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Chemical characterization and complement fixation of pectins from *Cola cordifolia* leaves

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ABSTRACT

Defatted leaves from the medicinal tree *Cola cordifolia* were extracted with 50% EtOH, 50 °C and 100 °C water. The polysaccharide rich extracts were fractionated and the structure of the polysaccharides elucidated. Linkage analysis of the polysaccharides indicates a rhamnogalacturonan type I backbone where both Rha and parts of GalA are substituted in position 3, indicating a highly branched polymer with short side chains. The purified fractions were tested for complement fixation, macrophage stimulating activity and anti-adhesion activity towards *Helicobacter pylori*. Here we report on complex and polydisperse types of pectins (M_w: 3–1300 kDa) as well as the presence of low M_w (<3 kDa) acidic oligosaccharides. The fractions showed a moderate complement fixing activity and no macrophage activating effects after LPS removal. Anti-adhesion activity towards *H. pylori* was not found.

1. Introduction

Cola cordifolia (Cav.) R. Br. (Malvaceae) is a large tree, widespread in the savannah from Senegal to Mali (Burkill, 2000). The tree is used in traditional medicine in Mali, Senegal and Gambia for multiple ailments. In Mali, leaves and bark are used to treat wounds, gastric ulcers, pain, fever and diarrhoea (Austarheim, Mahamane, et al., 2012; Grønhaug et al., 2008). Immunomodulating polysaccharides are suggested to be active components in treating ailments related to wounds and gastric ulcers (Cipriani et al., 2009; Matsumoto et al., 2002; Nergaard et al., 2005, 2004). *Helicobacter pylori* is well recognized as the main pathogen in gastric ulcer, and complementary polysaccharides has been shown to block tissue adhesion and colonization of the mucosa (Sharon & Ofek, 2002; Wittschier, Faller, & Hensel, 2007). Previously, pectins present in bark and leaves of *C. cordifolia* were found to have anti-ulcer activity in an experimental rat model (Austarheim, Mahamane, et al., 2012).

Bark harvesting harms the tree, but collection of leaves does not damage the tree in the same way as debarking (Zschocke, Rabe, Taylor, Jager, & van Staden, 2000). For several tree species, the bark and leaves contain the same active components, but before plant part replacement can be recommended for a specific plant, it is important to evaluate and compare the biological activity of the plant part to be substituted (Geldenhuys, 2007). Generally, the Department of Traditional Medicine (DMT) in Bamako, Mali, wants to promote the use of leaves instead of bark to create a sustainable harvesting of medicinal plants. This is crucial for providing enough and cheap medicines for the population who mainly depends on traditional medicine for their primary health care (Robison & Zhang, 2011).

Pectins, especially rhamnogalacturonan type I (RG-I), are found in the primary cell wall and provides structural support (Harholt, Suttangkakul, & Scheller, 2010). Considering that the bark and leaves have different biological functions, it is possibly that the pectins present are structurally dissimilar. It is therefore important to compare the pectins present before plant part substitution can be recommended since the pectins are thought to be part of the active components.

The aims of the present study were (i) to isolate, purify and structurally characterize pectins present in the leaves of *C. cordifolia*; (ii) assess complement fixation and induction of macrophages; (iii) compare characteristics of the leaf pectins to bark pectins.

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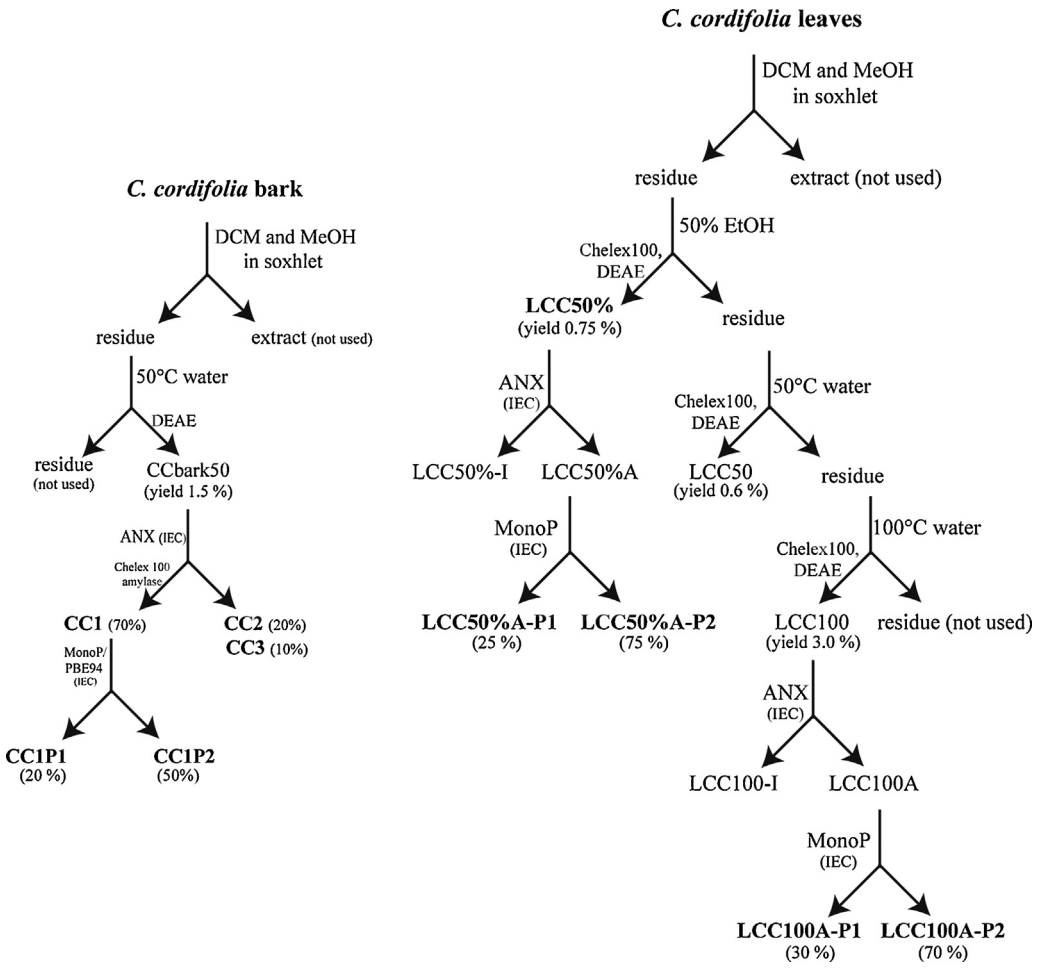

Fig. 1. Flow scheme. Fractionation of water extracts *Cola cordifolia* leaves. Numbers in parentheses indicate yield (%). Due to loss during fractionation, the yield for the purified fractions are not given as % of the plant material, but as % related to the fractions obtained in the same fractionation step. The flow scheme for bark fractions is included for comparison (Austarheim, Christensen, et al., 2012; IEC – ion exchange chromatography).

2. Experimental

2.1. Materials

The leaves of *C. cordifolia* (Cav). R Br, (Malvaceae, previously Sterculiaceae) were collected February 2009 in the garden of Department of Traditional Medicine, Bamako, Mali, and identified at the same department; a specimen is deposited at DMT under the number 1331. The plant material was dried indoors and subsequently pulverized in a mechanical grinder to a fine powder (0.1 mm).

2.2. Extraction and purification of polysaccharides

The powdered leaves of *C. cordifolia* (241 g) were successively extracted with dichloromethane (DCM) and methanol (MeOH) using a Soxhlet apparatus to remove lipophilic compounds. The residue (202 g) was then extracted twice for 1 h with 50% ethanol (EtOH) on reflux. The residue was then extracted with 5 L 50 °C MilliQ (Millipore Corporation) water for 1 h followed by centrifugation at 10,000 rpm (15,317 × *g*, Heraeus Multifuge 4KR). The residue left from the 50 °C water extraction, was subsequently extracted for 1 h with 5 L MilliQ water at 100 °C followed by centrifugation, see fractionation scheme in Fig. 1. The three extracts were dialyzed in membrane dialysis tubes, cut off 12–14 kDa (Spectra/pore, Spectrumlabs) for 48 h against MilliQ water with frequent changes to remove salts. For additional removal of coloured material, all extracts were passed through a short ion exchange column (IEC), DEAE Sepharose 5 cm × 3 cm (XK50, GE healthcare). Subsequent exchange of divalent ions with H^+ was carried out by passing the polysaccharide rich extracts through a Chelex 100 (Sigma–Aldrich) chelating resin column (2 cm × 20 cm, BioRad). The extracts were then dialyzed in membrane dialysis tubes (12–14 kDa) for 72 h against MilliQ water to remove salts and other low molecular weight compounds. The water was changed three times. The crude extracts were kept frozen at −18 °C until further use. The fractions resulting from 50% EtOH, 50 °C and 100 °C water extractions were named LCC50%, LCC50 and LCC100 respectively. LCC50 was not further purified due to the high viscosity and almost identical monosaccharide composition as the less viscous LCC100 (Table 1).

2.3. Fractionation of LCC50% and LCC100

250 ml LCC50% and LCC100 (1.5 mg/ml) were respectively applied on an ANX-Sepharose fast flow 4 anion exchange column (XK50, 5 cm × 30 cm) with Cl^- as counter ion, fitted into an ÄKTA FPLC system (GE healthcare). The column was first eluted with 1.5 column volumes (CV) degassed MilliQ water followed by a constant NaCl gradient (0–1 M in 1000 ml). Fractions of 15 ml were collected. The fractions that were eluted with water only, were named LCC50%-I and LCC100-I respectively. The carbohydrate profile was monitored by the phenol–sulfuric assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and 100 μl was transferred to a microtiterplate and the absorbance was read (470 nm). Elution of

Table 1a
Fractions obtained from extraction with 50% EtOH. Monosaccharide composition, protein content and molecular weights.

	LCC50%	LCC50%-I	LCC50% A	LCC50%A-P1	LCC50%A-P2	LCC50%A <3 kDa
Ara	13.3	13.9	10.4	16.1	1.6	22
Rha	15.3	16.5	16.2	15.8	19.8	9.8
Xyl	0.8	0	2.7	2.7	0	9.8
Gal	19.9	17.9	15.8	20.6	18.0	5.8
2-OMe-Gal	2.0	2.5	Trace	0	3.5	Trace
4-OMe-GlcA	19.4	17.2	13.3	8.2	21.5	6.4
GalA	25.1	26.8	38.8	34.6	34.2	34.7
Glc	4.2	5.2	2.8	2	1.5	11.5
Yariv	+	–	(+)	+	–	–
Protein (%):	0.8	n.d	n.d	0.1	0.9	n.d
LPS (%)	n.d	n.d	n.d	0.16	0.12	0.001
PDI	n.d	n.d	n.d	34	2.7	n.d
M_w (kDa)	n.d	n.d	n.d	1100	1240	<3
M_n (kDa)	n.d.	n.d	n.d	33	450	<3

PDI, poly dispersity index (PDI = M_w/M_n); n.d., not determined.

LCC50% gave one large, acidic peak named LCC50%A. LCC100 gave one small and one large peak. The largest peak was named LCC100A. The relevant fractions were pooled, dialyzed and lyophilized, see Fig. 1.

2.4. Fractionation of LCC50%A and LCC100A on MonoP

2.5 ml (1 mg/ml) sample was applied to a Mono P HR 5/20 (GE healthcare) column fitted in an ÄKTA FPLC system (GE-healthcare) coupled to a RI detector (Shimadzu). The column was first eluted with 4 ml degassed MilliQ water and then with a stepwise elution gradient from 0 to 1 M NaCl, step 1: 2 ml with a constant gradient to reach 0.4 M NaCl; step 2: isocratic elution for 4 ml; step 3: constant gradient to reach 1.0 M NaCl after additionally 8 ml; step 4: isocratic elution for 8 ml. The elution profile was determined by the phenol–sulfuric assay in addition to RI monitoring. LCC50%A gave rise to two baseline separated peaks named LCC50%A-P1 and LCC50%A-P2. LCC100A was baseline separated into LCC100A-P1 and LCC100A-P2. The relevant fractions were pooled, filtered 0.22 μm (Millipore) and dialyzed, see Fig. 1.

2.5. Ca^{2+} and Mg^{2+} quantification by atomic absorption spectrometry (AAS)

The quantification of calcium and magnesium was performed principally as described by (Welz & Sperling, 1999). Experimental details are described previously by Austarheim, Christensen, et al. (2012).

2.6. Monosaccharide composition

Methanolysis and TMS-derivatization followed by GC analysis were performed principally as described by Chambers and Clamp (1971) and the experimental details are described by Austarheim, Christensen, et al. (2012). 100 μg mannitol was added as internal standard. Based on standards for all the monomers present, the monosaccharides were identified and quantified.

2.7. Linkage determination by GC–MS

Prior to methylation (Ciucanu & Kerek, 1984), the uronic acids in the polysaccharides were reduced with $NaBD_4$ to their corresponding deuterated neutral sugars (Kim & Carpita, 1992). After deuterio-methylation using CD_3I, the samples were hydrolyzed by TFA (Kim & Carpita, 1992) or 90% formic acid (Bourne, Percival, & Paulsen, 1972), and subsequently reduced and acetylated (Kim & Carpita, 1992). The partially deuterio-methylated alditol acetates were analyzed by GC–MS using a GCMS-QP2010 instrument (Shimadzu), attached to a Restek Rxi-5MS column (30 m; 0.25 mm ID; 0.25 μm film thickness). The injector temperature was 280 °C, the ion source 200 °C and the interface 300 °C. The column temperature was 80 °C when injected, then increased with 10 °C/min to 140 °C, followed by 4 °C/min to 210 °C and then 20 °C/min to 300 °C. Helium was used as carrier gas (pressure control: 80 kPa). The quantification is based on the results from the monosaccharide composition (Section 2.6) and TIC (total ion current) from GC–MS. Effective carbon-response of each compound was applied according to Sweet, Shapiro, and Albersheim (1975).

2.8. Arabinogalactan type II (AG-II) precipitation

The presence of AG-II was detected by precipitation with β-glycosyl Yariv regents by single radial diffusion according to Van Holst and Clarke (1985). Samples of 16, 32 and 48 μg pectin were tested. This reagent interacts with polymers having 1→3,6 Gal in AG-II chains.

2.9. SEC-MALLS-VISC

The molecular weight distributions (and averages) of the purified fractions were determined as described previously (Christensen et al., 2001; Vold, Kristiansen, & Christensen, 2006). In brief, the system consisted of size exclusion chromatography columns and three online detectors: a multi-angle laser light scattering detector (MALLS) Dawn Heleos-II (Wyatt), a refractive index detector (Optilab DSP, Wyatt) and a viscometer (ViscoStar, Wyatt). LCC50%A-P1 and LCC50%A-P2 were analyzed using serially connected TSK G 4000PWXL and TSK G 3000PWXL columns (7.8 mm × 300 mm) and LCC100A-P1 and LCC100A-P2 were analyzed on TSK G 6000PWXL serially connected to TSK G 5000PWXL (7.8 mm × 300 mm). The columns and the detectors were attached to a SCL-10Avp system controller, a SIL-10AF autosampler, and a LC-10ADvp pump (Shimazu, Japan). Three standards were included for comparison and validation (not for calibration), pullulan P-1390 (Hayashibara Biochemical Laboratories, Japan), alginate 203 kDa (UP-LVG-603-04, FMC NovaMatrix, Norway) and the rhamnogalacturonan type I (RG-I) pectin CC1P1, [2→)[α-D-Gal(1→3)]α-L-Rha(1→4)α-D-GalA(1→]$_n$ (Austarheim, Christensen, et al., 2012). The column was eluted with aqueous 0.05 M Na_2SO_4/0.01 M EDTA, pH 6 at a flow rate of 0.5 ml/min. Pullulan P8 (8 kDa) was used for normalization of all the MALLS detectors. 100–150 μl sample solution (0.4–1.5 mg/ml) was injected. All samples were dissolved in MilliQ water 24 h prior to analysis, and diluted with concentrated eluent to reach eluent concentration before injection. A refractive index increment (dn/dc)

of 0.150 was used in the calculations. Intrinsic viscosities were obtained for each elution slice using an online viscosity detector. Owing to the extremely low concentration, the intrinsic viscosity may be approximated by η_{sp}/c. The same analysis and experimental approach have been applied to alginates (Vold et al., 2006) and *Sphagnum* pectins (Kristiansen, Ballance, Potthast, & Christensen, 2009). The molecular weight (M_w), number average weights (and M_n) and the weight based on viscosity (η_w) were calculated using the ASTRA 6 (Wyatt) software on the basis of exponential fits of log *M* versus V (elution volume).

2.10. Oligosaccharide separation on HPAEC-EC

LCC50%A and LCC100A were dissolved in MilliQ water and centrifuged in a Nanosep® (Pall) centrifugal device (3 kDa, grey), at 14,000 × *g* (Heraus, Fresco 21) for 10 min and analyzed on a CarboPac PA100 column (4 mm × 250 mm, Dionex) attached to a high performance anion exchange chromatography system with electrochemical detection (HPAEC-EC, Dionex ISO 3000). The column was equilibrated with 98% 100 mM NaOH/2% 1 M NaOAc in 100 mM NaOH, and a constant gradient of NaOH and NaOAc was applied after injection in the following procedure: 0.0–20 min; 98/2%–35/65% 100 mM NaOH/1 M NaOAc in 100 mM NaOH. The gradient was then held constant at 35/65% for 8 min before returning to initial conditions. A flow rate of 1 ml/min was applied.

2.11. Determination of acetyl- and methyl esterification

The infra-red (IR) spectra of LCC50% and LCC100 in a KBr tablet were registered and investigated for the presence of absorption bands at 1735 cm^{-1} and 1250 cm^{-1} corresponding to esters (Tomoda, Nakatsuk, & Satoh, 1974).

2.12. Protein quantification

The amount of protein in all fractions was determined by the Bio-Rad Protein assay according to the manufacturer's instructions. The standard curve was based on bovine serum albumin (BSA).

2.13. Analytical LPS (endotoxin) quantification

The amount of LPS was determined by GC–MS (de Santana et al., 2012). The standard curve was obtained by preparation of endotoxin from *Escherichia coli* O55:B5 (Sigma). The method is based on acetylation of 3-hydroxyl fatty acid methyl esters of the lipid A of the LPS molecule. 3-OAc-C14:0 ester was detected by selective ion monitoring, using 257 *m*/*z* as the target ion. Interference with polymers at the retention time for 3-OAc-C14:0 ester was not found.

2.14. Complement fixation activity

The effect on human complement was measured by method A as described by Michaelsen, Gilje, Samuelsen, Hagasen, and Paulsen (2000). PMII, isolated from the leaves *Plantago major*, was used as a positive control (Samuelsen et al., 1996). PMII is a RG-I type pectin with AG-II side chains, representing typical plant pectin with bioactivity in the relevant assays.

2.15. Induction of RAW 264.7 macrophages

RAW 264.7 macrophages are very sensitive towards LPS (Pugh et al., 2008). After quantification of LPS, samples with <0.02% LPS were preincubated for 2 h with polymyxin B, 10 μg/ml. Samples containing >0.02% were passed through a Polymyxin B (Sigma) agarose column (Detoxi-Gel, Pierce). To avoid ionic interactions with the column, a 0.2 M NH_4HCO_3 buffer, pH 6, was used as eluent. Equipment was washed with 1 M NaOH for 30 min to traces of LPS contamination.

RAW 264.7 mouse macrophage cells were seeded into 96 well plates (5×10^5 cells/ml), and incubated overnight with the LPS free polysaccharide test-solutions (100 μg/ml, 10 μg/ml and 1 μg/ml) in duplicates. LPS (500 ng/ml, *E. coli* O55:B5, Sigma–Aldrich) was used as a positive control. The amount of nitric oxide (NO) released by macrophages was quantified by the Griess reagent system (Austarheim, Christensen, et al., 2012).

2.16. Anti-adhesion studies on H. pylori

Anti-adhesion studies were carried on fractions LCC100A (see Fig. 1) and CC1 (from bark) according to Niehues and Hensel (2009) which focus on the direct interaction between human gastric epithelial (AGS) cells and *H. pylori*. Briefly, fluorescein isothiocyanate-labelled bacteria (*H. pylori* J99, ATCC 700,824) were pre-incubated with the pectin extracts (1 mg/ml) for 2 h at 37 °C. The AGS cells were grown in 6 well-plates to confluence of at least 80%. Labelled and pre-incubated bacteria were added to the wells for co-incubation with AGS cells at 37 °C. One hour later, the AGS cells were washed and trypsinized and the fluorescence intensities were read. A high fluorescence indicated high adhesion of bacteria to the AGS cells and therefore a low activity.

3. Results and discussion

3.1. Fractionation and purification of polysaccharides

The procedure for isolation and purification of polysaccharides were performed as described in Fig. 1. The yields for LCC50%, LCC50 and LCC100 were determined to be 0.75%, 0.6% and 3.0% respectively. The defatted leaves were extracted with 50% EtOH on reflux before extracting with 50 °C water as direct extraction with water gave a coloured extract with a high degree of polyphenols (Austarheim, Mahamane, et al., 2012). Due to high viscosity, the extracts were passed through a Chelex 100 column to remove divalent ions, creating ionic crosslinking of pectin chains. Passing LCC50 through a Chelex 100 column resulted in a reduction of Ca^{2+} from 0.9% to 0.3% and a reduction in Mg^{2+} from 1.3% to 0.6%. These were surprisingly low reductions in ionic contents compared to the 10 fold reduction in Ca^{2+} (residual amount 0.09%) and more than 400 fold reduction of Mg^{2+} (residual amount 0.006%) previously reported for aqueous bark extracts (Austarheim, Christensen, et al., 2012). The low decrease in divalent ion content might be due to high initial viscosity that will make the ions inaccessible to the resin.

As a result of loss during fractionation, the yield of the purified fractions are not given as % related to the plant material, but as % related to the fractions obtained from the same fractionation step, see Fig. 1. A fraction of LCC50% and LCC100 named LCC50%-I and LCC100-I respectively (Fig. 1) did not bind to the ANX-IEC although monosaccharide analysis showed an uronic acid content of 44% and 15.2% respectively (Tables 1a and 1b). This uncommon behaviour was also experienced by fractionation of polysaccharides from *C. cordifolia* bark (Austarheim, Christensen, et al., 2012). The reasons for this effect remain unclear, however crosslinking of the uronic acids with divalent ions may prevent the ionic interactions with the column. A significant part of LCC50%-I and LCC100-I precipitated in solution after eluting from the ANX-IEC. The precipitate did not dissolve under methanolytic or formolytic conditions. To investigate if the precipitate consisted of carbohydrate, the precipitate was washed five times with MilliQ water and two times with 50% EtOH to ensure that all soluble carbohydrate was removed. The possible presence of carbohydrates was

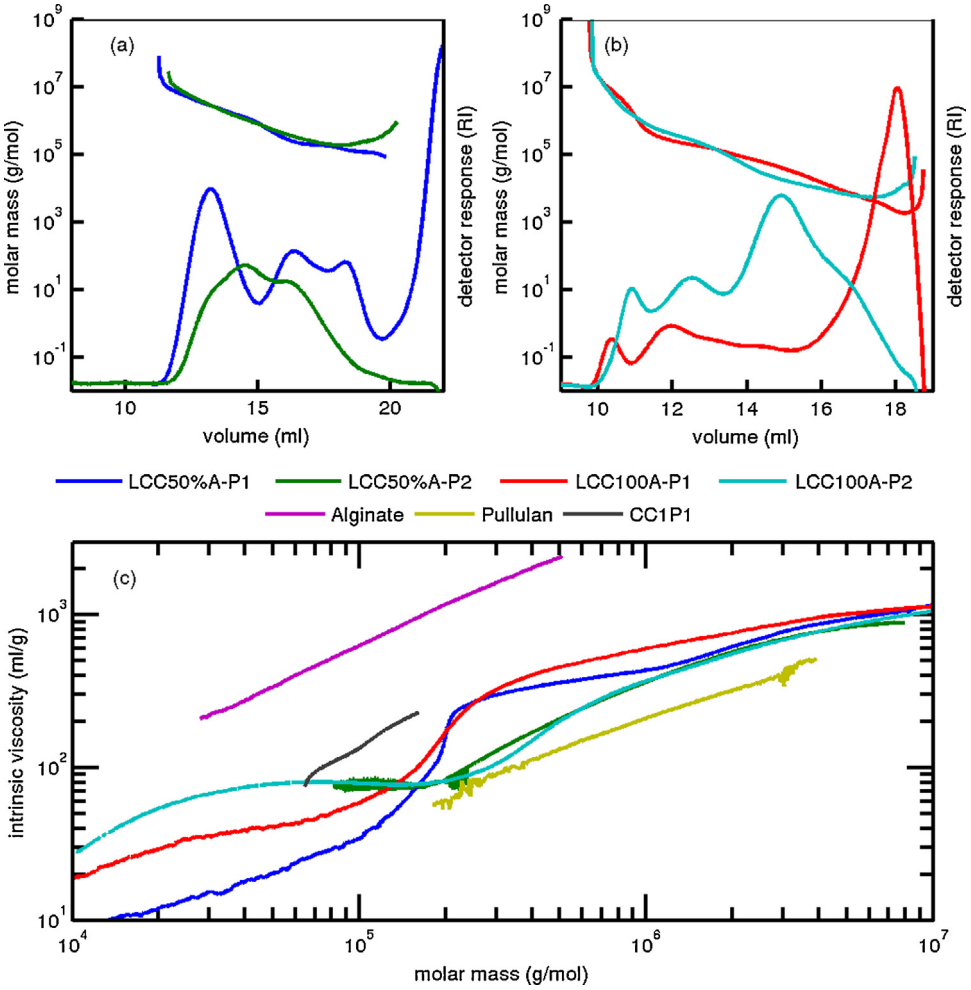

Fig. 2. (a) RI profiles and calibration curve of LCC50%A-P1 and LCC50%A-P2. SEC columns used TSK 6000 and 5000, (b) RI profile and calibration curve of LCC100A-P1 and LCC100A-P2. SEC columns used: TSK 4000 and 3000, (c) MHS plots of relationship between the intrinsic viscosity and the molecular weight of all fractions. For the sake of comparison, the homogenous standards alginate and pullulan were included, in addition to CC1P1 (Gal:Rha:GalA in ratio 1:1:1) (Austarheim, Christensen, et al., 2012). (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

detected by the phenol–sulfuric assay (Dubois et al., 1956). The yellow-orange colour that developed indicated the presence of sugars. The fact that normal hydrolysis conditions were not sufficient may indicate the presence of C-glycosides or carbohydrates linked to lignin (Bidlack, Malone, & Benson, 1992). Precipitation on the column might explain the reason for the high loss of material observed, approximately 50% of total weight. LCC100-I or LCC50%-I was not fractionated further due to the low solubility.

3.2. Monosaccharide composition and linkage analysis

The monosaccharide compositions of fractions from 50% EtOH extraction are listed in Table 1a, and composition of the 50 °C and 100 °C water extracts are listed in Table 1b. The results from the linkage analysis are presented in Table 2. IR spectra did not show any absorption in the relevant areas corresponding to the presence of esters. We therefore conclude that all uronic acids are non-esterified. Free uronic acids will give rise to possibilities for crosslinking with divalent ions resulting in increased viscosity.

LCC50%A-P1, LCC50%A-P2, LCC100A-P1 and LCC100A-P2, have all monosaccharides and linkages normally found in the RG-I backbone. In addition to the common branching point 1→2,4 linked Rha, the more unusual 1→2,3 linked Rha (Naran, Chen, & Carpita, 2008) was present in all fractions. Arabinan side chains are present in LCC50%A-P1, LCC100A-P1 and LCC100A-P2 as seen from the presence of 1→2,5 linked Ara in addition to terminal Ara, 1→5, and 1→3,5 (Table 2) (Pettolino, Walsh, Fincher, & Bacic, 2012). In addition to arabinan side chains, LCC50%A-P1 contains AG-II side chains as shown by the presence of 1→3,6 Gal, 1→3 Gal, 1→6 Gal and terminal Ara*f* in addition to a positive Yariv precipitation (Pettolino et al., 2012). The terminal and 1→4 linked Xyl are only present in the oligosaccharide fractions, comprising 9.8% Xyl (see Table 1). LCC50%A-P1 and LCC100A-P1 >3 kDa did not contain Xyl (results not shown).

Linkage analysis shows that LCC50%A-P2 contains a high degree of 1→3,4 linked GalA (12.7%). 1→3,4 GalA can be part of the homogalacturan back bone, however Capek, Rosik, Kardosova, and Toman (1987) and Renard, Crepeau, and Thibault (1999) have previously found alternating 1→3,4 linked GalA and 1→2,4 linked Rha in the RG-I backbone, with terminal GlcA linked to GalA. We therefore suggest that LCC50%A-P2 has similar structure elements with a 4-OMe-GlcA attached to GalA in the RG-I backbone.

3.3. Molecular weight distributions

The RI (concentration) profiles of LCC50%A-P1 and LCC50%A-P2 (Fig. 2a) show broad tetra- and trimodal distributions respectively, whereas LCC100A-P1 and LCC100A-P2 (Fig. 2b), show broad

Table 1b
Fractions obtained from 50 °C to 100 °C water extraction; monosaccharide composition, protein quantification and molecular weights.

	LCC50	LCC100	LCC100-I	LCC100 A	LC100A-P1	LCC100A-P2
Ara	4.9	7.0	31.3	10.0	8.2	12.5
Rha	18.0	20.1	6.4	10.1	12.1	11.1
Xyl	0	Trace	3.3	Trace	1.2	0
Gal	21.0	20.2	18.7	10.2	10.5	9.7
2-OMe-Gal	2.3	1.6	0	0.4	0	0
4-OMe-GlcA	13.3	13.9	4.1	4.5	2.3	5.1
GalA	37.1	34.2	11.1	63.1	63.5	57.9
Glc	3.3	3.0	25.2	1.7	2.2	3.7
Yariv	(+)	(+)	n.d.	(+)	(+)	–
Protein (%):	0	0.4	n.d.	0	0.1	0.1
LPS (%)	n.d.	n.d.	n.d.	n.d.	0.003	0.005
PDI	n.d.	n.d.	n.d.	n.d.	97	18
M_w (kDa)	n.d.	n.d.	n.d.	n.d.	462	318
M_n (kDa)	n.d.	n.d.	n.d.	n.d.	4.8	18

PDI, poly dispersity index (PDI = M_w/M_n); n.d, not determined.

tetramodal distributions. The calculated molecular weight data (log *M* vs. Volume) are not strictly linear, suggesting that all samples are heterogeneous (in terms of macromolecular geometries). A direct consequence of the broad and multimodal distributions is that LCC50%A-P1, LCC100A-P1 and LCC100A-P2 have high polydispersity indices (PDI = M_w/M_n) of 33.5, 96.7 and 17.5 respectively. LCC50%A-P2 is the least polydisperse fraction, with a PDI of 2.7. In comparison, polydispersity indices around 2.0 are often observed for chemically more homogeneous polysaccharides.

Relationship-analysis between the intrinsic viscosity and molecular weight, also called Mark–Houwink–Sakurada (MHS) plot (Fig. 2c) provides further information about the physical shape and extension of the polymers. The MHS plots of the pectin fractions all have a more or less sigmoid shape rather than the straight lines obtained for alginate and pullulan (included for comparison). This indicates that the pectin fractions are non-homogeneous, and consist of materials with widely different macromolecular geometries, as also indicated by the multimodal molecular weight distributions. Low intrinsic viscosities are obtained for molecular weights below 100 kDa, suggesting rather compact geometries which could be due to numerous long branches. The larger molecules (>100 kDa) have intrinsic viscosities shifted to higher values. This is probably due to a uniform distribution of shorter branches.

The oligomers present in LCC50%A-P1 and LCC100A-P1, shown by the appearance of a baseline separated peak at high elution volume, showed an average molecular weight of 7 kDa. LCC50%A-P1 contained significantly more oligosaccharides, probably due to good solubility in 50% EtOH. The <3 kDa oligomers present in LCC50%A-P1 and LCC100A-P1, were separated on a HPAEC-EC (elution profile not shown). Three large peaks and several smaller peaks were observed. The chromatograms from the two fractions were similar, indicating similar types/sizes of oligosaccharides present.

3.4. Structure-activity relationship

RG-I backbone with side chains of arabinans, AG-I and AG-II structures are often present in immunomodulating polysaccharide of plant origin (Paulsen & Barsett, 2005; Yamada & Kiyohara,

Table 2
Linkage analysis of the fractions LCC50%A-P1, LCC50%A-P2, LCC100A-P1 and LCC100A-P2.

	Linkage	LCC50%A-P1	LCC50%A-P2	LCC100A-P1	LCC100A-P2
Ara	T*f*	7.5	1.6	5.0	3.8
	1→2*f*	Trace	Trace	Trace	Trace
	1→3*f*	Trace	Trace	Trace	Trace
	1→5*f*[a]	4.4	Trace	1.0	5.9
	1→3,5*f*[a]	1.8	0	1.0	0
	1→2,5*f*[a]	2.4	0	1.2	2.8
Rha	T*p*	1.0	1.0	0	0
	1→2*p*	1.0	0.7	1.0	3.0
	1→3*p*	0.3	0.6	0.5	0.4
	1→2,3*p*	9.8	10.2	9.0	4.4
	1→2,4*p*	3.7	7.4	1.7	3.3
Xyl	T*p*	1.3	0	0.6	0
	1→4*p*	1.4	0	0.6	0
Gal	T*p*	8.0	7.5	7.2	4.6
	1→3*p*	1.3	2.1	1.0	1.4
	1→4*p*	6.0	7.1	1.1	3.7
	1→6*p*	1.3	0.4	Trace	Trace
	1→3,6*p*	3.9	0.9	1.2	0
2-OMe-Gal	T*p*	0	3.5	0	0
4-OMe-GlcA	T*p*	8.2	21.5	2.3	5.1
GalA	1→4*p*	29.0	19.5	58.5	52.4
	1→3,4*p*	4.0	12.7	4.0	4.0
	1→2,4*p*	1.6	2.0	1.0	1.5
Glc	1→4*p*	2.0	1.5	2.2	3.7

[a] It is not possible to distinguish between 1→5 linked Ara*f* and 1→4 Ara*p*, but normally Ara is present as furanose in pectins.

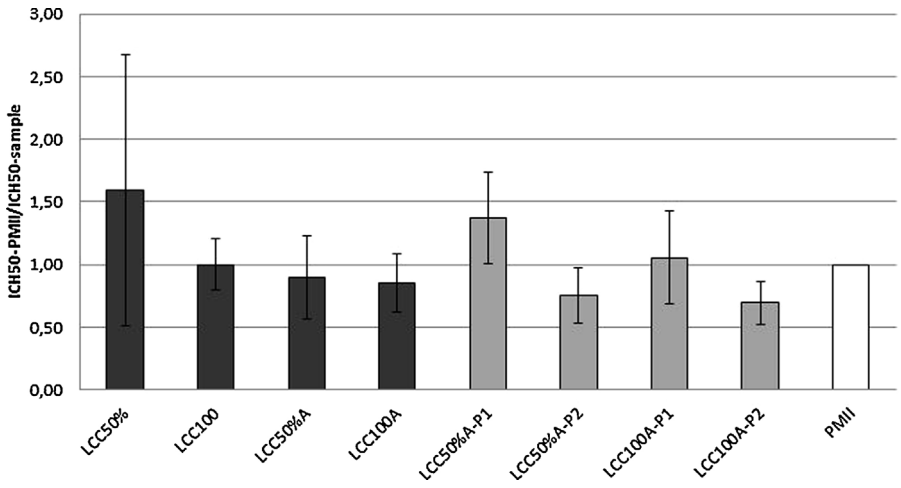

Fig. 3. Complement fixation assay. The figure shows the ratio ICH50-PMII/ICH50-sample and thus shows how active each individual test sample is compared to the positive control, PMII (130 ± 33 μg/ml). These results are based on three separate experiments.

2007). Most pectins reported to be active have a high molecular weight (>50 kDa), but mannans and glucans in the lower molecular weight range (5–15 kDa) have previously shown immunomodulating activity (Pieters, De Bruyne, & Vlietinck, 1999).

Of the purified fractions, LCC50%A-P1 displays the highest complement fixing activity, see Fig. 3. This fraction has a RG-I backbone with AG-II and arabinan side chains. LCC50%A-P2 show a lower activity, and this fraction is structurally different as it holds a high degree of branches with short, negatively charged side chains consisting of terminal 4-OMe-GlcA. Our results indicate that the abundant, short, negatively charged side results in a reduced complement fixing activity.

There is not a marked difference in the complement fixating activity between LCC100A-P1 and LCC100A-P2. This may be due to similarities between the structures, see Table 2.

The LCC50%A and LCC100A were separated in two fractions, >3 kDa and <3 kDa respectively for comparison of complement fixing activity (Fig. 4). The results showed that the oligosaccharides were inactive, and removing them from the original fraction resulted in an increase in activity of the original fraction.

Analytical determination of LPS content revealed that LCC50%A-P1 and P2 had a higher endotoxin level compared to LCC100A-P1 and P2, see Tables 1a and 1b. The reason may be that LPS is well dissolved in boiling 50% EtOH, and most of the LPS were therefore extracted in to the boiling 50% EtOH solution. Removing LPS from the fractions, and quenching the remaining LPS with pre-incubation of test solution with polymyxin B, resulted in no induction of RAW 264.7 cells. Pre-incubation of polymyxin B (3000 IU) is not very efficient, and can only be used on samples where the final LPS concentration is less than 20 ng/ml, see Fig. 5. We would like to stress the importance of LPS removal from fractions as RAW 264.7 cells are very sensitive to LPS.

Anti-adhesion studies towards *H. pylori* were carried out on the fraction LCC100A from leaves and CC1 from bark. We could not detect any anti-adhesion activity. Previously, pectins with a high degree of GlcA have shown a high activity in this assay (Lee et al., 2006; Lengsfeld, Titgemeyer, Faller, & Hensel, 2004). Our results indicate that methylation of the 4 positions of GlcA makes the polymers inactive. However, this can also be due to structural differences concerning the positioning of 4-OMe-GlcA. As we could not find any anti-adhesion activity, we conclude that the traditional use as an anti-ulcer remedy is not explained by inhibition of *H. pylori* colonization.

3.5. Comparing bark and leaf pectins

The use of leaves as a source of medicine instead of the bark is advantageous for sustainable harvesting and accessibility to medicines. Since the wound/ulcer healing properties of *C. cordifolia* is proposed to be due to the pectins present, the pectins in the leaves and bark are therefore compared (Austarheim, Christensen, et al., 2012).

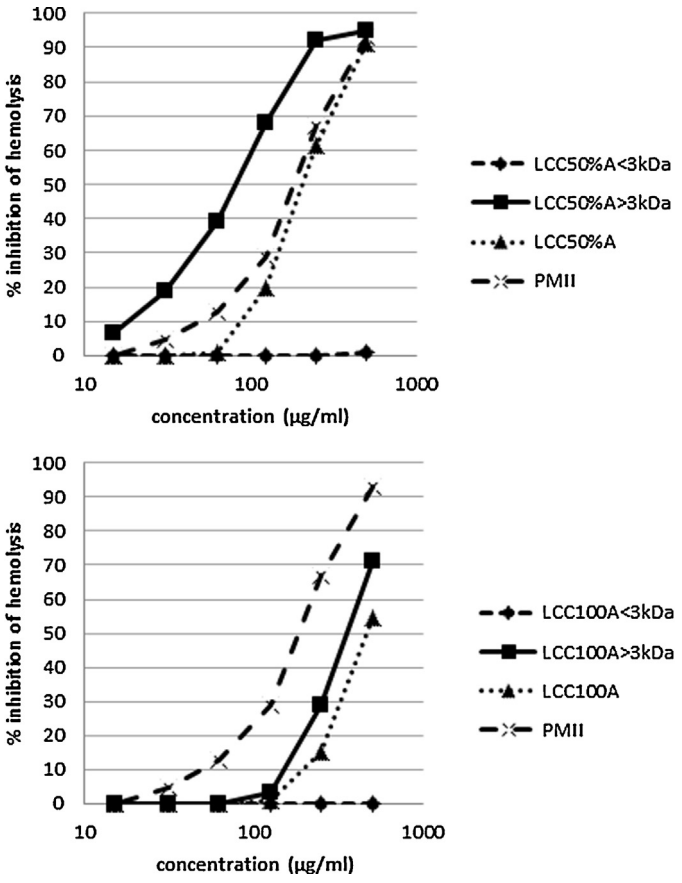

Fig. 4. Complement fixation assay. The figure shows the % inhibition of hemolysis due to complement fixation. The figure shows a representative test result.

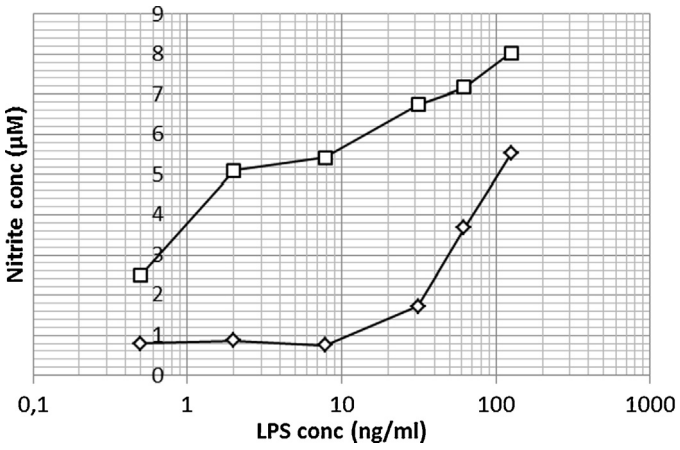

Fig. 5. NO production in RAW 264.7 macrophages (expressed as µM nitrite) stimulated with LPS from *E. coli* (□) and LPS preincubated with 3000 IU (10 µg/ml) polymyxin B for 2 h (◊). Results from a representative experiment.

The bark and leaves of *C. cordifolia* give water extracts with high viscosity, probably due to divalent crosslinking of uronic acids. This may be indirectly important for wound healing and healing of gastric ulcer as it may stick to membranes and skin (Matsumoto et al., 2002).

It was not possible to follow the exact same fractionation scheme for the two plant parts since CC1, the main bark fraction, was not found in the leaves (see Fig. 1). It is difficult to know if this will affect the medicinal potential. On average, extracts from both plant parts yield related pectin structures, with marked complement fixation and low macrophage activation.

The polysaccharides from the bark and the leaves of *C. cordifolia* contains pectins with RG-I backbone with 1→2,3 linked Rha in addition to high amounts of terminal 4-OMe-GlcA. Other similarities are the presence of 1→3,4 branching point of GalA, probably alternating with Rha 1→2,3 in the RG-I backbone, and also the relatively low overall presence of AG-II side chains. The amount 2-OMe-Gal is higher in bark than leaves, probably as a result of post modification of Gal in the slow growing bark.

CC2 was the only bark fraction with AG-II side chains, but this fraction has little in common with the AG-II rich leaf fraction LCC50%A-P1. CC2 has much longer side chains compared to LCC50%A-P1. However, CC1P2 (from bark) has clear similarities with LCC50%A-P2 when it comes to linkages, monosaccharide composition, molecular weight and polydispersity.

The purified fractions from the bark were generally more homogeneous and less polydisperse compared to those from the leaves. Oligosaccharides were not found in the bark. These differences can be due to the fact that the leaves are in constant construction, while the bark is more in a steady-state concerning biosynthesis of pectins. However, the oligosaccharides did not show activity in the complement test which may mean that they are not very important for the biological activity.

4. Conclusion

When comparing the pectins present in the leaves with bark pectins, it is clear that they are related when it comes to biological activity and structures, although some differences are present. However, before plant part replacement should be recommended, it is important to also address the secondary metabolites as possible active components.

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