3. Type of linkages present for the monosaccharides in the high molecular weight fractions isolated from berries and flowers of *S. nigra*

Monosacch.	Linkage type	SnBe 50-1	50W SnBe-1	100W SnBe-1	SnFl 50-1	50W SnFl-1	100W SnFl-1
Ara	**T_f**	18,4	5,2	10,5	13,8	9,2	10,0
	$1\rightarrow2_f,3_f$	3,6	3,2	4,2	2,5	0,9	1
	$1\rightarrow5_f$	2,1	1,9	3,4	2,6	3,4	1,5
	$1\rightarrow3,5_f$	0,7	0,8	2,2	0,8	1,3	0,8
	$1\rightarrow2,5_f$	0,2	1	0,3	0,2	0,1	0,1
SUM		25,0	12,1	20,6	19,9	14,9	13,4
Xyl	**Tp**	5,4	2,1	5,4	2,5	2,8	3,1
	$1\rightarrow4p$	n.d.	14,8	7,3	n.d.	n.d.	n.d.
	$1\rightarrow2,4p$	n.d.	0,3	0,5	n.d.	n.d.	n.d.
SUM		5,4	17,2	13,2	2,5	2,8	3,1
Rha	**Tp**	0,8	2,3	2,4	0,9	0,5	0,9
	$1\rightarrow2$	3,7	6,8	3,2	2,9	6,5	12,6
	$1\rightarrow3$	n.d.	n.d.	n.d.	0,2	n.d.	0,4
	$1\rightarrow2,4$	0,3	0,9	0,3	2,1	2,1	2,9
SUM		4,8	10,0	5,9	6,1	9,1	16,8
GlcA	**Tp**	2,2	n.d.	1,4	3,7	2,7	tr.
SUM		2,2	n.d.	1,4	3,7	2,7	n.d.
GalA	**Tp**	0,2	0,6	2,5	n.d.	n.d.	n.d.
	$1\rightarrow4$	16,1	23,4	21,2	32,3	41,7	41,6
	$1\rightarrow3,4$	0,2	n.d.	0,8	0,2	0,1	0,5
SUM		16,5	24,0	24,5	32,5	41,8	42,1
GlC	**Tp**	5,0	3,2	2,3	1	1,4	0,6
	$1\rightarrow4$	7,3	6,2	8,7	2,2	3,3	4,5
	$1\rightarrow6$	n.d.	1	n.d.	1,3	0,4	0,7
	$1\rightarrow4,6$	n.d.	2,8	1,7	n.d.	n.d.	n.d.
SUM		12,3	13,2	12,7	4,5	5,1	5,8
Gal	**Tp**	1,8	5,7	0,3	4,2	3,7	9,2
	$1\rightarrow3$	0,3	4,5	7,5	4,3	1,8	3,1
	$1\rightarrow6$	n.d.	1,2	n.d.	1,3	0,2	0,4
	$1\rightarrow3,4$	1,2	n.d.	0,1	1,3	0,4	0,4
	$1\rightarrow3,6$	21,3	3,2	4,6	15,6	14,1	2,8
SUM		24,6	14,6	12,5	26,7	20,2	15,9

n.d. = not detected; tr. = trace

All fractions have an increased activity after removal of low molecular weight compounds, and the systematic higher effect seen in the crude extracts from the flowers compared to those of the berries was again observed for the high molecular weight fractions (Fig. 3). This could be due to low or no activity of the xyloglucan hemicellulose part of the fractions. It also appears to be a substantial amount of inhibitory compounds that were removed as low molecular weight material especially in the flowers and most strikingly so in the material extracted with water of 100ºC.

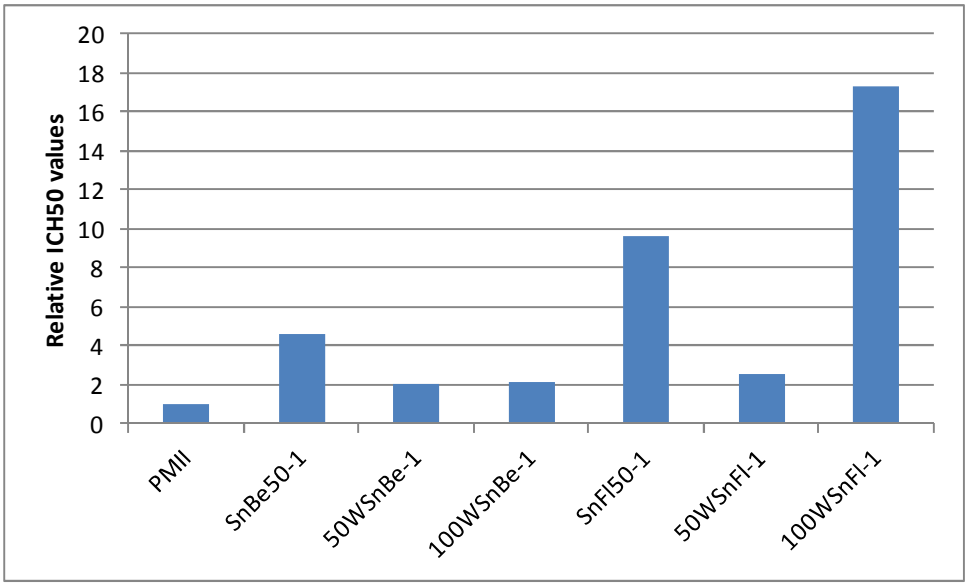

Fig. 3. Relative ICH_{50} values from the complement assay of fractions from *S. nigra* obtained after gel filtration on Biogel P6DG.

The results presented are from a typical set of the experiments performed.

The ability to activate mouse macrophages was also determined for the high molecular weight fractions from both berries and flowers. The production of nitric oxide (NO) was measured after treatment of the macrophages with the extracts for 24 hrs. LPS, a constituent of the outer membrane of gram negative bacteria, was utilised as a positive control, as it is a potent stimulator of cells of the monocytic lineage (Sweet and Hume, 1996). From Fig. 4 it is interesting to note that the fraction 100WSnFl-1 was the most active fraction in this assay while only the 50%ethanol-water extract form the berries (SNBe50-1) showed to induce production of NO. This effect was comparable to that of fraction 50WSnFl-1.

The extracts from berries and flowers of *S. nigra* all contained polysaccharides and they had varying effects in immunomodulating test systems. The fractions contained minor amounts of phenolic and protein material that not could be the reason for the differences in biological activity (results not shown). The polysaccharides from the flowers had the highest bioactivity in both systems tested for with the fraction obtained from the extract with water 100ºC being the most active. The structural analysis showed that the pectic elements in all the different fractions were similar. The most pronounced difference is the higher amount of rhamnose in the 100WSnFl-1, which indicates that the so called hairy region may play a role in the activity in the most active fraction. Higher amount of 1-2 linked rhamnose units compared the other fractions indicating longer regions in the Rha/GalA chain without any branches.

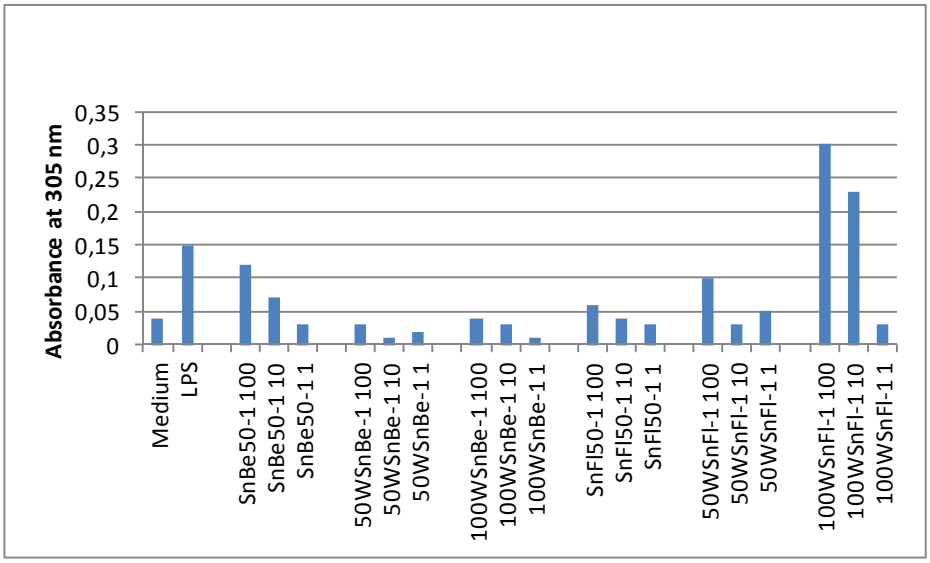

Fig. 4. NO production of the *S. nigra* high molecular weight fractions.
The concentration of LPS is 100ng/ml, the other samples range from 1-100µg/ml. The results presented are from a typical set of the experiments performed

4. CONCLUSION

Three different types of extracts of berries and flowers from *S. nigra* were prepared. The high molecular weight fractions of these extracts were investigated with respect to both carbohydrate and linkage composition, as well as the biological effects in the complement assay and the ability to induce mouse macrophage production of NO. The results clearly showed that the extracts from the flowers were more active than those from the berries, and part of this difference may be due to differences in the carbohydrate composition of the extracts. Based on this, one should encourage the drinking of juice made form flowers in the same way as has been done for the juice made from berries, since both may have a beneficial heath effect.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

Barak, V., Birkenfeld, S., Halperin, T., Kalickman, I. (2002). The effect of herbal remedies on the production of human inflammatory and anti-inflammatory cytokines. IMAJ, 4, 919-922.

Barros, L., Cabrita, L., Boas, M.V., Carvalho, A.M., Ferreira, I. (2011). Chemical, biochemical and electrochemical assays to evaluate phytochemicals and antioxidant activity of wild plants. Food Chem., 127, 1600-1608.

Barsett, H., Smestad Paulsen, B. (1991). Separation, isolation and characterization of acidic polysaccharides from the inner bark of *Ulmus glabra* Huds. Carbohydr. Polym., 17, 137-144.

Blumenthal, M., Goldberg, A., Brinckmann, J. (2000). Herbal Medicine: Expanded Commission E Monographs. Austin, American Botanical Council.

Cao, G.H., Prior, R.L. (1999). Anthocyanins are defected in human plasma after oral administration of an elderberry extract. Clin. Chem., 45, 574-576.

Chambers, R.E., Clamp, J.R. (1971). An Assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. Biochem. J., 125, 1009-1018.

Christensen, L.P., Kaack, K., Frette, X.C. (2008). Selection of elderberry (*S. nigra* L.) genotypes best suited for the preparation of elderflower extracts rich in flavonoids and phenolic acids. Eur. Food Res. Technol., 227, 293-305.

Ciucanu, I., Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res., 131, 209-217.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., 28, 350-356.

Fossum, G., Malterud, K., Moradi, A. (2008). *S. nigra* L., flos. Assessment report for the development of community monographs and for inclusion of herbal substance(s), preparation(s) or combinations thereof in the list. European Medicines Agency (EMA). from http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2009/12/WC500018238.pdf.

Hearst, C., McCollum, G., Nelson, D., Ballard, L.M., Millar, B.C., Goldsmith, C.E., Rooney, P.J., Loughrey, A., Moore, J.E., Rao, J.R. (2010). Antibacterial activity of elder (*S. nigra* L.) flower or berry against hospital pathogens. J. Med. Plant. Res., 4, 1805-1809.

Hilz, H., Bakx, E.J., Schols, H.A., Voragen, A.G.J. (2005). Cell wall polysaccharides in black currants and bilberries - characterisation in berries, juice, and press cake. Carbohydr. Polym., 59, 477-488.

Hilz, H., de Jong, L.E., Kabel, M.A., Verhoef, R., Schols, H.A., Voragen, A.G.J. (2007). Bilberry xyloglucan - novel building blocks containing beta-xylose within a complex structure. Carbohydr. Res., 342, 170-181.

Hoffman, M., Jia, Z.H., Pena, M.J., Cash, M., Harper, A., Blackburn, A.R., Darvill, A., York, W.S. (2005). Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. Carbohydr. Res., 340, 1826-1840.

Ishii, T., Thomas, J., Darvill, A., Albersheim, P. (1989). Structure of plant cell walls: XXVI. The walls of suspension-cultured sycamore cells contain a family of rhamnogalacturonan-I-like pectic polysaccharides. Plant Physiol., 89, 421-428.

Kaack, K. (2008a). Aroma composition and sensory quality of fruit juices processed from cultivars of elderberry (*S. nigra* L.). Eur. Food Res. Technol., 227, 45-56.

Kaack, K. (2008b). Processing of aroma extracts from elder flower (*S. nigra* L.). Eur. Food Res. Technol., 227, 375-390.

Krawitz, C., Abu Mraheil, M., Stein, M., Imirzalioglu, C., Domann, E., Pleschka, S., Hain, T. (2011). Inhibitory activity of a standardized elderberry liquid extract against clinically-relevant human respiratory bacterial pathogens and influenza A and B viruses. BMC Complement. Altern. Med., 11.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

McConville, M.J., Homans, S.W., Thomas-Oates, J.E., Dell, A., Bacic, A. (1990). Structures of the glycoinositolphospholipids from *Leishmania major*. A family of novel galactofuranose-containing glycolipids. J. Biol. Chem., 265, 7385-7394.

McNeil, M., Darvill, A.G., Albersheim, P. (1982). Structure of plant cell walls: XII. Identification of seven differently linked glycosyl residues attached to O-4 of the 2, 4-linked L-rhamnosyl residues of rhamnogalacturonan I. Plant Physiol., 70, 1586-1591.

Michaelsen, T.E., Gilje, A., Samuelsen, A.B., Hogasen, K., Paulsen, B.S. (2000). Interaction between human complement and a pectin type polysaccharide fraction, PMII, from the leaves of *Plantago major* L. Scand. J. Immunol., 52, 483-490.

Murkovic, M., Abuja, P.M., Bergmann, A.R., Zirngast, A., Adam, U., Winklhofer-Roob, B.M., Toplak, H. (2004). Effects of elderberry juice on fasting and postprandial serum lipids and low-density lipoprotein oxidation in healthy volunteers: a randomized, double-blind, placebo-controlled study. Eur. J. Clin. Nutr., 58, 244-249.

Nergard, C.S., Diallo, D., Michaelsen, T.E., Malterud, K.E., Kiyohara, H., Matsumoto, T., Yamada, H., Paulsen, B.S. (2004). Isolation, partial characterisation and immunomodulating activities of polysaccharides from *Vernonia kotschyana* Sch Bip. ex Walp. J. Ethnopharmacol., 91, 141-152.

Paulsen, B.S., Barsett, H. (2005). Bioactive pectic polysaccharides. Adv. Polym. Sci., 186, 69-101.

Peterson, G.L. (1979). Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem., 100, 201-220.

Rieger, G., Muller, M., Guttenberger, H., Bucar, F. (2008). Influence of altitudinal variation on the content of phenolic compounds in wild populations of *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*. J. Agric. Food Chem., 56, 9080-9086.

Rombouts, F.M., Thibault, J.F. (1986). Enzymatic and chemical degradation and the fine-structure of pectins from sugar-beet pulp. Carbohydr. Res., 154, 189-203.

Roschek, B., Fink, R.C., McMichael, M.D., Li, D., Alberte, R.S. (2009). Elderberry flavonoids bind to and prevent H1N1 infection *in vitro*. Phytochemistry, 70, 1255-1261.

Samuelsen, A.B., Paulsen, B.S., Wold, J.K., Otsuka, H., Kiyohara, H., Yamada, H., Knutsen, S.H. (1996). Characterization of biologically active pectin from *Plantago major* L. Carbohydr. Polym., 30, 37-44.

Schols, H.A., Bakx, E.J., Schipper, D., Voragen, A.G.J. (1995). A xylogalacturonan subunit present in the modified hairy regions of apple pectin. Carbohydr. Res., 279, 265-279.

Sims, I.M., Bacic, A. (1995). Extracellular polysaccharides from suspension cultures of *Nicotiana plumbaginifolia*. Phytochemistry, 38, 1397-1405.

Swain, T., Hillis, W.E. (1959). Phenolic constituents of *Prunus domestica*. I. Quantitative analysis of phenolic constituents. J. Sci. Food Agric., 10, 63-68.

Sweet, M.J., Hume, D.A. (1996). Endotoxin signal transduction in macrophages. J. Leukocyte Biol., 60, 8-26.

van Holst, G.J., Clarke, A.E. (1985). Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. Anal. Biochem., 148, 446-450.

Veberic, R., Jakopic, J., Stampar, F., Schmitzer, V. (2009). European elderberry (*S. nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. Food Chem., 114, 511-515.

Vlachojannis, J.E., Cameron, M., Chrubasik, S. (2010). A systematic review on the *Sambuci fructus:* effect and efficacy profiles. Phytother. Res., 24, 1-8.

Waknine-Grinberg, J.H., El-On, J., Barak, V., Barenholz, Y., Golenser, J. (2009). The immunomodulatory effect of sambucol on leishmanial and malarial infections. Planta Med., 75, 581-586.

Waldron, K.W., Faulds, C.B. (2007). Cell wall polysaccharides: composition and structure. In Kamerling J.P., Boons G.J., Lee Y.C., Suzuki A., Taniguchi N., Voragen A.G.J., (Eds.) Comprehensive Glycoscience, Elsevier: Oxford, 1, 181-201.

Weiss, R., Fintelmann, V. (2000). Herbal Medicine. Stuttgart, Thieme.

WHO. (2002). WHO monographs on selected medicinal plants. Geneva, World Health Organization.

Yamada, H., Kiyohara, H. (1999). Complement-activating polysaccharides from medicinal herbs. In Wagner H., (Ed.) Immunomodulatory agents from plants, Birkhäuser Verlag. Basel, 161-202.

Yamada, H., Kiyohara, H. (2007). Immunomodulating activity of plant polysaccharide structures. In Kamerling J.P., (Ed.) Comprehensive Glycoscience - From Chemistry to Systems Biology, 1st ed.; Elsevier: Oxford, 4, 663-694.

Yamada, H., Kiyohara, H., Matsumoto, T. (2009). Recent studies on structures and intestinal immunity modulating activities of pectins and pectic polysaccharides from medicinal herbs. In Schols H.A., Visser R.G.F., Voragen A.G.J., (Eds.) pectins and pectinases, Wageningen Academic Publishers. Wageningen, 293-304.

Zakay-Rones, Z., Thom, E., Wollan, T., Wadstein, J. (2004). Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. J. Int. Med. Res., 32, 132-140.

Zakay-Rones, Z., Varsano, N., Zlotnik, M., Manor, O., Regev, L., Schlesinger, M., Mumcuoglu, M. (1995). Inhibition of several strains of influenza virus *in vitro* and reduction of symptoms by an elderberry extract (*S. nigra* L.) during an outbreak of influenza B Panama. J. Altern. Complement. Med., 1, 361-369.

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