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Polysaccharides with immunomodulating properties from the bark of *Parkia biglobosa*

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ABSTRACT

The bark of *Parkia biglobosa* is used in traditional medicine to cure a wide range of illnesses. Polysaccharides were extracted from the bark with 50% ethanol–water, 50 °C and 100 °C water, and seven active fractions obtained by anion exchange chromatography and gel filtration. The complement fixation and macrophage stimulating activities of the different fractions were determined. The acidic fractions PBEII-I and PBEII-IV were the most active in the complement fixation assay, but the other fractions were also potent compared to the positive control BPII from *Biophytum petersianum*. Fractions PBEII-I and PBEII-IV were also the most potent fractions in stimulating macrophages to release nitric oxide. Structural studies showed that PBEII-I and PBEII-IV were pectic type polysaccharides, containing arabinogalactan type II structures. The observed differences in biological activities among the seven purified polysaccharide subfractions are probably due to differences in monosaccharide compositions, linkage types and molecular sizes.

1. Introduction

In traditional medicine plants are used to treat various types of ailments, and in developing countries about 75% of the population use traditional medicine based on medicinal plants for their primary healthcare. The demand for medicinal plants is increasing because of the high price of conventional medicines and a low availability of these in some rural areas. Even in developed countries, numerous traditional herbal products are being used for treating diseases which are hard to be cured only by conventional medicine (Bodeker, Ong, Grundy, Burford, & Shein, 2004).

A common way of preparing traditional medicine is to make a water decoction of plant material, and plant polysaccharides isolated from crude water extracts have shown effects related to the immune system by different *in vitro* and *in vivo* test systems (Paulsen & Barsett, 2005). The first report of a pure complex polysaccharide having biological activity came in 1984 (Stimpel, Proksch, Wagner, & Lohmann-Matthes, 1984), and after this many reports have been published on the chemical characteristic and biological activity of polysaccharides, especially polysaccharides from plants used in the treatment of wounds, ulcer and cancer (Austarheim, Mahamane et al. 2012; Lin et al., 2013; Samuelsen et al., 1996; Yamada & Kiyohara, 1999; Zong, Cao, & Wang, 2012).

Polysaccharides with biological activity often contain uronic acids (Paulsen & Barsett, 2005), like pectins. Pectins are generally known to be composed of linear homogalacturonan (HG) regions and branched rhamnogalacturonan (RG) I and II regions (Waldron & Faulds, 2007). The side chains of RG-I consist usually of arabinogalactan (AG) type I and II, as well as arabinan and galactan. The branched regions of the pectins are thought to be related to their immunomodulating activities (Yamada & Kiyohara, 2007).

Parkia is a pantropical genus of evolutionary interest with centers of distribution in South America, Africa and South-East Asia, comprising some 30 to 40 species which are pollinated by different groups of bats in different areas (Hopkins, 1983). *Parkia biglobosa*, sometimes called the African locust bean tree, and being up to 20 m high, is one of the grain legumes (Anderson & Pinto, 1985). In traditional medicine in Mali, the stem bark of *P. biglobosa* is used most frequently, followed by the leaves and seeds (Diallo et al., 2002; Grønhaug et al., 2008). It is used as a remedy to cure a wide range of illnesses (Inngjerdingen, Nergård, Diallo, Mounkoro, & Paulsen, 2004; Modupe, Noel, & John, 2011), such as external and

Abbreviations: AG-I, arabinogalactan type I; AG-II, arabinogalactan type II; Ara, arabinose; Gal, galactose; GalA, galacturonic acid; GC–MS, gas chromatography–mass spectrometry; Glc, glucose; GlcA, glucuronic acid; LPS, lipopolysaccharide; Man, mannose; NO, nitric oxide; RG-I, rhamnogalacturonan type I; RG-II, rhamnogalacturonan type II; Rha, rhamnose; Xyl, xylose; 4-OMe-GlcA, 4-*O*-methyl-glucuronic acid.

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internal wounds, headache, malaria, and cough. Most studies have focused on *P. biglobosa* seeds and seed products (Alabi, Akinsulire, & Sanyaolu, 2005; Chukwu, Orhevba, & Mahmood, 2010; Compaoré et al., 2011; Elemo, Elemo, Oladunmoye, & Erukainure, 2011; Labia, Bréhima, Tove, Jørn, & Mogens, 2007; Nnemeka, Moses, Martins, & Olobayo, 2009). Known bioactive compounds such as sterols and triterpenes from the bark of *P. biglobosa* have been reported (Araujo, Palma, Moutinho, & Abreu, 1995; Tringali, Spatafora, & Longo, 2000). However, reports on bioactive polysaccharides from the bark of *P. biglobosa* are rare. As mentioned above, polysaccharides have been shown to have effects that can be associated with wound healing. In this paper, we aim to find details of the relationship between the chemical characteristics of the polysaccharides from the 50% ethanol–water extract, the 50 °C and 100 °C water extracts of *P. biglobosa* and their biological activities.

2. Materials and methods

2.1. Plant material

The bark of *P. biglobosa* was collected in Mali, and identified by the Department of Traditional Medicine (DMT), Mali. The bark was washed, cut into small pieces, dried and pulverized to a fine powder by a mechanical grinder. A voucher specimen is deposited at the herbarium of DMT (Voucher No. 0285/DMT).

2.2. Extraction and purification of polysaccharides

2.2.1. Method of extraction

In order to remove low molecular weight compounds, the powdered bark of *P. biglobosa* was pre-extracted with hexane, ethyl acetate and 96% methanol, respectively. The residue was further extracted twice with 50% ethanol–water at 50 °C for 2 h and centrifuged. The supernatant was filtered through Whatman filter paper, concentrated, and precipitated with concentrated ethanol. The precipitate, denominated PBE, was lyophilized and subjected for further studies (Fig. 1). The residue was then extracted with distilled water of 50 °C, and after filtration the residue was extracted with distilled water of 100 °C. After each extraction the water extracts were subjected to ultrafiltration with cut off 5000 Da, and the high molecular weight (HMW) fraction was subjected to dialysis with cut off 3500 Da, lyophilized and kept for further studies. The crude water extracts were denominated PB50 and PB100 (Fig. 1).

2.2.2. Ion exchange chromatography

The crude extracts PBE, PB50 and PB100 were applied to an anion exchange column packed with ANX Sepharose™ 4 Fast Flow (high sub) (GE Healthcare). To obtain the neutral fractions, PBEN, PB50 N and PB100 N, the column was first eluted with distilled water. Elution with a linear NaCl gradient (0–1.5 M) in water was used to obtain the acidic fractions. The carbohydrate elution profiles were determined using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The related fractions were pooled, dialyzed at cut-off 3500 Da against distilled water for removal of NaCl, and lyophilized.

2.2.3. Gel filtration chromatography

The acidic fractions marked in Fig. 1 were dissolved in elution buffer (10 mM NaCl), filtered through a Millipore filter (0.45 μm), and applied to a Hiload™ 26/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta, Amersham Pharmacia Biotech). Fractions were pooled based on their elution profiles, as determined by the phenol–sulfuric acid method, dialyzed and lyophilized.

2.3. Determination of monosaccharide composition

The monosaccharide compositions of the isolated fractions were determined by gas chromatography of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained after methanolysis with 3 M hydrochloric acid in anhydrous methanol for 24 h at 80 °C (Austarheim, Christensen et al. 2012; Barsett, Paulsen, & Habte, 1992; Chambers & Clamp, 1971). Mannitol was used as an internal standard. The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy).

2.4. Molecular weight determination

The homogeneity and molecular weight of the different polysaccharides were determined by size exclusion chromatography on a Hiload™ 16/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta, Amersham Pharmacia Biotech). Dextran polymers (Pharmacia) B512 (5.6 kDa), T8360 (19 kDa), T250 (233 kDa), T500 (475 kDa) and D1740 K (1740 kDa) were used as calibration standards. Approximately 5 mg of the samples were dissolved in 2 mL of 10 mM NaCl buffer and filtered through a Millipore filter (0.45 μm) and applied to the column. The samples were eluted with 10 mM NaCl at 0.5 mL/min, collecting 2 mL fractions. The eluent was detected with a Shimadzu RI detector. The retention volume was converted to molecular weight by using the standards.

2.5. Precipitation with the Yariv β-glucosyl reagent

Precipitation with the Yariv β-glucosyl reagent was performed on the samples as described by van Holst and Clarke (1985). The Yariv β-glucosyl reagent forms a colored precipitate with compounds containing AG-II structures. A solution of Arabic gum in water (1 mg/mL) was used as a positive control.

2.6. Determination of phenolic content

The total amount of phenolic compounds in the polysaccharide fractions was quantitatively determined according to the Folin–Ciocalteu assay (Singleton & Rossi, 1965). Two hundred microliters of lyophilized sample (1 mg/mL) dissolved in water (three replicates) was added the same amount of Folin–Ciocalteu's phenol reagent (1:1 in water, Merck/Kebo), mixed and left for 3 min at room temperature. Two hundred microliters of 1 M Na_2CO_3 was added, the tubes were mixed and allowed to stand for 1 h. The absorbance was measured at 750 nm. A standard curve was plotted using ferulic acid. The total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

2.7. Determination of protein content

The protein content of the polysaccharide fractions was determined by the Bio-Rad protein assay, based on the method of Bradford (Bio-Rad, CA, USA; Bradford, 1976). The standard procedure for microtiter plates was used with bovine serum albumin (BSA) as a protein standard in the concentration range 15 μg/mL–500 μg/mL. The Bio-Rad protein assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs.

2.8. Determination of glycosidic linkages

Glycosidic linkage elucidation was performed by methylation studies. Prior to methylation, the free uronic acids were reduced

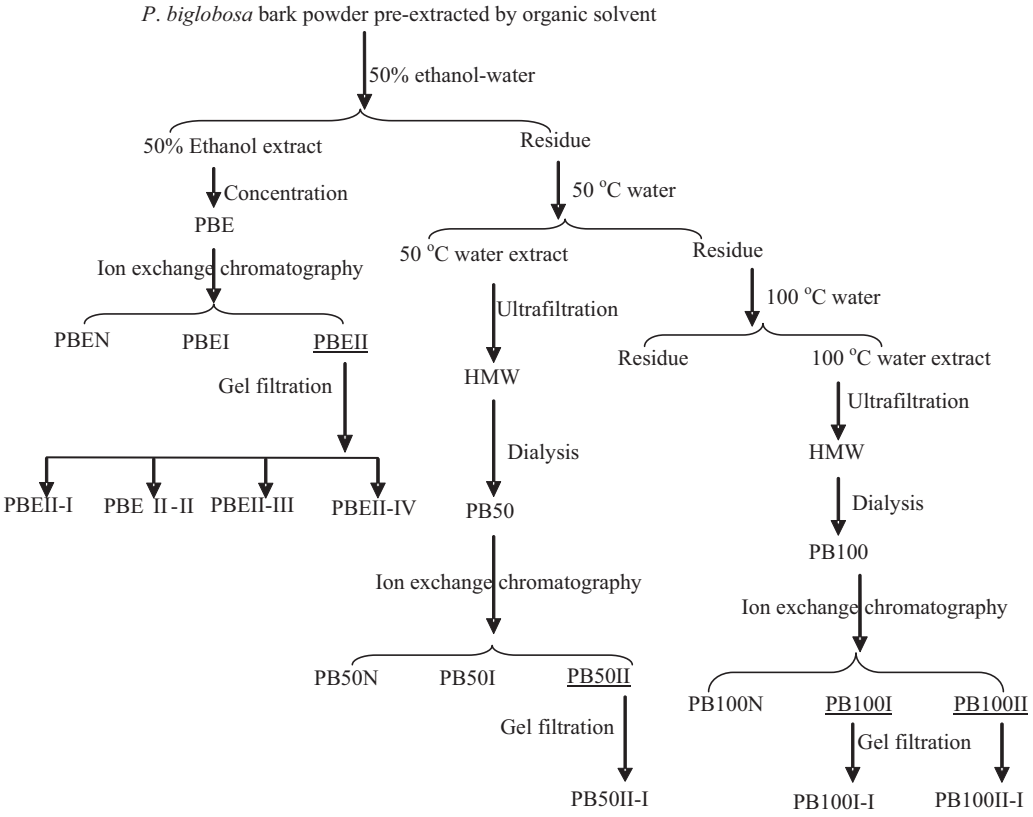

Fig. 1. Extraction and fractionation scheme of the polysaccharides from the bark of *P. biglobosa* (underlined acidic fractions had high complement fixation activity and were fractionated for further studies).

with $NaBD_4$ to their corresponding neutral sugars. After reduction of the polymers, methylation, hydrolysis, reduction and acetylation (Kim & Carpita, 1992) were carried out. The derivatives were analyzed by GC–MS using a GCMS-QP2010 (Shimadzu) attached to a Restek Rxi-5MS (30 m; 0.25 mm i.d.; 0.25 μm film) column. The injector temperature was 280 °C, the ion source temperature 200 °C and the interface temperature 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 the carrier gas (pressure control: 80 kPa). The compound at each peak was characterized by an interpretation of the retention times and the characteristic mass spectra. The estimation of the relative amounts of each linkage type was related to the total amount of each monosaccharide type as determined by methanolysis. Effective carbon-response factors were applied for quantification (Sweet, Shapir, & Albersheim, 1975).

2.9. Complement fixation assay

The complement fixation test is based on inhibition of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera as described by Michaelsen, Gilje, Samuelsen, Hagasen, & Paulsen (2000) (Method A). BPII, a highly active pectic polysaccharide from the aerial parts of *Biophytum petersianum* Klotzsch (Grønhaug et al., 2011), was used as a positive control. 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 (ICH_{50}). A low ICH_{50} value means a high complement fixation activity. The activity of all the polysaccharide fractions are given as the ICH_{50} value of the positive control BPII divided on the ICH_{50} value of the sample.

2.10. Activation of macrophages

The capacity of the polysaccharides to stimulate macrophages to produce nitric oxide (NO) was performed as earlier described, using a colorimetric assay with Griess reagents (Inngjerdingen et al., 2012). The mouse macrophage cell line Raw 264.7 was used in this assay. Briefly, macrophages at a density of 5×10^5 cells/mL were seeded into 96-well flat-bottomed plates (5×10^4 cells/well) and stimulated for 24 h in duplicates with increasing concentrations of samples, medium alone, and as positive controls LPS (from *Pseudomonas aeruginosa* 10, Sigma–Aldrich) and the polysaccharide fraction Oc50A1.I.A from *Opilia celtidifolia* (Grønhaug et al., 2010). Cells were then centrifuged at 1400 rpm for 2 min and the supernatants were harvested. The culture supernatants were mixed with Griess reagents, and the absorbance measured at 540 nm. A serial dilution of nitrite ($NaNO_2$) was used as a standard reference curve. The results are expressed as the mean ± SD. The difference between the control, medium alone, and the test samples was tested for statistical significance by Dunnett's multiple comparison test. A value of $p < 0.05$ was considered as statistical significant.

3. Results

3.1. Extraction and purification of polysaccharide fractions from *P. biglobosa*

The bark of *P. biglobosa* was further extracted with 50% ethanol–water after pre-extraction with organic solvents, and the extracts were precipitated with concentrated ethanol. The precipitate PBE was applied to an anion exchange column, and one neutral fraction, PBEN, and two acidic fractions, PBEI and PBEII, were obtained. The acidic fraction PBEII was chosen for further

work as PBEN and PBEI were low in activity in the complement fixation assay. PBEII was further fractionated by gel filtration and led to the isolation of four fractions, PBEII-I, PBEII-II, PBEII-III and PBEII-IV (Fig. 1). The fractions PB50II, and PB100I and PB100II, obtained after ion-exchange chromatography of the 50 °C and 100 °C water extracts, were subjected to further studies based on their activities in the complement fixation assay. PB50II, PB100I and PB100II were further fractionated by gel filtration and three active fractions were obtained: PB50II-I, PB100I-I and PB100II-I (Fig. 1).

3.2. Chemical composition of the polysaccharides

After methanolysis, the monosaccharide compositions of the polysaccharides were analyzed by GC and the results are shown in Table 1. The monosaccharides present in PBEII were mainly arabinose (Ara) (23.1 mol%), rhamnose (Rha) (14.3 mol%), galactose (Gal) (25.4 mol%) and GalA (22.4 mol%), constituting more than 85 mol% of the monomer content. The monosaccharide compositions of the four purified subfractions were quite different from the composition of PBEII. The highest molecular weight fraction PBEII-I contained mainly Ara (10.7 mol%), Gal (42.9 mol%), glucose (Glc) (22 mol%) and glucuronic acid (GlcA) (14.3 mol%), while PBEII-II contained mainly Ara (30.2 mol%), Gal (34.2 mol%), GlcA (12 mol%) and GalA (13.2 mol%). The monosaccharide compositions of the two lower molecular weight fractions PBEII-III and PBEII-IV were quite similar, the major constituents being Ara, Rha and Gal, making up over 80 mol% of the total monosaccharide compositions.

The fraction PB50II-I contained a high amount of GalA (26.2 mol%), in addition to Gal (22.8 mol%), GlcA (18.3 mol%), Glc (10.1 mol%), Ara (9.7 mol%) and Rha (8.5 mol%). The chromatogram of PB100I-I showed a high content of Ara (26.6 mol%), followed by Xyl, Gal, and Rha. The fraction PB100II-I was found to be rich in GalA (30.1 mol%), Ara (21.2 mol%), Gal (18 mol%) and GlcA (10.5 mol%). The differences in monosaccharide compositions between the purified water soluble fractions suggest that structural differences exist among them. Negative results of the iodine test indicated absence of starch in all fractions.

3.3. Protein and phenolic content

According to the Bio-Rad protein assay and Folin–Ciocalteu assay, the fraction PBEII-IV contained small amounts of protein (1.2%) and phenolic compounds (2.4%). The other three purified fractions from PBEII had no protein or phenolic compounds present according to the assays used. The fractions isolated from the 50 °C and 100 °C water extracts were not applied for protein and phenolic compound determination, due to lack of material.

3.4. Precipitation with the Yariv reagent

A positive reaction with the Yariv reagent showed that the fractions PBEII-I and PBEII-II contained AG-II structures. The heavier the color of the reddish circle, the higher amount of AG-II is present. AG-II might represent branch points in the pectic backbones of the fractions, most probably with longer sections containing 1,3 Gal linkages (Kitazawa et al., 2013). The fraction PBEII-I showed a higher degree of precipitation with the Yariv reagent than PBEII-II and the positive control (Gum Arabic). PBEII-II only gave a small reddish circle in reaction with the Yariv reagent. Fractions PB50II-I, PB100I-I and PB100II-I were not tested for AG-II content due to lack of material.

3.5. Molecular weight distribution

Size exclusion chromatography using dextran standards was employed to determine the average *Mw* of the purified fractions. The molecular weights of PBEII-I, PBEII-II, PBEII-III, PBEII-IV, PB50II-I, PB100I-I and PB100II-I were determined to be 303.7, 91.6, 23.3, 5.9, 275.4, 37.5 and 746.5 kDa, respectively.

3.6. Linkage analysis of the polysaccharide fractions

In order to determine the nature of the linkages for the different monosaccharides in the seven purified fractions, permethylation of the reduced polymers was performed, partially *O*-methylated alditol acetate standards (PMAAs) were prepared and subjected to GC–MS. The ratios of the linkages present were calculated based on the monosaccharide compositions (Table 1) and the areas of the methylated, as well as effective carbon-response factors (Table 2).

Arabinans are probably present in all fractions as seen from the presence of 1 → 5 linked Ara, in addition to terminal Ara, 1 → 3,5 and 1 → 2,5 linked Ara (Table 2), only fractions PB100I-I and PB100II-I contain 1 → 2,5 linked Ara.

Terminally, 1 → 3 linked, 1 → 6 and 1 → 3,6 linked units of Gal were present in all fractions, albeit in different amounts. AG-II structures consist of a backbone of 1 → 3 linked Gal with branching points at 1 → 3,6 Gal, which can either be bound to Gal or Ara units. The fractions PBEII-I and PBEII-II contain high amounts of 1 → 3 and 1 → 3,6 linked Gal, which indicates the presence of AG-II structural units. Although only fractions PBEII-I and PBEII-II were tested and showed positive reactions in the Yariv test, other fractions also contain typical AG-II linkages. The Gal linkages found in the fractions isolated from the 50 °C and 100 °C water extracts are less complex compared to the fractions from the 50% ethanol–water extract. In addition to AG-II, fractions PBEII-I and PB100I-I probably also contain AG-I, seen by the presence of 1 → 3,4 and 1 → 4 linked Gal. The presence of AG-I in the other purified fractions is unlikely, as no or only trace amounts of 1 → 4 linked Gal was detected.

Fractions PBEII-II, PBEII-III, PBEII-IV, PB50II-I and PB100II-I, all contain monosaccharides and linkages normally found in polymers with RG-I backbones, including the common branching point 1 → 2,4 linked Rha. The carboxylic acids in fraction PB100I-I were not reduced to free uronic acids, therefore the linkage patterns of the acidic monosaccharides are not available. However, fraction PB100I-I contain GalA, 1 → 2 and 1 → 2,4 linked Rha, in addition to fair amounts of Gal and Ara, which may indicate the presence of RG-I with AG-II side chains.

Glucuronic acid appeared in all the fractions (not reduced in fraction PB100I-I) mainly as 1 → 4 linked units, and with smaller amounts of terminally linked units. Terminal GlcA might be directly linked to position 3 of 1 → 4 linked GalA in the RG-I backbone, or may also be a part of the AG-II side chains (Capek, Rosik, Kardosova, & Toman, 1987; Renard, Crepeau, & Thibault, 1999).

3.7. Complement fixation activity

The native fraction PBEII from the 50% ethanol–water extract and its isolated subfractions PBEII-I, PBEII-II, PBEII-III and PBEII-IV, and the sub-fractions PB50II-I, PB100I-I and PB100II-I from the water extracts showed strong human complement fixation activity *in vitro* (Fig. 2). From the 50% ethanol extract, fractions PBEII-I and PBE II-IV were the most potent. The water soluble fractions PB50II-I and PB100I-I also exhibited strong activity. All these fractions had a higher activity than the positive control BPII. The native fraction PBEII showed a complement fixation activity comparable to BPII.

3.8. Nitric oxide release from macrophages

Based on the results of the complement fixation assay, the purified fractions were investigated for their capacity to induce a dose-dependent release of NO from macrophages, as measured by the quantification of its breakdown production nitrite. The mouse

Table 1
Characterization of polysaccharide fractions from *P. biglobosa* bark.

	PBEII	PBEII-I	PBEII-II	PBEII-III	PBEII-IV	PB50II-I	PB100I-I	PB100II-I
Ara[a]	23.1	10.7	30.2	32.9	25.8	9.7	26.6	21.2
Rha[a]	14.3	2.6	8.5	25.6	29.1	8.5	8.6	7.3
Xyl[a]	0.9	0.1	0.3	n.d.	n.d.	0.3	24.1	0.2
Man[a]	1.0	n.d.	0.5	1.1	3.4	1.5	2.8	n.d.
Gal[a]	25.4	42.9	34.2	27.6	25	22.8	14.1	18.0
Glc[a]	5.2	22.0	0.9	2.5	8.4	10.1	4.7	6.1
GlcA[a]	7.8	14.3	12.0	4.4	3.4	18.3	5.8	10.5
GalA[a]	22.4	7.4	13.2	5.9	4.8	26.2	5.0	30.1
4-*O*-Me-GlcA[a]	Trace	Trace	Trace	Trace	Trace	1.5	5.5	1.3
Protein (% w/w)		n.d.	n.d.	n.d.	1.2			
Phenols (% w/w)[b]		n.d.	n.d.	n.d.	2.4			
Presence of AG-II[c]		++	+	n.d.	n.d.			
Mw (kDa)[d]		303.7	91.6	23.3	5.9	275.4	37.5	746.5

n.d. not detected
[a] mol% related to total content of the monosaccharides Ara, Rha, Xyl, Man, Gal, Glc, GlcA, 4-*O*-Me-GlcA and GalA.
[b] The total phenolic content is expressed as ferulic acid equivalents.
[c] The presence of arabinogalactans type II (AG-II) was identified by precipitation with the β-glycosyl Yariv reagent. +, AG-II present; ++, AG-II present in high amounts.
[d] The molecular weight (*Mw*) was determined by size exclusion chromatography.

macrophage cell line Raw 264.7 was used for these experiments and lipopolysaccharide (LPS) and Oc50A1.I.A from *O. celtidifolia* were utilized as positive controls. The test was performed in three independent experiments.

No statistical significant NO production was observed in macrophages cultured with fractions PB50II-I, PB100I-I and PB100II-I (data not shown). As shown in Fig. 3, fractions PBEII-I, PBEII-II and PBEII-IV showed statistical significant stimulating effects on NO release from macrophages at a concentration of 100 μg/mL. The fraction PBEII-IV induced the strongest release of NO, followed by PBEII-I and PBEII-II. LPS, a potent stimulator of cells of the monocytic lineages, is a well-known contaminator in biological material. Fraction PBEII-III did not show any activity in this test system, and would probably contain LPS in similar concentrations as the active fractions due to the same isolation and purification procedures being used. It is therefore reasonable to anticipate that LPS is not responsible for the effect seen in the active fractions.

4. Discussion

P. biglobosa bark is used in Malian traditional medicine to treat a wide range of diseases such as external and internal wounds, headache, malaria, and cough. Medicinal plants used for wound healing often appear to have in common that they are rich in polysaccharides, which may be responsible of their wound healing properties (Paulsen, 2001). It was therefore of interest to study the

Table 2
The linkages (mol%) of the monosaccharides present in the purified fractions from the bark of *P. biglobosa*, determined by GC–MS after methylation.

	PBEII-I	PBEII-II	PBEII-III	PBEII-IV	PB50II-I	PB100I-I	PB100II-I
T-Ara*f*	4.5	12.4	19.7	14.8	3.1	11.0	7.8
1 → 2 Ara	n.d.	n.d.	1.0	1.5	n.d.	n.d.	n.d.
1 → 3 Ara	n.d.	0.7	1.4	1.8	n.d.	0.7	n.d.
1 → 5 Ara	4.5	14.0	8.3	5.3	5.5	9.1	10.0
1 → 3,5 Ara	1.3	2.7	2.4	2.3	0.6	3.6	2.3
1 → 2,5 Ara	n.d.	n.d.	n.d.	n.d.	n.d.	2.2	1.0
T-Rha	1.0	3.1	8.7	7.2	2.4	Trace	1.7
1 → 3 Rha	n.d.	n.d.	2.7	n.d.	n.d.	5.3	n.d.
1 → 2 Rha	n.d.	n.d.	9.1	16.1	5.3	Trace	4.0
1 → 2,4 Rha	1.6	5.1	5.2	5.8	0.7	3.3	1.6
T-Xyl	n.d.	n.d.	n.d.	n.d.	n.d.	3.9	n.d.
1 → 4 Xyl	n.d.	n.d.	n.d.	n.d.	n.d.	20.1	n.d.
T-GlcA	0.9	0.6	1.5	0.9	Trace	n.d.	3.4
1 → 4 GlcA	13.4	11.4	2.9	2.5	18.6	n.d.	7.1
T-Glc	2.6	0.6	n.d.	1.4	1.9	0.9[a]	1.4
1 → 3 Glc	n.d.	n.d.	1.0	1.8	n.d.	3.8	n.d.
1 → 4 Glc	0.7	n.d.	0.5	2.0	5.7	n.d.	3.0
1 → 6 Glc	6.6	n.d.	n.d.	0.6	0.8	n.d.	0.6
1 → 4,6 Glc	10.8	n.d.	0.6	2.6	1.6	n.d.	1.1
1 → 2,6 Glc	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T-GalA	1.7	n.d.	n.d.	n.d.	5.4	n.d.	5.5
1 → 4 GalA	5.7	10.5	4.6	3.9	18.3	n.d.	23.6
1 → 3,4 GalA	n.d.	2.3	1.3	0.6	2.4	n.d.	1.0
T-Gal	1.3	2.3	4.4	2.9	7.2	1.9	6.9
1 → 4 Gal	0.5	n.d.	0.7	n.d.	n.d.	4.5[b]	n.d.
1 → 3 Gal	14.3	5.3	3.0	2.4	6.8	3.0	4.1
1 → 6 Gal	1.9	4.7	3.2	3.6	3.2	0.6	3.0
1 → 2,4 Gal	n.d.	n.d.	1.8	n.d.	n.d.	n.d.	n.d.
1 → 3,4 Gal	12.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1 → 3,6 Gal	11.8	18.6	11.3	7.6	5.6	4.2	4.0
1 → 3,4,6 Gal	0.8	2.5	2.9	7.8	n.d.	n.d.	n.d.

n.d., not detected.
[a] May contain trace T-GlcA.
[b] May contain trace 1 → 4 GalA.

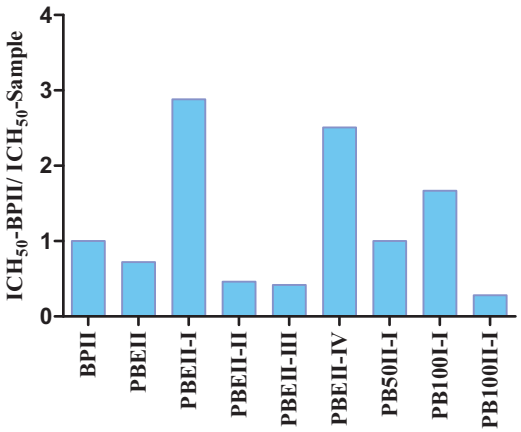

Fig. 2. Complement fixation assay. The figure shows the ratio ICH_{50}–BPII/ICH_{50}–sample and thus shows how active each individual test sample is compared to the positive control BPII (polysaccharide from *B. petersianum*).

structure and biological activity of polysaccharides from *P. biglobosa* bark.

The crude extracts PBE, PB50 and PB100 were fractionated by ion exchange chromatography and gel filtration as described, and led to the isolation of seven biologically active subfractions with different molecular weights. The monosaccharide compositions of these seven subfractions were quite different (Table 1), but all contained monosaccharides typical for pectic type polysaccharides.

The seven purified sub-fractions exhibited strong human complement fixation activities, fractions PBEII-I, PBEII-IV and PB100I-I exhibiting the most potent activities. Regarding the ability to stimulate macrophages in releasing NO, fractions PBEII-I, PBEII-IV and PBEII-II showed statistically significant activities.

Previously it has been reported that acidic polysaccharides with high molecular weights appear to be more active in the complement fixation assay than those with low molecular weights (Grønhaug et al., 2010; Nergård et al., 2005; Togola et al., 2008). However, among the seven purified fractions in the present study, the highest molecular weight fraction exhibited the lowest activity. The lowest molecular weight fraction, PBEII-IV, showed higher macrophage stimulating activity compared to the other fractions.

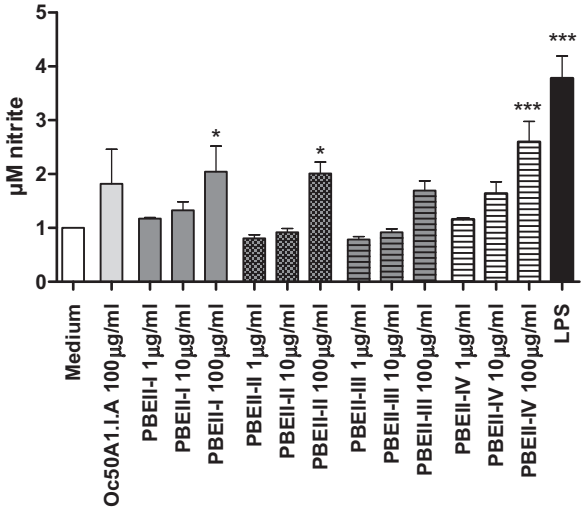

Fig. 3. Measurement of nitric oxide release from macrophages after stimulation with isolated polysaccharide fractions from 50% ethanol–water extract of *P. biglobosa* bark. The polysaccharide fraction Oc50A1.I.A from *Opilia celtidifolia* and LPS were used as positive controls, and medium alone as negative control. Data are presented as the mean of three independent experiments ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

In addition to molecular weight differences, the type of monosaccharide linkages might be a reason for influence on biological activity. According to the results of glycosidic determination most fractions are composed of RG-I structures, PBEII-I only containing a short RG-I chain. Previously RG-I containing polysaccharides with side chains of AG-I and/or AG-II have shown high immunomodulating effects (Grønhaug et al., 2011). Fractions PBEII-I and PB100I-I may contain AG-I due to the presence of 1 → 3,4 and 1 → 4 Gal units, while only small amounts or absence of 1 → 4 Gal units indicate the absence of AG-I in other purified fractions. All of the fractions contain typical AG-II linkages, 1 → 3 Gal, 1 → 6 Gal and 1 → 3,6 Gal, and polysaccharides rich in AG-II have shown effects in a number of biological assays (Grønhaug et al., 2010; Paulsen & Barsett, 2005; Yamada & Kiyohara, 1999, 2007).

The fraction PB100I-I contains considerable amounts of terminal Xyl, 1 → 4 Xyl and 1 → 3 Rha, which indicates a different linkage pattern compared to the other fractions. The presence of terminal Xyl may indicate the presence of xylogalacturonan (Inngjerdingen, Coulibaly, Diallo, Michaelsen, & Paulsen, 2006). The polysaccharides isolated from *Artemisia sphaerocephala* also contain high amount of 1 → 3 Rha which had high complement fixation activity (Batbayar et al., 2008).

Various immunomodulating polysaccharides isolated from plants (*Opilia celtidifolia*, *Vernonia kotschyana*, *Brassica oleracea*) also contain protein and phenolic compounds (Inngjerdingen et al., 2012; Samuelsen et al., 2007; Togola et al., 2008). The fraction PBEII-IV was the only fraction where small amounts of protein and phenolic compounds were detected, which may explain some of the observed immunomodulating activity in the lowest molecular weight subfraction.

5. Conclusions

Seven purified polysaccharide fractions were isolated from the 50% ethanol–water, 50 °C and 100 °C water extract from the bark of *P. biglobosa*. These fractions had slight different monosaccharide compositions and molecular sizes, but all contained monosaccharides typical for pectic type polysaccharides. All fractions exhibited strong human complement fixation activities, while only fractions PBEII-I, PBEII-IV and PBEII-II from the 50% ethanol–water extract showed statistically significant macrophage stimulation activities. The highest complement fixation activity was found in fraction PBEII-I, containing AG-I, AG-II and arabinan structural units. Fraction PBEII-IV, containing an RG-I backbone with side chains of arabinan and AG-II, had very high complement fixation activity and was the most potent in stimulating macrophages to release NO. The presence of protein and phenolic compounds in fraction PBEII-IV may explain some of detected activities. The high yield and biological activity of fractions isolated from the 50% ethanol–water extract suggests that this extract could be more related to the medicinal activity than the 50 °C and 100 °C water extracts. In future work it would be of interest to analyze the difference in bioactivities due to physiological parameters.

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