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Bioactive arabinogalactans from the leaves of *Opilia celtidifolia* Endl. ex Walp. (Opiliaceae)

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The leaves of the tree *Opilia celtidifolia* have a long tradition for being used in Mali and other West African countries against various ailments such as for wound healing. Previous studies on polysaccharides from these leaves showed the presence of pectic-like polymers with an effect on the human complement system as well as the ability to activate macrophages. The present study shows that bioactive arabinogalactans isolated by water of 50°C could be separated into two acidic fractions, Oc50A1 and Oc50A2. The former could, by gel filtration on Sephacryl S-400, be separated into two fractions, which were further purified on a Superdex 200 column to give the fractions Oc50A1.I.pur and Oc50A1.II.pur. These fractions were subjected to chemical and biological studies. The polysaccharides consisted mainly of heavily branched type II arabinogalactans and minor amounts of rhamnogalacturonan I regions. The isolated polymers had a high human complement-fixating ability, as well as the ability to stimulate rat macrophages and dendritic cells (DCs) and to induce B cell proliferation. These effects were especially pronounced for the higher molecular weight fraction of Oc50A1.I.pur. The fractions Oc50A1.I.pur and Oc501.II.pur stimulated secretion of pro-inflammatory cytokines from purified B cells or DCs. Collectively, these results indicate that the arabinogalactan type II polymers present in the leaves of *O. celtidifolia* may be used to develop medical devices for regulating inflammatory processes.

Keywords: B cells/cytokines/dendritic cells/immunomodulating activity/*Opilia celtidifolia* Endl. ex Walp.

Introduction

In traditional medicine, plants have been used to treat various types of ailments. Many of these medicinal plants contain polysaccharides showing biological effects related to the immune system by different test assays in vitro and in vivo (Wagner et al. 1999; Yamada and Kiyohara 1999; Paulsen and Barsett 2005). Arabinogalactans from the Brazilian plants *Phyllanthus niruri* and *Maytenus ilicifolia* have been shown to be the active principles for treatment of ailments in the gastric system (Cipriani et al. 2006; Mellinger et al. 2008). According to Yamada and Kiyohara (1999), 1984 was the first year that a pure complex polysaccharide having biological activity was reported. Stimpel et al. (1984) showed that polysaccharides from *Echinacea purpurea* had granulocyte- and macrophage-stimulating activity and that the polysaccharides enhanced production of IL-1. Since then, many reports have been published on the biological activity of polysaccharides, especially polysaccharides from plants used for the treatment of external wounds and dermal ailments, as well as those used against ulcer and tumors, were early in focus. In developing countries, the population use medicinal plants for treatment of ailments and to improve their state of health. Traditional medicine is a significant element in the cultural patrimony, and its use has further increased with the increase in price of conventional medicine in the local currency.

Polysaccharides with biological activity are often charged, i.e. they contain uronic acids, e.g. D-galacturonic acid as in the pectic type polymers (Paulsen and Barsett 2005). Different types of polysaccharides, isolated from plants used in traditional medicine, are identified based on their activities on the complement system, e.g. arabinans, arabinogalactans, and rhamnogalacturonans (Yamada and Kiyohara 1999). The arabinogalactans are divided into two types, arabinogalactan type I (AG-I) and arabinogalactan type II (AG-II). The first is recognized by having β-D-(1→4)-Gal units as the main core, while the latter is characterized by having galactose as both β-D-(1→3)-Gal and β-D-(1→6)-Gal units with β-D-(1→3,6)-Gal as branching points. The 1→3,6Gal unit is a main feature that is recognized by the ability to form a colored complex with the Yariv reagent. The rhamnogalacturonans are also divided into type I (RG-I) and type II (RG-II). RG-I has the main core alternating α-D-(1→4)-GalA residues and α-L-(1→2)-Rha units. Parts of the rhamnosyl units are branch points, mainly on position 4. RG-II has a quite different structure. It has a α-D-(1→4)-GalA backbone which is substituted with five different oligosaccharides consisting of several rare monosaccharides, and this

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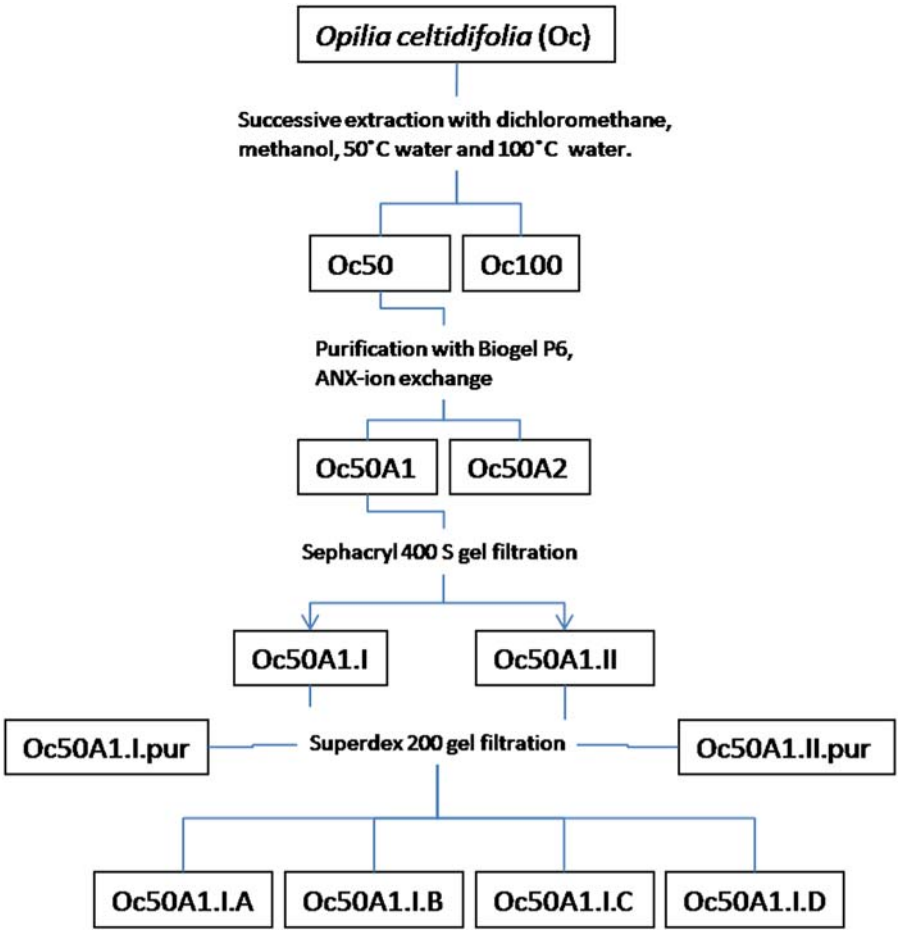
structure is basically well conserved, i.e. appears to be similar in all polymers where it has been found. Several of these types of polymers have effects on macrophages, T lymphocytes and NK cells. The majority of these polysaccharides also exhibit a mucoadhesive effect since they bind to the surface of cells and thus can cause local effects seen in certain experiments (Schmidgall et al. 2000). Characterization of pectic polysaccharides with immune modulating activity has been reported from different medicinal plants from West Africa in several recent papers, effects varying from complement fixation to activation of macrophages and dendritic cells (DCs) (Samuelsen et al. 1996; Michaelsen et al. 2000; Diallo et al. 2001; Nergard et al. 2005; Inngjerdingen et al. 2006; Togola et al. 2007; Inngjerdingen et al. 2008).

Opilia celtidifolia Endl. ex Walp. (Opiliaceae) is a tree used in traditional medicine in the treatment of a wide variety of diseases in West Africa. A leaf decoction of *O. celtidifolia* is commonly used against dermatitis in Mali (Togola et al. 2005; Grønhaug et al. 2008). The plant is, in Mali, also reported to be used against malaria, as an appetite-enhancing agent, against abdominal pain, internal worms, and as a wound healing remedy (Diallo et al. 2002; Inngjerdingen et al. 2004; Togola et al. 2005). In Senegal a leaf macerate from the same tree is used to treat edema, and a decoction of the leaves is used as a gargle for dental abscesses. A leaf decoction is taken as a febrifuge in Ivory Coast. *O. celtidifolia* is also used to treat sleeping sickness and leprosy (Burkill 1997).

A few studies have been focused on low molecular weight compounds from methanolic extracts of *O. celtidifolia*. Crespin et al. (1993) found six triterpenoid saponins in the MeOH:H_2O (4:1) extract, and Druet et al. (1991) also reported several triterpenoid compounds in *O. celtidifolia*. Shihata et al. (1977) studied the pharmacological properties of saponin fractions from the plant and reported the fractions to be intestinal antispasmodic, uterine stimulant, hypotensive, and to depress the coronary outflow.

Togola et al. (2007) studied the carbohydrate composition, the complement-fixating ability, and the ability to activate macrophages of acidic polysaccharide fractions extracted from the leaves of *O. celtidifolia*. They reported that the polymers contained high amounts of arabinogalactan II. Enzymatic digestion with endo-polygalacturonanase revealed little differences in monosaccharide composition, effect in the complement-fixating assay, and activation of macrophages. Initial studies also gave similar results. The authors indicated that *O. celtidifolia* is a medicinal plant of great potential. On



Scheme 1. Extraction and fractionation scheme used on *O. celtidifolia* leaves. After ion-exchange chromatography, two acidic fractions were isolated, Oc50A1 and Oc50A2, with A2 being more acidic than A1. Gel filtration of Oc50A1 yielded the two subfractions, Oc50A1.I and Oc50A1.II. Further purification on another gel filtration column yielded the fractions Oc50A1.I.pur and Oc50A1.II.pur. The peak which gave Oc50A1.I.pur was also divided into four subfractions, Oc50A1.I.A–D, to investigate polydispersity in the fraction.

Table I. Monosaccharide composition and protein content of the acidic fractions from the leaves of *Opilia celtidifolia*

	Oc50A1.I	Oc50A1.I.pur	Oc50A1.I.A	Oc50A1.I.B	Oc50A1.I.C	Oc50A1.I.D	Oc50A1.II	Oc50A1.II.pur
Monosaccharide composition[a]								
Ara	27.4	40.1	38.9	44.7	53.2	35.2	25.8	41.4
Rha	7.6	3.1	4.2	2.6	3.8	5.7	10.9	3.7
Man	5.5	3.4	5.8	3.5	3.8	4.0	5.2	3.4
Gal	27.1	36.4	30.9	40.0	16.6	36.7	26.2	36.0
Glc	4.5	2.5	5.4	Trace	2.4	4.3	12.9	3.6
GlcA	–	2.4	Trace	Trace	3.3	2.2	–	2.5
GalA	24.7	8.7	11.5	4.8	11.9	9.0	19.0	6.3
4-OMe-GlcA	3.2	3.4	3.3	4.7	5.1	3.0	Trace	3.2
Total protein content (%, w/w)	11.6	3.2	5.5	1.2	1.6	3.4	17.2	3.6

[a]% of total carbohydrate content.

the basis of these studies, we wanted to further purify acidic polysaccharide fractions and test them in additional biological assays. The chemical and physical properties of the purified fractions were further investigated to gain more detailed information on the bioactive structure able to interact with different elements of the immune system.

Results

Purification of polysaccharides

The leaves of *O. celtidifolia* were extracted with dichloromethane (DCM) and methanol (MeOH), as described in Materials and methods, to remove lipids and low molecular weight compounds. The plant material was then extracted with water at 50°C, and after volume reduction and partial removal of small molecules with Bio Gel P6 DG, this fraction was named Oc50. Purification of the crude water extract Oc50 on an anion-exchange chromatography column resulted in two acidic fractions, Oc50A1 and Oc50A2. Oc50A1 was further purified on a Sephacryl S-400 gel filtration column with the resulting fractions Oc50A1.I and Oc50A1.II, having different molecular weights (Scheme 1). The monosaccharide compositions of these two subfractions were determined and are given in Table I. With the intention of further purification of the fractions Oc50A1.I and Oc50A1.II, they were applied on a Superdex 200 gel filtration resulting in the fractions Oc50A1.I.pur and Oc50A1.II.pur. The peak designated Oc50A1.I.pur was further divided into four subfractions Oc50A1.I.A, Oc50A1.I.B, Oc50A1.I.C, and Oc50A1.I.D as described in Figure 1A and Scheme 1. Figure 1B shows the elution profile of each of the subfractions when rechromatographed on an analytical Superdex 200 column. The rechromatography procedure revealed that the original fraction Oc50A1.I.pur was heterogeneous with regards to molecular size or conformation.

Determination of carbohydrate composition

The monosaccharide composition of the different fractions is shown in Table I. The major constituents of Oc50A1.I are arabinose (Ara; 27.4%), galactose (Gal; 27.1%), and galacturonic acid (GalA; 24.7%). The same distribution was seen for Oc50A1.II, where Gal (26.2%), Ara (25.8%), and GalA (19.0%) were the major constituents of the polysaccharide. The main differences between Oc50A1.I and Oc50A1.II were found in the amount of glucose (Glc; 4.5 and 12.9%, respectively) and 4-*O*-methyl glucuronic acid (3.2% and trace amounts, respectively). Other monosaccharides were also present in minor amounts (Table I). The carbohydrate composition of Oc50A1.I.pur is quite different from the composition of Oc50A1.I. The purified fraction has relatively much higher content of Ara and Gal, and lower content of uronic acid than the parent fraction. This is the same for the Oc50A1.II.pur versus Oc50A1.II. The monosaccharide composition of the purified polysaccharide fractions, Oc50A1.I.pur and Oc50A1.II.pur, was nearly identical (Table I) with Ara (40.1 and 41.4%, respectively) and Gal (36.4 and 36.0%, respectively) making up over 75% of the total monosaccharide composition.

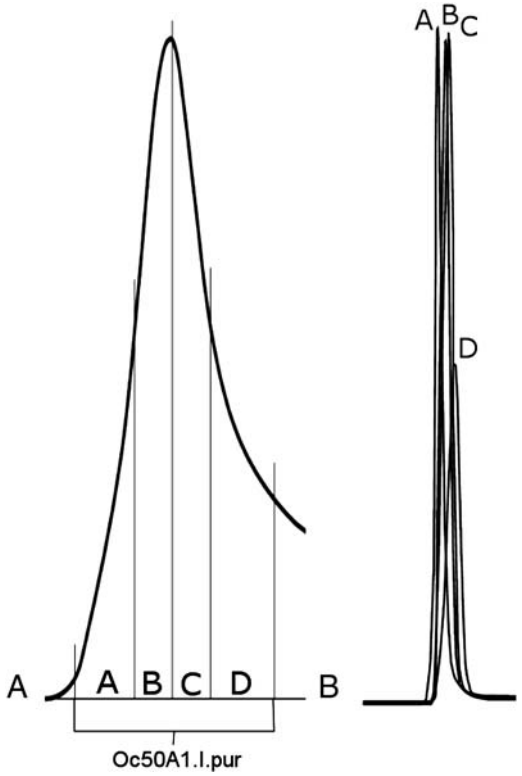


Fig. 1. (**A**) The figure shows the elution profile of Oc50A1.I.pur from Superdex 200 gel filtration and how the peak was divided into the four subfractions. (**B**) The figure shows the polydispersity of sample Oc50A1.I.pur after rechromatography of each subfraction.

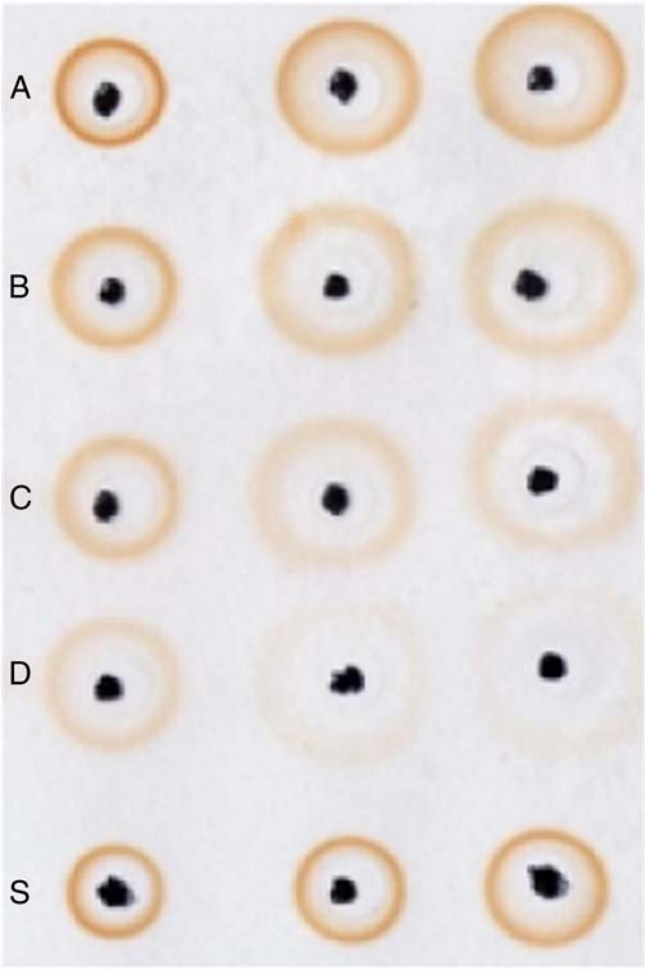


Fig. 2. Detection of precipitated arabinogalactan type II structures in agarose gel containing Yariv β-glucosyl reagent (0.1 mg). (**A**–**D**) Oc50A1.I.A–D (8 μg/μL), (**S**) positive control (gum arabic, 1 μg/μL). There were 2, 4, and 6 μL of each sample was added to three wells in a row.

There were minor differences in the monosaccharide composition of the subfractions Oc50A1.I.A, Oc50A1.I.B, Oc50A1.I.C, and Oc50A1.I.D. The content of Ara was higher in the two fractions Oc50A1.I.C and Oc50A1.I.B (53.2 and 44.7%, respectively) compared to Oc50A1.I.A and Oc50A1.I.D (38.9 and 35.2%, respectively). The amount of Gal varied between the fractions, Oc50A1.I.A (30.9%), Oc50A1.I.B (40.0%), Oc50A1.I.C (16.6%), and Oc50A1.I.D (36.7%). The three fractions Oc50A1.I.A, Oc50A1.I.C, and Oc50A1.I.D contained around twice as much GalA as Oc50A1.I.B. The composition of the other monosaccharides also varied to a minor extent (Table 1). These differences between the subfractions Oc50A1.I.A, Oc50A1.I.B, Oc50A1.I.C, and Oc50A1.I.D further strengthen the notion of some structural differences between them.

Precipitation with the Yariv reagent

All four fractions showed a positive reaction with the Yariv β-glucosyl reagent indicating the presence of AG-II in all four fractions (Figure 2) with a decline in precipitation from

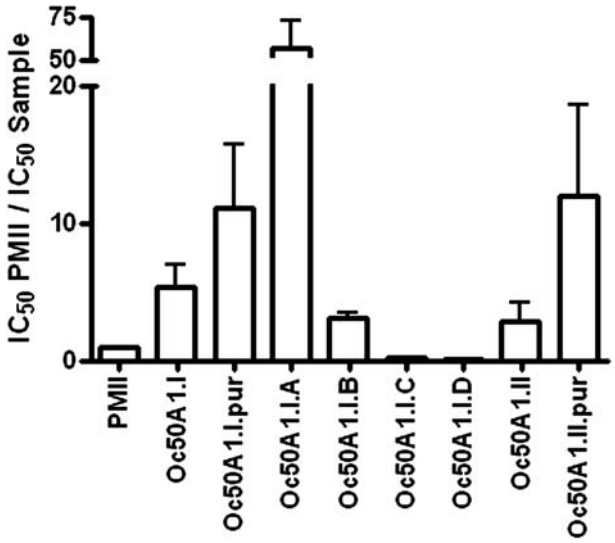


Fig. 3. Complement-fixating assay. IC_{50} values are the calculated concentration of the test material able to inhibit 50% of the lytic activity in the assay system. The figure shows the ratio of IC_{50} PMII/IC_{50} test sample and thus shows how active each individual test sample is compared with the internal positive control, PMII.

Oc50A1.I.A to Oc50A1.I.D indicating structural heterogeneity in the fraction Oc50A1.I.pur.

Determination of glycosidic linkages present in the polysaccharides

The main monosaccharides in all fractions are Ara and Gal. The Ara monomers are mainly found as either T*f* Ara or 1→5*f* Ara. 1→5*f* Ara monomers are the building blocks of the arabinan backbone. The arabinans can be substituted in position 2 and/or 3 and in our fractions as these branching points are present. The Gal moieties are mainly composed of 1→3*p* Gal and 1→3,6*p* Gal. AG-II consists of a backbone of 1→3*p* Gal with branching points at 1→3,6*p* Gal which can either be bound to Gal or Ara. This is in accordance with the results using the Yariv reagent. The low amounts of T*p* Rha, 1→2*p* Rha, and 1→2,4*p* Rha (2.4–5.2%) present indicate a short backbone of RG-I, and based on the remaining amount of 1→4*p* GalA, after having subtracted the part to be included in the RG-I region, only small amounts of homogalacturonan chains may be present in all the fractions.

Determination of protein content and amino acid composition

As seen in Table I, the purification of Oc50A1.I and Oc50A1.II on the Superdex 200 column resulted in reduction in the protein content in fractions Oc50A1.I.pur and Oc50A1.II.pur, which both now contained almost the same amount of protein (3.2 and 3.6%, respectively). Table I also shows that Oc50A1.I.A (5.5%) had the highest amount of protein while Oc50A1.I.B and C contained a smaller amount of protein (1.2 and 1.6%, respectively), Oc50A1.I.D contained 3.4% protein.

The amino acid composition was determined for the fractions Oc50A1.I and Oc50A1.II. The fractions contained mostly alanine (29.2 and 26.8% of total protein content, respectively), serine (15.3 and 17.0%), threonine (12.0 and 12.2%), glycine

(10.2 and 10.9%), and glutamate (9.3 and 9.5%). Other amino acids were present in minor amounts.

Polydispersity and molecular weight determination

Figure 1B shows the elution profiles for each of the Oc50A1.I.A–D fractions when rechromatographed on an analytical Superdex 200 column. The fractions were eluted in the same order as they originally were divided in, with I.A first, then I.B, I.C, and I.D. Molecular weights determined by ultracentrifugation were found to be 150 ± 10 kDa for I.A and I.B, 120 ± 10 kDa for I.C, and 72 ± 2 kDa for I.D. These results show that the polysaccharides in Oc50A1.I.pur are of a polydisperse nature.

Human complement-fixating assay

The fractions Oc50A1.I, Oc50A1.I.pur, Oc50A1.II, and Oc50A1.II.pur showed strong human complement-fixating activity in vitro compared to the positive control PMII (Figure 3). For the subfractions Oc50A1.I.A–D, Oc50A1.I.A and Oc50A1.I.B showed strong activities, while Oc50A1.I.C and Oc50A1.I.D showed much less. The activity was dose dependent, and all the fractions, except Oc50A1.I.C and Oc50A1.I.D, were more active on weight basis than PMII. The subfraction Oc50A1.I.A was the most active one, and it has a calculated IC_{50} value of 0.6 μg/mL, as compared to that of PMII being 35.5 μg/mL (60 times more active).

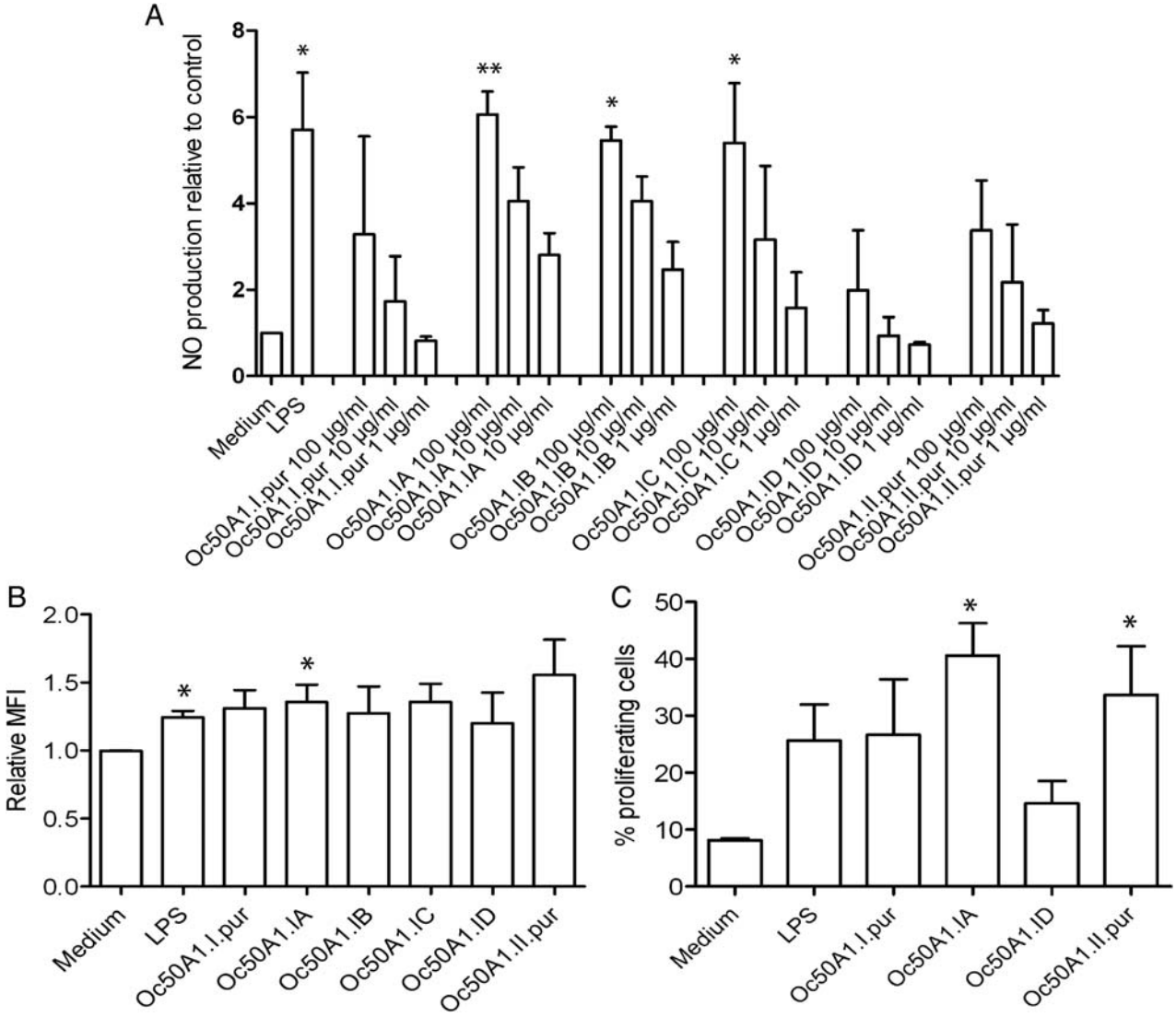


Fig. 4. Leukocyte stimulatory activities of *O. celtidifolia*. (**A**) Measurement of nitric oxide release from Raw 264.7 macrophages after overnight stimulation with different concentrations of *O. celtidifolia* fractions or 250 ng/mL LPS as positive control. NO production from stimulated macrophages was related to production from cells in control medium only. Data are presented as the mean of three independent experiments ± SEM. **, $P < 0.001$; *, $P < 0.05$. (**B**) Maturation of immature dendritic cells with *O. celtidifolia* fractions. Bone marrow-derived dendritic cells were incubated for 24 h with the indicated pectic fractions at 50 μg/mL, 250 ng/mL LPS as positive control, or medium alone as negative control. The expression level CD86 was evaluated by flow cytometry. The fold change in the median fluorescence intensity (MFI) of samples compared to that of untreated cells is presented. Data are presented as the mean of three independent experiments ± SEM. **, $P < 0.006$; *, $P < 0.05$. (**C**) B cell proliferation induced by *O. celtidifolia* fractions. CFSE-stained splenic B cells were cultured in the presence of 100 μg/mL polysaccharide fractions, 250 ng/mL LPS, or medium alone for 5 days. Proliferation was measured by flow cytometry as the percent decrease in CFSE fluorescence intensity in the B cell population. Data are presented as the mean of three independent experiments ± SEM. *, $P < 0.05$.

Activation of rat macrophages

Production of nitric oxide (NO) from macrophages as a test of activation was measured after treatment of the macrophages with the fractions Oc50A1.I.pur, Oc50A1.II.pur, and Oc50A1.I.A–D. The well-characterized mouse macrophage cell line Raw264.7 was used for these experiments. Lipopolysaccharide (LPS), a constituent of the outer membrane of gram-negative bacteria, was utilized as a positive control, as it is a potent stimulator of cells of the monocytic lineages (Sweet and Hume 1996). Release of NO was observed from macrophages stimulated with several of the polysaccharide fractions. Oc50A1.IA and Oc50A1.IB induced the most brisk NO production, followed by Oc50A1.IC (Figure 4A). NO production was variable in response to Oc50A1.I.pur or Oc50A1.II.pur, and no production was observed in macrophages cultured with Oc50A1.ID.

Maturation of rat DCs

DC maturation typically leads to up-regulation of the co-stimulatory molecule CD86. Bone marrow-derived immature DCs were stimulated for 24 h with either LPS as positive control (250 ng/mL) or 50 μg/mL of Oc50A1.I.pur, Oc50A1.II.pur, or Oc50A1.I.A–D fractions. The expression levels of CD86 on the cells were analyzed by flow cytometry. The median fluorescence intensity (MFI) of CD86 expression of the stimulated samples relative to the MFI of untreated cells was calculated. We observed a significant increase in CD86 expression of DCs treated with Oc50A1.IA (1.3-fold) and of the positive control LPS (1.25-fold) (Figure 4B). While we observed a trend towards up-regulation of CD86 expression by the other fractions, this was not significant.

Rat B cell proliferation

It was next tested whether the polysaccharide fractions could induce B cell proliferative activities. As LPS is a well known T-cell independent mitogen, we included LPS as a positive control. Splenic B cells were stained with the intracellular dye carboxyfluorescein succinimidyl ester (CFSE) and cultured in the presence of pectic fractions or LPS for 5 days. Flow cytometric analysis was applied to calculate proliferation as the loss of CFSE fluorescence intensity in the populations, as cell divisions will result in dilution of the CFSE fluorescence from the starting population. We observed a robust proliferative response of both the main fraction Oc50A1.II.pur, as well as of Oc50A1.IA (Figure 4C). LPS tended to induce proliferating cells, but the percentage proliferating cells was not significant from the negative control.

Cytokine secretion from rat DCs and B cells

Production and secretion of cytokines is a typical event following activation of leukocytes. We therefore tested the ability of the pectic fractions to induce the release of cytokines, to further confirm their stimulatory properties. As LPS is known to promote cytokine release from both DCs and B cells, it was included as a positive control. The cells were stimulated for 24 h in the presence of either medium alone, 250 ng/mL LPS, or 50 μg/mL of the fractions Oc50A1.I.pur or Oc50A1.II.pur. Utilizing a multiplex cytokine assay, we could simultaneously measure the presence of IL-1α, IL-6, IL-10, IL-12p70, IL-18, TNF-α, MCP-1, MIP-1α, and IP-10 in the medium of stimulated cells. We found that B cells secreted IL-10 and TNF-α in response to both Oc50A1.I.pur and Oc50A1.II.pur, while stimulation with Oc50A1.II.pur also induced the release of IL-6 (Figure 5A). LPS induced the release of TNF-α, albeit at somewhat lower levels than Oc50A1.I.pur and Oc50A1.II.pur. DCs cultured for 24 h in the presence of 50 μg/mL of Oc50A1.I.pur released IL-1α, IL-6, and TNF-α comparably to LPS stimulation (Figure 5B). Oc50A1.II.pur stimulation led to significant secretion of only TNF-α. IL-10 secretion

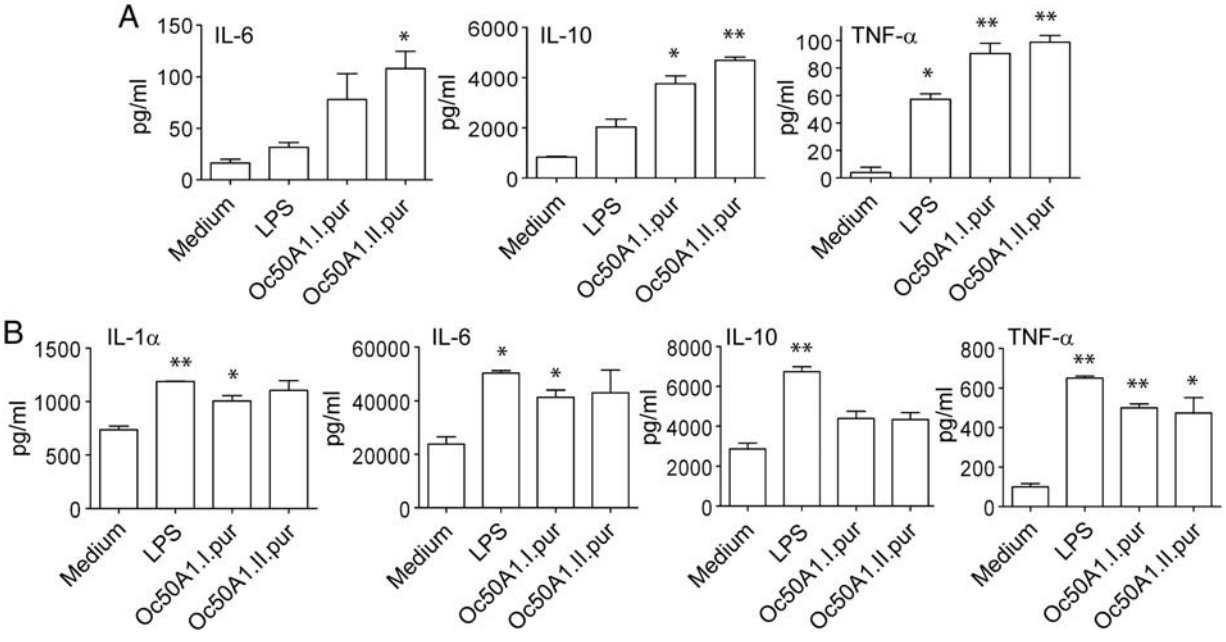


Fig. 5. Induction of cytokine secretion from (**A**) B cells and (**B**) dendritic cells. Cells were stimulated in duplicates with Oc50A1.I.pur or Oc50A1.II.pur at 50 μg/mL, 250 ng/mL LPS as positive control, or medium alone as negative control. The data presented represent the mean ± SEM of duplicate measurements from one of two independent experiments. **, $P < 0.005$; *, $P < 0.05$.

Table II. Linkage composition of the acidic fractions from the leaves of *O. celtidifolia*

Type of linkage	Oc50A1.I.pur	Oc50A1.I.A	Oc50A1.I.B	Oc50A1.I.C	Oc50A1.I.D	Oc50A1.II.pur
T*f* Ara	27.8	21.8	25.7	37.2	21.4	24.7
1→3*f* Ara	1.0	1.6	1.6	1.0	1.8	1.6
1→5*f* Ara	9.7	11.9	13.2	13.7	10.2	12.9
1→2,5*f* Ara	0.2	0.4	0.5	0.2	0.4	0.3
1→3,5*f* Ara	1.4	3.2	2.7	1.1	1.5	2.0
1→2,3,5*f* Ara	–	–	1.1	–	–	–
T*p* Xyl	Trace	Trace	Trace	Trace	Trace	Trace
T*p* Rha	1.4	1.0	0.6	0.9	0.5	1.7
1→2*p* Rha	1.0	1.4	1.0	2.1	3.3	1.3
1→3*p* Rha	0.2	0.7	0.1	–	0.5	0.1
1→2,4*p* Rha	0.5	1.2	0.8	0.8	1.4	0.6
Man	3.4	5.8	3.5	3.8	4.0	3.4
T*f* Gal	–	0.6	0.1	–	0.3	0.2
T*p* Gal	2.0	2.6	1.8	0.7	2.8	2.4
1→3*p* Gal	6.5	9.9	7.6	2.3	6.0	6.0
1→4*p* Gal	–	2.9	–	–	1.5	1.4
1→6*p* Gal	2.7	2.2	3.2	1.0	3.3	3.1
1→3,6*p* Gal	25.5	12.7	27.4	12.6	22.8	22.9
T*p* GalA	–	–	0.4	–	–	–
1→4*p* GalA	8.7	11.5	4.4	11.9	9.0	6.3
T*p* Glc	2.5	1.5	Trace	1.8	3.2	2.4
1→4*p* Glc	–	3.8	–	0.6	1.1	1.2
T*p* GlcA	2.4	Trace	Trace	3.3	1.6	2.1
1→4*p* GlcA	–	–	–	–	0.6	0.4
T*p* 4-OMe-GlcA	3.4	3.3	4.7	5.1	3.0	3.2

was only significantly induced by LPS, and not by either Oc50A1.I.pur or Oc50A1.II.pur.

Discussion

O. celtidifolia is used in traditional medicine in Mali to treat different kinds of skin disorders. Infectious diseases and wounds are two of the major health problems in Mali, and they are often treated by using hot water extracts of medicinal plants. It is therefore of interest to study the structure and biological activity of water soluble high molecular weight compounds isolated from these extracts. Togola et al. (2007) reported that the pectic polysaccharide fractions $Oc50A_1$ and $Oc50A_2$ isolated from the leaves of *O. celtidifolia* had high complement-fixating ability and macrophage stimulation activity. Further investigation of the polysaccharide fractions from the leaves of this tree was therefore of interest.

Carbohydrate composition and structural features of the Oc50A1 fractions

Isolation of the fraction Oc50 from a 50°C water extract of the leaves of *O. celtidifolia* was performed after several purification steps as described in Materials and methods. Oc50 was further purified on an anion-exchange chromatography column giving two acidic fractions, Oc50A1 and Oc50A2, which correspond to the two acidic fractions, $Oc50A_1$ and $Oc50A_2$, obtained by Togola et al. (2007). Gel filtration of the acidic fraction Oc50A1 on a Sephacryl S-400 column gave the two different molecular weight fractions Oc50A1.I and Oc50A1.II. The results of the carbohydrate composition analysis of fractions Oc50A1.I and Oc50A1.II are quite similar to the results that Togola et al. (2007) found for $Oc50A_1$. The main difference is that the fractions -A1.I and -A1.II contain more GalA (24.7 and 19.0%, respectively) than $Oc50A_1$ (5.3%) (Togola et al. 2007). In addition, $Oc50A_1$ contained small amounts of Fuc and Xyl. Fuc was not present in our samples while trace amounts of Xyl were detected in the methylation analysis.

Further purification of the two fractions on Superdex 200 gave the fractions Oc50A1.I.pur and Oc50A1.II.pur. This purification step led to products that compared to the fractions OC50A1.I and Oc50A1.II, had higher contents of Ara and Gal, and was concomitant with a decrease in Rha, GalA, Glc, and protein (Table I), meaning that this step was important for the purification of the bioactive product. The polysaccharides are typical AG-II polymers as can be seen from the presence of 1→3,6Gal as the major branching point in the polymers (Table II), also with the presence of 1→3Gal and 1→6Gal units in chains and a substantial amount of Ara in furanose form as terminal units.

After purification on a Superdex 200 column, the peak Oc50A1.I.pur was divided in four subfractions: I.A, I.B, I.C, and I.D (Figure 1A). The results from the carbohydrate composition and linkage analysis showed that these fractions are similar, but not identical. Rechromatography of each of the fractions confirmed the presence of molecular size or conformational differences among the subfractions. Molecular weight analysis by ultracentrifugation confirmed that the subfractions had different molecular weights, I.A and I.B had higher molecular weights than I.C and I.D.

Precipitation with the Yariv reagent showed that fractions I.A–I.D all contain AG-II. This is in agreement with the results from the linkage analysis where all four fractions were found to contain 1→3,6Gal, which is a characteristic of AG-II. Polysaccharides containing AG-II structures have shown effects in a number of biological assays (Yamada and Kiyohara 1999; Paulsen and Barsett 2005; Yamada and Kiyohara 2007).

The amino acid composition found for fractions Oc50A1.I and Oc50A1.II was quite similar to those found for other polysaccharides rich in AG-II structures, e.g. those found in

the Malian medicinal plant *Glinus oppositifolius* (Inngjerdingen et al. 2005).

Biological activity

The fractions I, I.pur, II, and II.pur all had high complement-fixating activity compared to the positive control PMII. Interestingly, when the four subfractions, I.A, I.B, I.C, and I.D, were tested, there were large differences in the complement-fixating ability between them. I.A had a high activity, while I.B had minor activity. I.C and I.D, on the other hand, had little or no activity. The similar pattern but less pronounced was observed when we analyzed NO production by macrophages in response to the fractions I.pur, II.pur, and the four subfractions of I.pur. Fractions I.A and I.B were the most potent inducers of NO, while fraction I.D induced less NO production. Again, fraction I.A was more potent than either I.pur or II.pur. We also studied the ability of the pectic fractions to activate DCs, which are also derived from the monocytic lineage. DCs are critically important for inducing adaptive immune responses and are activated by numerous stimuli, including bacterial products such as LPS. This activation leads to up-regulation of MHC class II, CD80, and CD86, along with induction of cytokine production. We observed that all fractions, including I.pur, its subfractions, and II.pur led to a modest increase in CD86 expression by DCs, where fractions I.A and II.pur were the most potent. When assessing B cell proliferative capacities, fraction I.A induced the highest B cell proliferative activity, while fraction I.D in comparison had little activity. In addition, fraction II.pur induced proliferative activity, in contrast to fraction I.pur.

The medical effect of the pectic fractions' influence on the immune system remains to be determined, although our data suggest that they may participate in boosting innate immune system through the complement system of cascade reaction and the activity of antigen-presenting cells, such as macrophages and DCs. Also, our analysis of cytokine secretion suggests that the pectic polysaccharides may promote inflammation, as we observe potent production of the pro-inflammatory cytokines IL-1α, IL-6, and TNF-α. However, as B cells also produce high amounts of the anti-inflammatory cytokine IL-10 in response to the pectic polysaccharides, the polysaccharides may also contribute to dampening or regulating the strength of the inflammatory processes.

Collectively, the biological assays point to Oc50A1.I.A as the fraction with the highest bioactivity. These results are interesting since all four subfractions originate from the peak I.pur, and it seems that what causes the bioactivity of I.pur is found in the first part of the fraction (I.A/I.B). All the fractions contain AG-II and seem to be fairly similar when comparing monosaccharide composition and linkage pattern. Since the precipitation with Yariv reagent and the linkage analysis showed that all fractions contain AG-II, there must be something else that decides whether the fractions are active or not. The differences in molecular size, shape/conformation, or polymerization may be one of the reasons that influence biological activity. It would be interesting to analyze the different subfractions by atomic force microscopy to see if there are any visible differences due to macromolecular folding, dimerization, or aggregation that may explain the differences in bioactivity between the fractions.

Materials and methods

Plant material

The leaves of *O. celtidifolia* were collected in Mali and identified by the Department of Traditional Medicine (DMT), Bamako, Mali. A voucher specimen of the leaves is deposited at the herbarium at DMT, file nos. 2052 and 2477.

Extraction and purification of polysaccharides

In order to remove lipid material and low molecular weight compounds, the leaves of *O. celtidifolia* (4.560 kg) were pre-extracted by Soxhlet extraction with DCM and MeOH. Exchange of extraction solvent was determined by the color of the solution. When the DCM extract was light green, MeOH extraction was started. Soxhlet extraction with MeOH was ended when no more colored material was extracted, and the weight of the dried plant material was then 3.307 kg.

Subsequently, the dried plant material was extracted twice with 17-L water at 50°C for 4 h and filtered through a fine nylon mesh. The filtrate was centrifuged on a Multifuge 4 KR (Heraeus, Germany) at 4000 rpm for 30 min, and the supernatant was then elutriated from the sediment. This yielded the crude extract Oc50. Due to massive foam formation, it was not possible to concentrate Oc50 by evaporation under reduced pressure. To remove ions and other low molecular weight compounds, 1 kg Bio Gel P6 DG (Bio-Rad, USA) was added to 8–9-L Oc50 extract. The solution was frequently stirred. The supernatant was elutriated from the gel through a fine mesh. After this procedure, the crude extract, Oc50, was kept at −18°C until further use.

Oc50 was fractionated by anion-exchange chromatography on an ANX Sepharose 4 Fast Flow column (Amersham Bioscience, CV 981.25 cm^3, Sweden) with chloride as a counterion. The column was coupled to a Perimax pump (Spetec, USA). The extract was filtrated (5 μm) before application on the column. Neutral polysaccharides were eluted with distilled water (2 mL/min), while acidic polysaccharides were eluted with a NaCl gradient (0–1.5 M, 2 mL/min). Fractions of 25 mL were collected using a Superfrac fraction collector (GE Healthcare, Sweden). The phenol-sulphuric acid assay was used to determine the carbohydrate elution profile (Dubois et al. 1956). Oc50 was separated into two fractions. The first fraction was eluted at 0.65 M NaCl, and the second fraction was eluted at 0.85 M NaCl. The two acidic polysaccharide fractions were pooled, dialyzed, concentrated, and called Oc50A1 and Oc50A2, respectively.

Oc50A1, 500 mL, was filtrated and further fractionated by gel filtration on a Sephacryl S-400 HR column (GE Healthcare, CV 2 L, Sweden). The column was coupled to a Perimax pump (Spetec, USA) and eluted with purified distilled water. Fractions of 15 mL were collected by a Superfrac fraction collector (GE Healthcare, Sweden). The phenol-sulphuric acid assay was used to determine the carbohydrate elution profile. Oc50A1 was separated into two different molecular weight fractions. The first polysaccharide fraction was eluted at molecular weight 100 ± 5 kDa, and the second fraction was eluted at molecular weight 68 ± 1 kDa. The molecular weights of the fractions were determined by ultracentrifugation. The two acidic polysaccharide fractions were pooled, concentrated, and called Oc50A1.I and Oc50A1.II, respectively.

The two acidic fractions, Oc50A1.I and Oc50A1.II, were applied on a High Load 26/60 Superdex 200 pg gel filtration column (GE Healthcare, Sweden) coupled to an ÄKTA P920 FPLC system (Amersham Pharmacia Biotech, Sweden) for further purification. The samples were filtrated (0.22 μm) prior to injection and eluted with 10 mM NaCl (1 mL/min). Samples of 5.1 mL were collected with a fraction collector, and the elution profile was monitored with a RID-10A refractive index detector (Shimadzu). Both the fractions Oc50A1.I and OC50A1.II gave two distinct fractions, one high molecular weight fraction and one intermediate/low molecular weight fraction. The high molecular weight fraction was eluted around the void volume, and this purified polysaccharide fraction was called Oc50A1.I.pur and Oc50A1.II.pur, respectively. To investigate the polydispersity, the peak which yielded the fraction Oc50A1.I.pur was divided and collected in four subfractions Oc50A1.I.A–D as described in Figure 1A.

Determination of carbohydrate composition

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

Determination of glycosidic linkages present in the polysaccharides

Prior to methylation of the polymers, the unesterified uronic acids were reduced to their corresponding neutral sugars. The free uronic acids were activated with carbodiimide and reduced with $NaBD_4$ as described by Sims and Bacic (1995). The reduced polymers were methylated with the method of Ciucanu and Kerek (1984), modified by McConville et al. (1990). The methylated polysaccharides were hydrolyzed with trifluoroacetic acid, the monomers reduced with 1 M $NaBD_4$ in 2 M NH_4OH (Sims and Bacic 1995), and the resulting partly methylated alditols acetylated to partially methylated alditol acetates, PMAA, by adding 200 μL 1-methylimidazole and 2 mL acetic acid anhydride. The samples were mixed and dissolved by sonication and allowed to stand for 10 min. Excess of reagent was destroyed by adding 10 mL distilled water, mixed, and allowed to stand for another 10 min. The PMAA were extracted over using 2 × 1 mL DCM (CH_2Cl_2). The DCM phase was extracted with 2 × 5 mL distilled water before the DCM was evaporated under N_2. The PMAA were dissolved in 100 μL MeOH prior to gas chromatography–mass spectrometry (GC–MS) analysis. The derived PMAA were analyzed by GC–MS on Fisons GC 8065, USA using split injection and a Fisons fused silica column (30 m × 0.2 mm i.d.), USA with a film thickness of 0.20 mm coupled with a Hewlett-Packard Mass Selective Detector 5970 as described by Inngjerdingen et al. (2006). The compounds at each peak were identified by interpretation of the retention time and the characteristic 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 amount of each PMAA present in the mixture.

Precipitation with the Yariv reagent

The presence of AG-II structures was detected in the acidic fractions by single radial diffusion in an agarose gel containing the β-glucosyl Yariv reagent, 1,3,5-tri-(4-β-D-glucosopyranosyloxyphenyl-azo)-2,4,6-trihydroxybenzene, which specifically interacts with and precipitates compounds containing type II arabinogalactan structures (van Holst and Clarke 1985). The polysaccharide fractions were dissolved at a concentration of 5 mg/mL in distilled water and gum arabic, used as positive control, at 1 mg/mL. There were 2, 4, and 6 μL of each sample applied into wells with 4 μm of diameter made in agar gel. The plate was incubated for 24 h at room temperature in an air tight container with a moistened filter paper. A positive reaction was identified by a reddish circle (halo) around the well into which the samples had been applied.

Determination of protein content and amino acid composition

The protein content of the samples was detected using the protein assay of Lowry et al. (1951), modified by Peterson (1979). Prior to amino acid analysis, the samples were dissolved in H_2O to concentration 10 μg/μL, and 10 μL was subjected to gaseous phase hydrolysis. After hydrolysis, 200 μL loading buffer was added to the tube to dissolve amino acids, and the solution was centrifuged. The precipitate was removed from the sample, and 20 μL was injected to the amino acid analyser. Chromatograms were recorded at 570 nm and 440 nm.

Polydispersity and molecular weight determination

Polydispersity and molecular weight determination of the acidic polysaccharides were determined by gel filtration on a Superdex 200 10/30 GL (GE Healthcare, Sweden) coupled to a RI-detector and by analytical ultracentrifugation. For the gel filtration, the samples (1 mg) were dissolved in 0.5 mL 10 mM NaCl and applied to the column. The samples were eluted with 10 mM NaCl at 0.5 mL/min (1.5 × column volume), collecting 1 mL fractions. For the analytical ultracentrifugation, samples were run at 16,000 rpm on a Beckman Optima XLI (Beckman Instruments, Palo Alto, USA) until equilibrium was reached (~24 h); the resultant interference pattern was analyzed using the MSTAR algorithm (Harding 2000; Harding 2005).

Human complement-fixating assay

The complement-fixation test is based on inhibition of hemolysis of antibody-sensitized sheep red blood cells by human sera as described by Michaelsen et al. (2000) (Method A). PMII, a highly active pectin fraction from the leaves of *Plantago major*, was used as a positive control (Samuelsen et al. 1996; Togola et al. 2007). Inhibition of lysis induced by the test samples was calculated by the formula $[(A_{control} - A_{test}) / A_{control}] \times 100\%$. From these data, a dose–response curve was created to calculate the concentration of test sample giving 50% inhibition of lysis (IC_{50}). A low IC_{50} value means a high complement-fixating activity. Due to some day to day variation in the observed IC_{50} values, we calculated the IC_{50} PMII/IC_{50} fraction ratio, which were less prone to variation.

Animals

Eight- to 12-week-old rats of the PVG.7B strain (which possesses a CD45 allotype [RT7.2] but is otherwise used interchangeably with the standard PVG strain [RT7.1]) have been maintained at the Institute of Basic Medical Sciences for more than 20 generations. Rats were housed in compliance with guidelines set by the Experimental Animal board under the Ministry of Agriculture of Norway and The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The laboratory animal facilities are subject to a routine health-monitoring program and tested for infectious organisms according to a modification of Federation of European Laboratory Animal Science Associations recommendations.

Cells and cell culture

Splenic mononuclear cells were obtained by crunching the spleen through a 70-μm cell strainer (Falcon; Becton Dickinson, USA), followed by layering onto Lymphoprep (Axis-Shield, Scotland) and spinning for 20 min at 700 × *g*. For purification of B cells, the mononuclear cells were incubated with sheep anti-rat IgG Dynabeads (Invitrogen Dynal, Oslo, Norway) for 30 min at room temperature (70 μL beads/2 × 10^7 cells). The cell/bead conjugates were resuspended in complete RPMI (cRPMI; RPMI 1640, 10% fetal calf serum [FCS], 1% penicillin/streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 2-mercaptoethanol [all from Invitrogen, Paisley, UK]) and incubated overnight at 37°C in humidified atmosphere with 5% of CO_2. The resulting bead-free cell suspension was routinely 90% OX12 positive (IgG receptor). Bone marrow was obtained from the femurs, and cells were isolated by passing the marrow through a 70-μm cell strainer. The resulting cell suspension was layered onto Nycoprep (Axis-Shield, Scotland) and spun at 650 × *g* for 20 min. Cells were harvested and cultured for 7 days in 100 mm Petri dishes (Nunc, Roskilde, Denmark) at 1 × 10^6 cells/mL in cRPMI containing 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, UK). Cells were fed every second day with fresh GM-CSF. The cells developed the phenotype of DCs, and the purity was routinely 80–90% CD11c positive cells (using the antibody CD11c-FITC, BD Biosciences Europe, Belgium). The mouse macrophage cell line Raw264.7 was cultured in cRPMI and split every second day.

Measurement of NO release

Macrophages were plated at a density of 5 × 10^5 cells/mL in 96-well flat-bottomed plates (5 × 10^4 cells/well) and cultured with the indicated concentrations of polysaccharides, LPS (from *Pseudomonas aeruginosa* 10, Sigma-Aldrich), or medium alone. Cells were incubated for 24 h and then centrifuged at 1300 rpm for 2 min. The supernatant was harvested, and the amount of NO 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.

Rat B cell proliferation assay

B cells were stained with 5 μM CFSE (Molecular Probes Invitrogen, Paisley, UK) in PBS supplemented with 2% FCS for 10 min/37°C. After washes in cRPMI, cells were cultured in 96-well plates (0.2 million cells/well) with indicated concentrations of polysaccharides, LPS as positive control, or medium alone as negative control. Proliferation was assessed after 5 days, by analyzing the dilution of CFSE in the population by flow cytometry. Data are presented as the relative decrease in CFSE intensity of samples compared to the negative control. Dead cells were excluded by staining the cells with propidium iodide (Molecular Probes Invitrogen, Paisley, UK).

Maturation of rat DCs

DCs were isolated by gentle pipetting. One hundred microliters of cells was added to 96-well flat-bottomed plates (1 × 10^6 cells/mL). Polysaccharide extracts or LPS were added in the indicated concentrations. The cells were incubated for 24 h, and acquisition of a mature phenotype was analyzed by co-staining the cells with CD11c-FITC and PE-conjugated rat CD86 (BD Biosciences Europe, Belgium). An acquisition gate was set for cells that were positive for CD11c. The samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences Europe, Belgium).

Rat cytokine measurements

B cells (1 × 10^5 cells/mL) or DCs were stimulated overnight in the presence of polysaccharide fractions, LPS, or medium as negative control in 100 μL total volume of cRPMI. The supernatants were harvested and frozen at −80°C until assayed. Concentrations of released cytokines were measured using a multiplex cytokine immuno-assay (Milliplex^{TM} MAP, rat cytokine/chemokine kit, USA) in duplicates of 25 μL undiluted supernatants according to the protocol supplied by the manufacturer. The presence of IL-1α, IL-6, IL-10, IL-12p70, IL-18, TNF-α, MCP-1, MIP-1α, and IP-10 was measured simultaneously. The samples were read and analyzed using the Luminex xMAP platform (Bio-Rad, Hercules, CA). The cytokine concentrations were determined from a standard curve assayed at the same time with defined cytokine reference samples using Bio-Plex Manager 4.1 software.

Statistical analysis

Experimental values were expressed as mean ± SEM. A parametric statistical analysis of multiple comparisons for matched data was applied with one-way repeated-measures ANOVA. The means were compared with the Tukey's multiple comparisons test, where values of $P < 0.05$ were considered to be statistically significant. Analysis was performed with the GraphPad 5.0 software, San Diego, CA, USA.

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Conflict of interest statement

None declared.

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Abbreviations

AG-I, arabinogalactan type I; AG-II, arabinogalactan type II; Ara, arabinose; CFSE, carboxyfluorescein succinimidyl ester; DCM, dichloromethane; DCs, dendritic cells; DMT, Department of Traditional Medicine; FCS, fetal calf serum; Gal, galactose; GalA, galacturonic acid; GC–MS, gas chromatography–mass spectrometry; Glc, glucose; GlcA, glucuronic acid; LPS, lipopolysaccharide; Man, mannose; MeOH, methanol; MFI, median fluorescence intensity; NO, nitric oxide; PMAA, partially methylated alditol acetates; RG-I, rhamnogalacturonan type I; RG-II, rhamnogalacturonan type II; Rha, rhamnose; Xyl, xylose.

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