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Bioactive pectic polysaccharides from *Glinus oppositifolius* (L.) Aug. DC., a Malian medicinal plant, isolation and partial characterization

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Abstract

Glinus oppositifolius (L.) Aug. DC. (Aizoaceae) is a Malian medicinal plant used against various types of illnesses related to the immune response, like joint pains, inflammations, fever, malaria and wounds. Two pectin type polysaccharides, GOA1 and GOA2, being isolated from a 50 °C water extract from the aerial parts of *Glinus oppositifolius* were investigated for their activity towards the complement system and different leukocyte subsets because of the assumed effects on conditions related to the immune system. The polysaccharide polymer in GOA1 was shown to contain considerable amounts of the neutral sugars arabinose (26.4 mol%) and galactose (42.9 mol%), and methylation analysis indicated the presence of arabinogalactans type I (AG-I) and type II (AG-II). GOA2 was rich in galacturonic acid (68.3 mol%), along with rhamnose, arabinose and galactose. Structural studies indicated that rhamnose and galacturonic acid might constitute a rhamnogalacturonan backbone, often found in pectic substances, with side chains consisting of arabinose and galactose. Both GOA1 and GOA2 were shown to exhibit potent dose-dependent complement fixating activities, and induced chemotaxis of macrophages, T cells and NK cells.
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Keywords: *Glinus oppositifolius* (L.) Aug. DC.; Pectin; Arabinogalactan; Immunomodulation

1. Introduction

Glinus oppositifolius (L.) Aug. DC. (Aizoaceae) is a slender spreading or ascending annual herb with stems up to 40 cm long, the leaves being opposed two by two, and with white-green flowers located axially. The plant grows on damp sandy sites, occurring across West Africa from Senegal to South Nigeria, and is widely distributed in the tropics and sub-tropics generally (Burkhill, 1985). In Mali, West Africa, the plant can be found in the Gourma, Diré and Hauossa areas, and in the region near Lac Horo.

In Malian traditional medicine *Glinus oppositifolius* is used in the treatment of various types of ailments. Dried stems with leaves are ground into a fine powder, added to

Abbreviations: AG-I, arabinogalactan type I; AG-II, arabinogalactan type II; DMT, Department of Traditional Medicine; FPLC, fast protein liquid chromatography; FCS, fetal calf serum; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; GC, gas chromatography; LPS, lipopolysaccharide; Man, mannose; MS, mass spectroscopy; PBMC, peripheral blood mononuclear cells; PBS, Dulbecco's phosphate buffered saline; PMII, pectin fraction from the leaves of *Plantago major* L. (positive control); RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II; Rha, rhamnose; SEC/MALLS, size-exclusion chromatography coupled to multi-angle laser light scattering; SRBC, sheep red blood cells; Xyl, xylose

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food, and used for treating abdominal pain and jaundice. A decoction of a fine powder of the aerial parts is used in the treatment of malaria (Diallo et al., 1999). A maceration of pounded plant material with oil or with water is used as a wound healing remedy (Debes, 1998). *Glinus oppositifolius* has in addition been reported by traditional healers for treating joint pains, inflammations, diarrhoea, intestinal parasites, fever, boils and skin disorders (Debes, 1998; Diallo, 2000). An effective immune response is necessary to recover from these diseases, and therefore, it was of interest to look for immunomodulating compounds in the plant. Several papers have recently reported immunomodulating effects of plant polysaccharides from hot water extracts (Samuelsen et al., 1996; Wagner et al., 1999; Yamada and Kiyohara, 1999; Nergard et al., 2004), and the presence of such compounds could partly be responsible for the assumed effects of *Glinus oppositifolius*. As the hot water extract of the aerial parts of *Glinus oppositifolius* is a frequently used preparation (Debes, 1998), it was relevant to look for bioactive high molecular weight compounds in this extract.

The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response. It allows the host to better defend itself against invading microorganisms during the course of infection, and this is attractive because it allows for enhanced host-derived mechanisms to take part in the immune response and it does not involve the use of organism-specific therapeutics such as antibiotics (Tzianabos, 2000). The targets which can be considered for any interaction with polysaccharides range from the prostaglandin metabolism, NO-mediators, and endocrinal systems to complement receptors, adhesion molecules and leukocyte chemotaxis (Wagner and Kraus, 2000).

The isolated polysaccharide fractions from *Glinus oppositifolius* were tested for immunomodulation by complement fixation activities, and induction of chemotaxis of leukocytes. In general, the complement system plays an important role as a primary defense on bacterial invasions and viral infections, and appears to be intrinsically associated with several immune reactions such as the chemotactic attraction of leucocytes, immune adherence, modulation of antibody production and increased local vascular permeability. Agents which improve or stimulate leukocyte locomotion are of interest, as the capacity of leukocytes to respond by chemotaxis is part of an optimal host defence against infection. Very often, chronic and recurrent infections, cancer and rheumatoid arthritis are associated with diminished chemotaxis in vitro (Wagner and Jurcic, 1991).

There are no references available in the literature on studies performed on polysaccharides from *Glinus oppositifolius*, but triterpenoid saponins have been isolated and characterized (Diallo, 2000; Traore et al., 2000). A chloroform extract of the aerial parts of *Glinus oppositifolius* has previously been shown to possess antimalarial activity (Traore-Keita et al., 2000; Traore et al., 2000). Antioxidant-activity by a methanol extract of the whole plant has been revealed. In addition, a methanol and a dichloromethane extract have shown inhibition against *Candida albicans*, larvicidal activity against *Anopheles gambiae* and *Culex quinquefasciatus* and a molluscicide effect on three types of snails, *Biomphalaria glabrata*, *Biomphalaria pfeifferi* and *Bulinus truncates* (Diallo et al., 2001).

Partial structural characterization and biological activity of isolated pectic polysaccharides from *Glinus oppositifolius* is presented in this study. *Glinus oppositifolius* was chosen after primary ethnopharmacological field research in Gourma, Mali, West Africa, 1989–1991 and 1998 (Diallo et al., 1999).

2. Materials and methods

2.1. Plant material

The aerial parts of *Glinus oppositifolius* were collected in Diré, Mali, in April 1996, and identified by the Department of Traditional Medicine (DMT), Bamako, Mali. A voucher specimen is deposited in the herbarium at DMT.

2.2. Extraction and purification of polysaccharides

In order to remove low molecular weight compounds, powdered aerial parts of *Glinus oppositifolius* (400 g) was pre-extracted with dichloromethane (DCM) (2.4 l) for 3×24 h under reflux and methanol (MeOH) (2.4 l) for 3×24 h under reflux. As the material was still coloured, further extraction with 80% ethanol (EtOH) (1.5 l) for 3×1 h was performed, before filtration through Whatman GF/A glass fibre filter. Subsequently, the dried residue was extracted twice with water (5 l) at 50 °C for 2 h, and filtered through gauze and Whatman GF/A glass fibre filter. Prior to further separation and isolation, the aqueous extract was dialysed, first against tap water, then distilled water in a Spectra/Por® Membrane dialysis tube (Spectrum) with a molecular weight cut off at 3500 Da. A gel complex was formed during the dialysing procedure, probably caused by pectins complexing with Ca^{2+}-ions from the tap water. The crude 50 °C water extract, GO (yield 15 g, 3.8%), after filtration to remove the gel complex, gelGO (yield 1.11%), was kept at −18 °C until further use.

330 ml GO (equalling 1452 mg dry material), after centrifugation and filtration (5 μm) to remove another gel complex, was separated by anion-exchange chromatography on a DEAE Sepharose fast flow column (Pharmacia) with chloride as counter ion. The column was coupled to an IKA PA-SF digital pump (IKA). The neutral polysaccharides were eluted with distilled water (1 ml/min), while the acidic polysaccharides were eluted with a NaCl gradient (0–2 M) at 2 ml/min. Fractions of 10 ml were collected in a Pharmacia LKB Superfrac fraction collector. The carbohydrate elution profile was determined using the phenol–sulphuric acid assay (Dubois et

al., 1956). The relevant fractions were pooled, dialysed and lyophilized.

The acidic fractions thus obtained were further applied on a Superose 6 column (HR 10/30, 25 ml, Pharmacia) coupled to a FPLC system (Pharmacia) for a further separation. The injection-loop was 500 μl; 600–900 μg of the isolated fractions were applied onto the column. The column was eluted with distilled water (30 ml/h), and fractions of 0.5 ml were collected with a Fraction Collector Frac-100 (Pharmacia). The eluent was monitored with a Shimadzu RID-10A Refractive Index Detector. The phenol–sulphuric acid assay was used to determine the carbohydrate elution profile.

2.3. Determination of carbohydrate composition and content

The polysaccharide samples (1 mg) were subjected to methanolysis using 4 M HCl in anhydrous methanol at 80 °C for 24 h. Mannitol was added as internal standard. After the 24 h reaction time, the reagents were removed with nitrogen and the methyl-glycosides dried in vacuum over P_2O_5 for 1 h prior to conversion into the corresponding trimethyl silyl ethers (TMS-derivates). The samples were analyzed by capillary gas chromatography on a Carlo Erba 6000 Vega Series 2 chromatograph with an ICU 600 programmer (Chambers and Clamp, 1971; Barsett et al., 1992).

Investigations for the presence of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), monosaccharides present in rhamnogalacturonan II (RG-II) type polysaccharides were performed by the thiobarbituric acid assay (TBA assay) (Karkhanis et al., 1978).

2.4. Determination of protein content and amino acid composition

The polysaccharide samples were subjected to hydrolysis under vacuum in 6 M HCl at 110 °C for 24 h. After removal of HCl under reduced pressure, the amino acid composition was determined using a Biocal JC 5000 automatic amino acid analyser. The protein content of the fractions was determined from the amino acid composition analysis or by the protein assay of Lowry et al. (1951) modified by Peterson (1979).

2.5. Precipitation with the Yariv β-glucosyl reagent

Precipitation with the Yariv β-glucosyl reagent was performed as described by van Holst and Clarke (1985). The Yariv β-glucosyl reagent forms a coloured precipitate with compounds containing arabinogalactan type II structures. A positive reaction was identified as a reddish circle around the well. A solution of Arabic gum in water (1 mg/ml) was used as a positive control.

2.6. Homogeneity and molecular weight determination

Homogeneity and molecular weights of the acidic polysaccharide fractions were determined by size-exclusion chromatography (SEC) coupled to refractive index (RI) and multi-angle laser light scattering (MALLS) detectors, and by analytical ultracentrifugation, the sedimentation equilibrium technique as described by Hokputsa et al. (2003). The samples were dissolved in phosphate/chloride buffer, pH 7, ionic strength 0.1 at a concentration of 1 mg/ml and left until completely dissolved.

2.7. Determination of the glycosidic linkage composition of the polysaccharides

Prior to methylation the uronic acids of the polymer fractions were reduced to the corresponding neutral sugars. Carboxyl esters were first reduced with sodium borodeuteride in imidazole buffer to generate 6,6-dideuteriosugars. The free uronic acids were activated with a carbodiimide (*N*-cyclohexyl-*N′*-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluenesulfonate, Sigma–Aldrich) and reduced with sodium borodeuteride (Kim and Carpita, 1992).

After reduction of the polymers methylation was carried out after the method of Ciucanu and Kerek (1984). The methylation procedure was followed by hydrolysis of the glycosidic linkages, reduction of the partially methylated monosaccharides to alditols, and acetylation, as described by Kim and Carpita (1992). The derived partially methylated alditol acetates were analysed by GC–MS. The gas chromatograph, GC–MS Fisons GC 8065 (Fisons Instruments), was fitted with a split–splitless injector, used in the split mode and a SPB-1 fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. The injector temperature was 250 °C, the detector temperature 300 °C and the column temperature was 80 °C when injected, then increased with 20 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then 30 °C/min to 300 °C. Helium was the carrier gas with a flow rate of 0.9 ml/min. EI mass spectra were obtained using Hewlett-Packard Mass Selective Detector 5970 with a Hewlett-Packard GC. The compound at each peak was characterised by an interpretation of the characteristic mass spectra and retention times in relation to the standard sugar derivatives.

2.8. Immunomodulating activities of the polysaccharides

2.8.1. Complement fixating activity

The complement fixation test is based on inhibition of hemolysis of antibody sensitized SRBC (sheep red blood cells) by human sera as described by Michaelsen et al. (2000). The pectin fraction PMII from the leaves of *Plantago major* L. (Samuelsen et al., 1996) was used as positive control.

2.8.2. Isolation of leukocyte subsets

Buffy coats from healthy human volunteers (The Red Cross Blood Bank, Ullevaal Hospital, Oslo, Norway) were underlaid with Lymhoprep (Nycomed Pharma, Oslo, Norway) in order to generate peripheral blood mononuclear cells (PBMC). After density centrifugation at 1700 rpm for

30 min, interface cells were collected and washed three times with RPMI 1640 supplemented with 2% fetal calf serum (FCS). Monocytes were isolated by adherence for 1–2 h at 37 °C to plastic flasks suspended in AIM-V medium at 7×10^6 cells/ml. Non-adherent cells were removed by rigorous washes with RPMI 1640, after which macrophages were generated by incubating monocytes in AIM-V medium containing 40 ng/ml M-CSF for 6–8 days. The cells were fed medium supplemented with M-CSF after 3–4 days. The non-adherent cells were further incubated on nylonwool to remove B cells and residual monocytes. T cells were separated from the nylonwool non-adherent cells after incubation with CD3-coated Dynabeads (Dynal, Oslo, Norway) for 30 min at 4 °C. The T cells were separated in a magnetic field, and incubated overnight in RPMI 1640 containing 10% FCS in order to induce release of the Dynabeads prior to functional analysis. NK cells were generated by incubating the nylonwool non-adherent cells overnight in AIM-V supplemented with 500 IU/ml IL-2. Non-adherent cells were removed, and the adherent NK cells incubated for a further 14 days in AIM-V containing 500 IU/ml IL-2. The phenotypes of macrophages, T cells and NK cells were all confirmed to be more than 90% pure populations by analysis in flow cytometry against their respective markers CD14, CD3 and CD56. Human M-CSF was obtained from R&D Systems (UK). CD14-FITC and CD56-PE antibodies were from Immunotools (Friesoythe, Germany), while the anti-CD3 antibody OKT3 were generated from its hybridoma.

2.8.3. Chemotaxis assays

Chemotaxis of cells was assayed using a 48-well transwell chemotaxis chamber, with either 5 μm (macrophages, NK cells) or 3 μm (T cells) pore-sized polyvinylpyrrolidone-free filters (NeuroProbe, Gaithersburg, MD). The bottom chambers were filled with 28 μl IMDM containing 0.5% BSA with or without increasing concentrations of the polysaccharide samples or as positive control the chemokine CXCL12 (100 ng/ml), and lipopolysaccharide (LPS) as negative control. Cells (1×10^5) in 50 μl were loaded in the upper chambers. After 2 h incubation at 37 °C and 5% CO_2, transmigrated cells were harvested from the bottom chambers and counted in a Bürker chamber. All experiments were performed in triplicate. CXCL12 was purchased from PeproTech (Rocky Hill, NJ) and LPS from *Salmonella typhosa* was from Sigma (St. Louis, MO).

2.8.4. Nitric oxide measurement

1×10^5 cells were seeded into 96 well plates, and stimulated overnight in duplicates with increasing concentrations of samples and LPS. The plate was afterwards spun and 50 μl of supernatants were transferred to a fresh plate and added 50 μl of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphtyl)ethylenediamine and 2.5% phosphoric acid). The samples were left for 10 min at room temperature protected from light, and the absorbance was measured at 540 nm. A dilution series of nitrite ($NaNO_2$) was performed as a standard reference curve.

3. Results

3.1. Extraction and purification of the polysaccharides

The yield of polymeric material (GO) obtained after extraction with water at 50 °C was 3.8%, based on dried and powdered aerial parts of *Glinus oppositifolius*. GO was fractionated by anion-exchange chromatography on a DEAE Sepharose fast flow column, and separated into one neutral (GON, 0.002%) and two acidic (GOA1, 0.02% and GOA2, 0.05%) polysaccharide fractions. The yields given in brackets are based on the dried, powdered aerial parts of *Glinus oppositifolius*. GOA1 was eluted in the range of 0.6–0.9 M NaCl and GOA2 at 0.9–1.06 M NaCl (Fig. 1). Size-exclusion chromatography on a Superose 6 column did not lead to further separation of GOA1 and GOA2.

3.2. Carbohydrate composition and content

The carbohydrate content of the crude extract GO, and gelGO formed during dialysis after the extraction with water at 50° C, were determined to be 46 and 43%, respectively. Galacturonic acid, galactose, arabinose and rhamnose were the main carbohydrate components of GO, while 85.2% of the carbohydrate in gelGO was galacturonic acid.

The monosaccharide compositions of the polysaccharide fractions obtained after ion-exchange chromatography are shown in Table 1. Arabinose, galactose and glucose were the main sugar components of the neutral polysaccharide fraction GON. The total carbohydrate content was determined to be 65.7% by methanolysis. GON contained considerable amounts of glucose, but gave a negative reaction for starch when a solution of iodine was added.

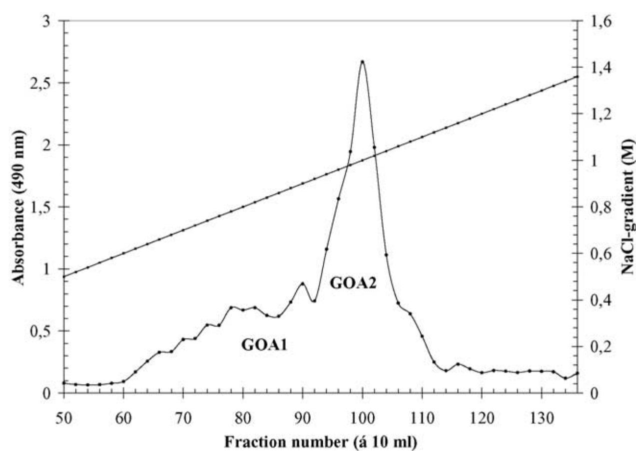

Fig. 1. Separation of the 50 °C water extract, GO, by anion-exchange chromatography on a DEAE Sepharose fast flow column.

Table 1
Characterisation of the polysaccharide fractions obtained after ion-exchange chromatography of the crude extract GO

	GON	GOA1	GOA2
Composition			
Total carbohydrate content (%, w/w)	65.7	52.6	45
Protein content (%, w/w)	n.d.	10	1.5
Monosaccharide composition[a]			
Ara	36.1	26.4	5.5
Rha	Trace	4.2	10.3
Fuc	0.5	–	1.3
Xyl	2.6	3.9	0.5
Man	4.6	4.3	0.6
Gal	32.5	42.9	9.7
Glc	23.7	3.5	3.3
4-*O*-Me-GlcA	–	2.9	0.4
GalA	–	12.1	68.3

n.d.: not determined (due to lack of material).
[a] mol% of total carbohydrate content.

Table 2
Amino acid composition (mol%) of GOA1 and GOA2

Amino acid	GOA1	GOA2
Aspartic acid	6.8	12.3
Hydroxyproline	19.3	3.8
Glutamic acid	8.3	13.7
Serine	11.7	11.2
Glycine	7.9	12.3
Arginine	1.5	2.5
Threonine	8.8	7.2
Alanine	14.7	10.4
Proline	4.1	5.4
Tyrosine	1	1.8
Valine	4.6	5.4
Methionine	2.6	0.9
Cystein	1.3	1.2
Isoleucine	1.7	2.7
Leucine	3	4.9
Phenylalanine	1.3	2.4
Lysine	1.6	2.1

GOA1 determined to have a total carbohydrate content of 52.6%, contained large amounts of the neutral sugars arabinose and galactose (26.4 and 42.9 mol%, respectively). Minor amounts of rhamnose, xylose, mannose, glucose, galacturonic acid and 4-*O*-Me-glucuronic acid were also present. GOA2 contained a total of 45% carbohydrate, and the monosaccharide composition is one typical for pectins. The carbohydrate is rich in galacturonic acid, rhamnose, galactose and arabinose in decreasing order (Table 1).

Both GOA1 and GOA2 gave a negative reaction in the thiobarbituric acid assay, indicating that the monosaccharides 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), and thereby rhamnogalacturonan type II (RG-II), are not present in the fractions.

3.3. Determination of protein content and amino acid composition

The crude extract, GO, contains 2.8% protein. The protein content and amino acid composition of GOA1 and GOA2 are shown in Tables 1 and 2, respectively. GOA1 has a high content of hydroxyproline, alanine and serine, and the total protein content was determined to be 10%. GOA2 was shown to contain small amounts of hydroxyproline compared to GOA1, and the total protein content was considerably lower, 1.5%.

3.4. Precipitation with the Yariv β-glucosyl reagent

A positive reaction with the Yariv β-glucosyl reagent indicated the presence of an arabinogalactan type II structure in GOA1 (Fig. 2). GOA2 also gave a positive reaction with the Yariv reagent, but bound weaker to the reagent compared to GOA1.

3.5. Molecular weight determination

Molecular weight determined by SEC/MALLS showed that GOA2 has relatively large refractive index (RI), but small light scattering peaks (Fig. 3B). This would suggest that the fraction consist of low molecular weight polysaccharide. According to the RI peaks GOA2 has a shoulder on the high molecular weight side (Fig. 3B). This indicates the presence of more than one species. Although these species could not be separated with the system used, and therefore, individual molecular weight could not be obtained, the average molecular weight over the whole peak area was determined. The weight average molecular weights of GOA2 obtained from SEC/MALLS and sedimentation equilibrium were approximately 39 and 30 kDa, respectively. GOA1 contains significant amount of low molecular weight material

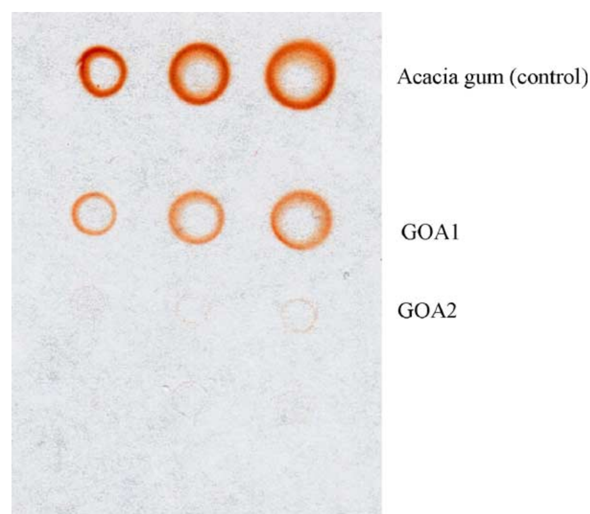

Fig. 2. Detection of arabinogalactan type II structures in agarose gels containing β-glucosyl–Yariv reagent (0.1 mg). Volume of samples (2 mg/ml) and control (acacia gum, 1 mg/ml) are 2, 4 and 6 μl in each well, respectively.

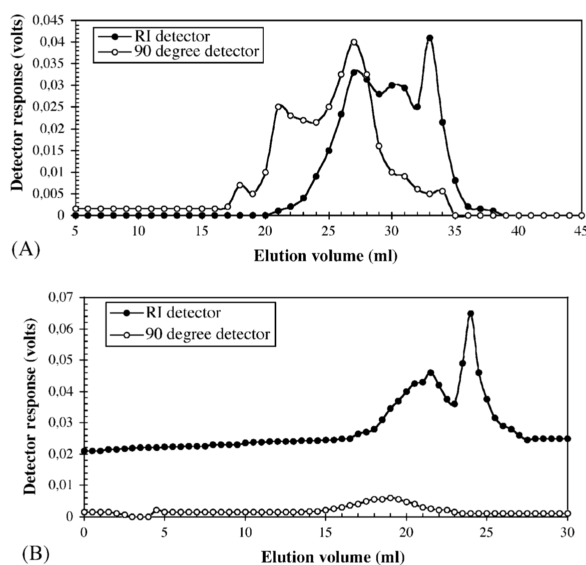

Fig. 3. Light scattering and refractive index profiles from size-exclusion chromatography coupled to multi-angle laser light scattering of GOA1 (A) and GOA2 (B).

Table 3
The linkages of GOA1 and GOA2 (mol%). Determined by reduction, methylation and GC–MS

Sugar	Type of linkage	Fraction	
		GOA1	GOA2
Arabinose	T*f*	11.7	3.4
	1,3*f*	3.5	0.5
	1,5	8.4	1.6
	1,3,5	2.4	–
	1,2,5	0.4	–
Rhamnose	T	2.1	1.7
	1,2	0.7	7.5
	1,2,4	1.4	0.9
Fucose	T		
Xylose	T	2.0	0.5
	1,4	1.4	–
	1,2,4	0.5	–
Mannose	1,2	4.3	0.6
Glucose	T	0.8	1.3
	1,4	2.0	1.7
	1,4,6	0.7	0.3
Galactose	T*f*	0.4	0.7
	T*p*	3.6	4.0
	1,3	6.5	3.2
	1,4	5.1	–
	1,6	7.6	0.8
	1,4,6	0.2	–
	1,3,4	0.6	–
	1,3,6	15.7	1.0
	1,3,4,6	2.9	–
4-*O*-Me-glucuronic acid	T	2.0	Trace
	1,4	0.9	–
Galacturonic acid	T	2.3	2.3
	1,4	9.8	64.5
	1,3,4	-	1.4

(Fig. 3A), the molecular weight was determined to be 70 kDa by SEC/MALLS.

3.6. Linkage analysis of the isolated fractions

Methylation and GC–MS analysis were performed in order to determine the nature of the linkages of the different monosaccharides in GOA1 and GOA2. The results are presented in Table 3.

According to the methylation results, the polymer in GOA1 contains a galactan part that is relatively branched, 36.6% of the galactose units are 1,3,6-linked. Significant amounts of 1,3-linked, 1,4-linked and 1,6-linked galactose units are also present. Arabinose is found mainly as terminally linked units, while the rest are 1,3-linked, 1,5-linked, 1,3,5-linked and 1,2,5-linked (Table 3). This indicates the presence of arabinogalactans type I and II. The galacturonic acid exist as 1,4-linked and terminally linked units, while rhamnose is 1,2-linked with branching in 4-position.

The carbohydrate in GOA2 is composed mainly of 1,4-linked galacturonic acid (64.5 mol%). There is also a significant amount of 1,2-linked rhamnose units present in the polymer, with branching in 4-position. The galactose units exist as 1,3-linked, 1,6-linked and 1,3,6-linked, while arabinose is terminally and 1,5-linked. Rhamnose and galacturonic acid might constitute a rhamnogalacturonan backbone, with sidechains consisting of arabinose and galactose.

3.7. Immunomodulating activities

3.7.1. Complement fixating activity

The complement fixating activities of the neutral and the acidic polysaccharide fractions were determined in vitro. Inhibition of hemolysis is a measure of the samples use of complement.

The activity for all the fractions was concentration dependent at the range of concentrations studied. The neutral fraction, GON, was shown to exhibit a low complement fixating activity compared to the acidic fractions (Fig. 4),

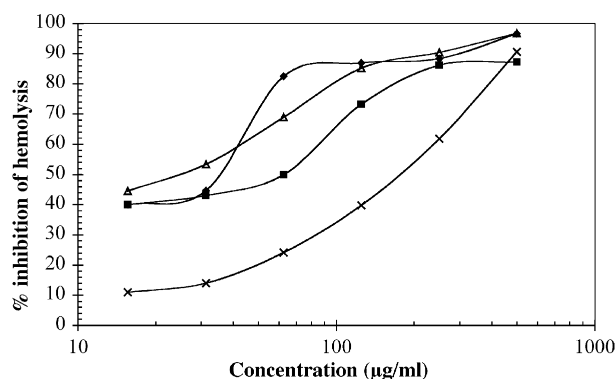

Fig. 4. Concentration-dependent effect of GON (×), GOA1 (⧫) and GOA2 (■) on the inhibition of hemolysis. PMII from *Plantago major* L. (△) was used as a positive control.

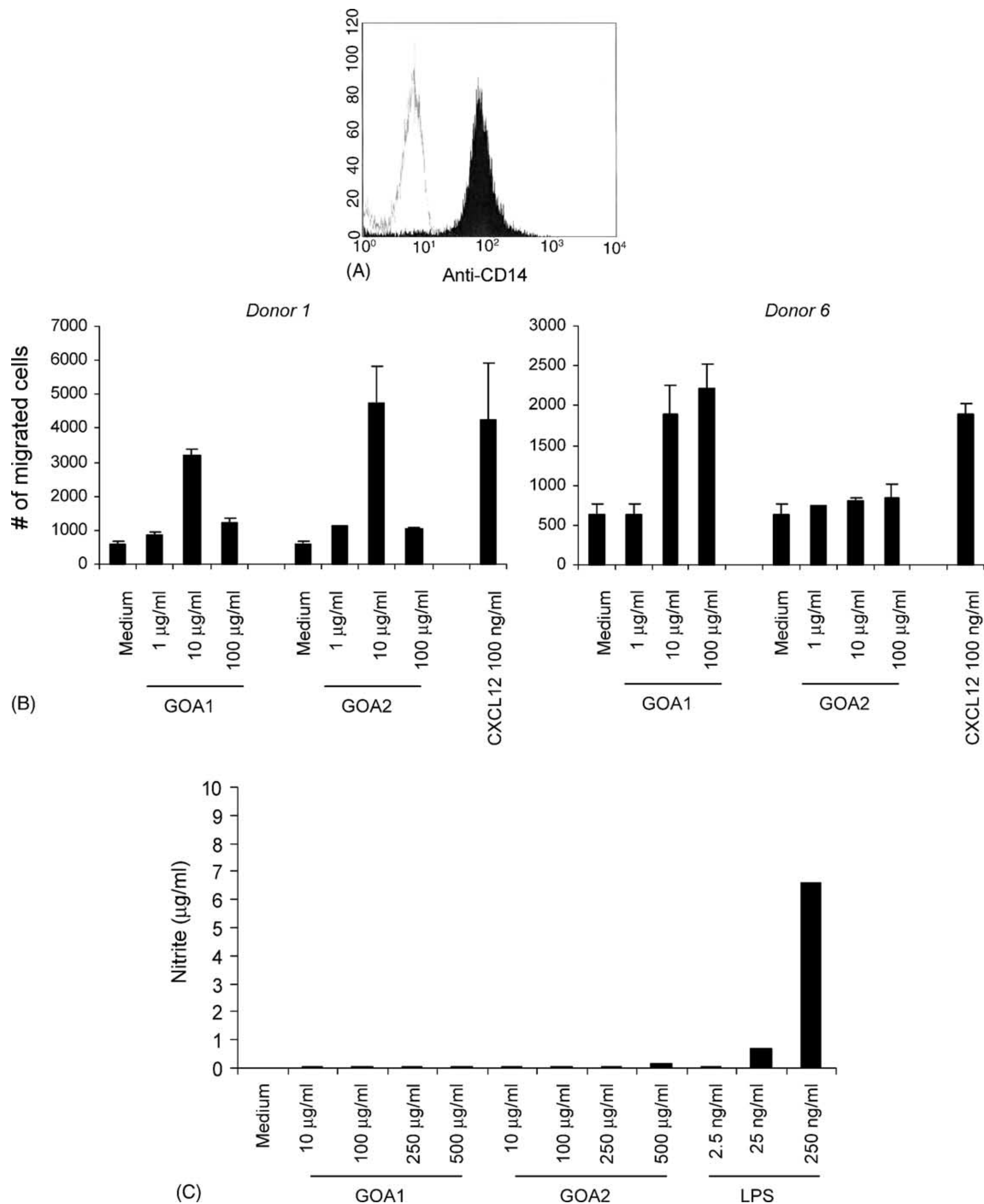

Fig. 5. Activation of macrophages. Expression of the monocyte/macrophage marker CD14 by macrophages (panel A). GOA1- and GOA2-induced chemotaxis of macrophages, two representative donors shown out of six tested. The chemokine CXCL12 was used as positive control (panel B). Nitric oxide measurement of GOA1 and GOA2 stimulated macrophages. LPS used as positive control (panel C).

having an ICH_{50} (the lowest concentration of sample needed to give 50% inhibition of hemolysis of antibody-sensitized SRBC) of about 200 µg/ml. The pectin fraction PMII from the leaves of *Plantago major* L., previously shown to be highly active in the complement fixation assay (Samuelsen et al., 1996), gave an ICH_{50} of 25 µg/ml in the same system. GOA1 possess the same level of activity as PMII, with an ICH_{50} at 34 µg/ml, indicating this polymer to be highly biological active. GOA2 with an ICH_{50} at 60 µg/ml, was also showing a potent complement fixating activity (Fig. 4).

3.8. Chemotaxis of macrophages, NK cells and T cells

Fig. 5B indicates that both GOA1 and GOA2 were active in eliciting chemotaxis of macrophages. Macrophages from two out of six donors did not react to GOA2 (as shown in Fig. 5B, right panel), while the other donors reacted to GOA2 with

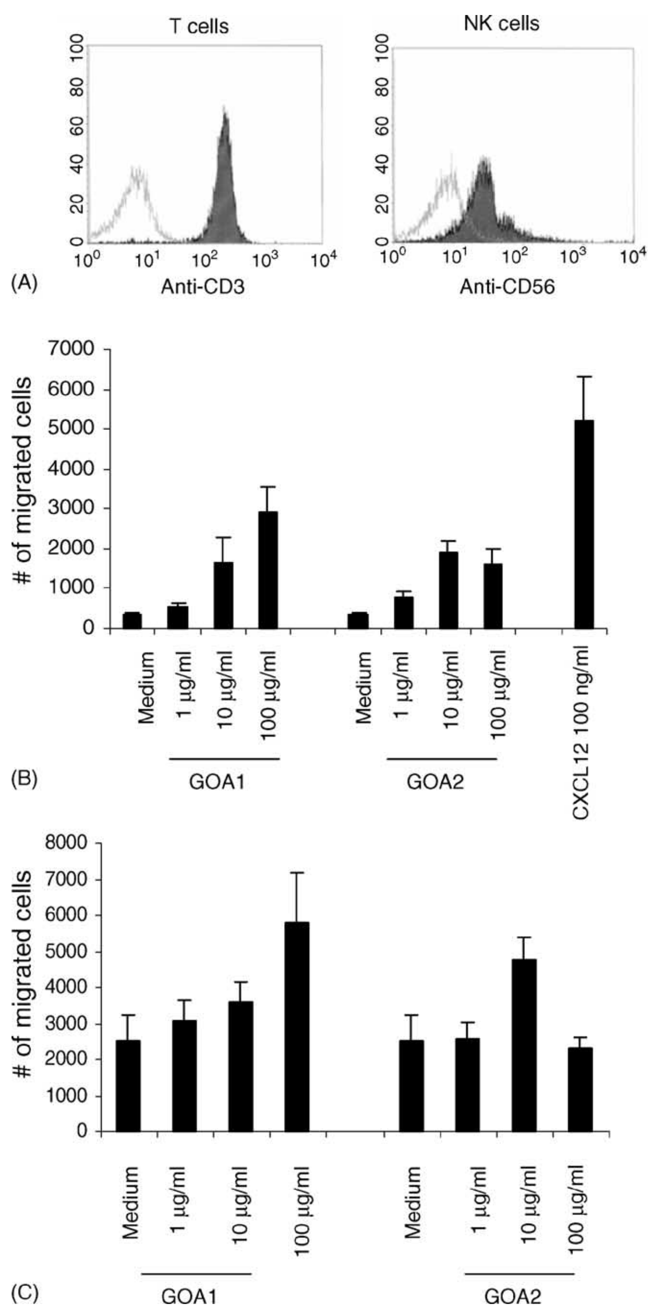

Fig. 6. Chemotaxis of T cells (panel B) and IL-2 activated NK cells (panel C). The chemokine CXCL12 was used as positive control. Purity of the T cell and NK cell populations is demonstrated with CD3 and CD56 staining, respectively (panel A).

maximum chemotaxis at 10 and 100 μg/ml. All macrophage preparations tested responded to 10 and 100 μg/ml of GOA1 (Fig. 5B). The ability of GOA1 and GOA2 to chemoattract T cells and NK cells was also tested. As shown in Fig. 6B, GOA1 and GOA2 induced chemotaxis of T cells with optimal concentrations at 10 and 100 μg/ml. Also, IL-2 activated NK cells were chemoattracted towards the same concentrations of GOA1 and GOA2 (Fig. 6C). The chemokine CXCL12 was used as a positive control for migration. CXCL12 binds the receptor CXCR4, which is expressed by all three leukocyte subsets (Hamon et al., 2004).

3.9. Nitric oxide measurement

The ability of macrophages to induce the release of nitric oxide (NO) was tested, measured through nitrite, which is a stable breakdown product of NO. We were unable to detect nitric oxide release under a wide range of concentrations of GOA1 and GOA2, while a positive control of LPS led to some nitrite formation (Fig. 5C).

4. Discussion and conclusions

Glinus oppositifolius is used in Malian traditional medicine for treating conditions of infectious origin and ailments related to the immune system. Infectious diseases and chronic wounds are among the major health problems in Mali, and are often treated by using medicinal plants. It was, therefore, of importance to evaluate possible effects of *Glinus oppositifolius*, as demonstration of bioactivity, which corresponds to its traditional application, can support the traditional medical use of the plant. During the last years, plant polysaccharides have been shown to act as potent immunomodulating agents, and a water extract prepared from the aerial parts of *Glinus oppositifolius* at 50 °C was fractionated, and isolated into one neutral (GON) and two acidic (GOA1 and GOA2) carbohydrate polymers. These polymers were further partially characterized, and their activity towards the complement system and leukocyte migration elucidated.

According to methylation analysis, GOA1 seems to be a complex pectic polysaccharide, galactose and arabinose comprising 70 mol% of the monosaccharide constituents in the carbohydrate. The presence of 1,3-linked, 1,6-linked, 1,3,6-linked, 1,4-linked and 1,3,4-linked galactose units, in addition to terminally linked units of arabinose, suggests arabinogalactans being a major part of the polymer. The arabinogalactans are normally relatively large sidechains on hydroxyproline present in hydroxyproline rich peptides or in other cases on the rhamnose units of rhamnogalacturonans (Paulsen, 2002). They can be subdivided into two main structural types. Type I pectic L-arabino-D-galactans are arabinose-substituted derivatives of linear 1,4-linked β-D-galactose units. Arabinose and galactose units are linked via *O*-3 along the main chains. No association with protein has been reported for this group. The second group, type II, compromise a highly branched polysaccharide with ramified chains of 1,3-linked and 1,6-linked β-D-Gal*p* units, the former predominantly in the interior and the latter in the exterior chains. The arabinose units might be substituted to the galactan through *O*-3 of the 1,6-linked galactan sidechains. In addition to arabinose and galactose, type II arabinogalactans contain a range of other monosaccharides, including glucuronic acid and its 4-*O*-methyl ether and galacturonic

acid (Huisman et al., 2001). The positive interaction with the Yariv-β-glucosyl reagent further indicates the presence of an arabinogalactan type II in GOA1. The protein content of the fraction was determined to be 10%, as estimated from the amino acid analysis. The protein was rich in hydroxyproline, alanine and serine, and this is typical for type II arabinogalactans (Paulsen, 2002). The rhamnose and galacturonic acid present in GOA1 may constitute a rhamnogalacturonan backbone to which AG-I and AG-II are attached, probably through *O*-4 of the rhamnose units.

The carbohydrate in GOA2 contains significant amounts of galacturonic acid (68.3 mol%), being mainly 1,4-linked, and with some branching in 3-position. Rhamnose exists as 1,2-linked units, with branching in 4-position. Rhamnose and galacturonic acid may constitute a rhamnogalacturonan backbone, often found in pectic substances. Native pectins are believed to consist of a backbone in which "smooth" galacturonan regions of α-(1 → 4)-linked D-galacturonosyl residues are interrupted by ramified ("hairy") rhamnogalacturonan regions consisting of a backbone of alternating α-(1 → 2)-linked L-rhamnosyl and α-(1 → 4)-linked D-galacturonosyl residues (rhamnogalacturonan I, RG-I). Neutral side chains are predominantly attached to *O*-4 of the rhamnosyl residues, and composed of D-galactosyl and L-arabinosyl residues. The proportion of "smooth" to "hairy" regions can vary greatly depending on the type of tissue or its development stage (Voragen et al., 2000). In GOA2, about 30% of the galactose units are 1,3-linked, 9% are 1,6-linked and 10% are 1,3,6-linked. Over half of the arabinose units occupy a terminal position, and 30% is 1,5-linked.

A minor component of plant cell wall is rhamnogalacturonan II (RG-II), which has an extremely complex structure (Voragen et al., 2000). Several medicinal plants from various parts of the world have been found to contain rhamnogalacturonan type II, being composed of eleven different glycosyl residues, including the unusual monosaccharides 3-deoxy-D-*manno*-2-octulosonic acid (KDO), 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), apiose and 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid) (Whitcombe et al., 1995). A RG type II polymer has been isolated from the leaves of *Panax ginseng* and showed IL-6 enhancing properties as well as a potent secretion enhancing activity of the nerve growth factor (NGF) (Yamada, 2000). GOA1 and GOA2, however, do not seem to contain RG-II, as determined by the thiobarbituric acid assay (TBA assay) (Karkhanis et al., 1978).

Size-exclusion chromatography (SEC) coupled to refractive index (RI) and multi-angle laser light scattering (MALLS) detectors were used to assess the homogeneity and molecular weight of GOA1 and GOA2, and in addition, the analytical ultracentrifugation was used for molecular weight determination of GOA2. SEC separates molecules according to their hydrodynamic volume, provided that there are no non-size exclusion mechanisms interfering with the separation. For a homologous series, this results in a separation according to decreasing molecular mass. Dual detection with in-line refractive index and light scattering detectors allows the determination of absolute M_w (Hokputsa et al., 2003). The molecular weight of GOA2 obtained from sedimentation equilibrium was lower than that obtained from SEC/MALLS, 30 and 39 kDa, respectively. This is probably due to concentration dependence, the higher concentration, the lower the molecular weight obtained from sedimentation equilibrium experiment. A second factor that may influence the SEC/MALLS results is the fact that light scattering signals were very small, thus increasing the error on the calculated weight average molecular weight values. The DCDT+ software used for obtaining these values allows determination of the sedimentation coefficient distribution and fitting of Gaussian to these distributions. The presence of different species, therefore, become more apparent and their sedimentation coefficients may be obtained. The sedimentation coefficient distribution and their fits clearly indicate the presence of two species in GOA2. This result is in good agreement with the result of the RI chromatograms. It can be concluded that GOA2 contains more than one species and is polydisperse.

During the last years, several polysaccharides isolated from plants used in phytotherapy and traditional medicine have been tested for complement modulating properties. Especially pectins, type II arabinogalactans, arabinans and other heteroglycans like glucuronoarabinoxylan have turned out to stimulate the complement system (Yamada and Kiyohara, 1999). The complement system is an important component of the immune defence against infections, and proteolytic cleavage of the complement components leads to generation of biologically active complement activation products that may increase local vascular permeability, attract leucocytes (chemotaxis), mediate immune adherence and modulate antibody production (Wagner and Jurcic, 1991).

The acidic pectic polysaccharide fractions isolated from *Glinus oppositifolius*, GOA1 and GOA2, both showed dose-dependent, potent complement fixating activities. The complement fixation test, however, does not differentiate between activation and inhibition of the system, but shows that the complement system is affected by the presence of certain polysaccharides. Complement activators result in a decrease of hemolysis due to the complement titer by the activation of the complement system, while complement inhibitors result in the inhibition of hemolysis due to the inhibition of a certain step in the complement system by the coexistence of the inhibitors in the assay system (Yamada and Kiyohara, 1999). While an activation of the complement system will contribute to inflammatory responses and to immunological defence reactions, the consumption of complement by polysaccharide due to fixating of complement could be a good therapeutic strategy for treating inflammatory diseases.

Bacterial LPS are structures on the cell membrane of gram negative bacteria. LPS consist of a highly biologically active lipid anchor, lipid A, which is linked to the bacterial membrane and hydrophilic carbohydrate part protruding from the cell surface. Free LPS are known to activate the complement system. To avoid false-positive results from these tests, the LPS content of GOA1 and GOA2 was determined using the

Limulus amebocyte lysate pyrogen (LAL) test. The contamination was found to be <0.025% in GOA1 and <0.005% in GOA2. LPS from *Escherichia coli* and *Neisseria meningitidis* were subjected to the complement fixation test but had no activity in the concentrations tested (3–750 μg/ml) (Samuelsen et al., 1999).

Investigations of the possible mechanisms of action behind the noted inflammatory activity of polysaccharides have revealed that, beside influence on the complement cascade, endocrinal functions and cytokine induction, the effect on chemotaxis of leukocytes has to be considered as one important factor. Regarding wound healing, platelet aggregation at the injury site is followed by infiltration of leukocytes, including neutrophils and macrophages, into the wound site during the inflammatory phase (Ishida et al., 2004). Their function is to engulf particles, including infectious agents, internalize them and destroy them. To achieve this purpose, chemotaxis of phagocytes to inflammatory site following a concentration gradient of chemokine is the first step essential for host defence (Hsu et al., 2003). Besides their phagocytic function, they are also an important source of growth factors (DiPietro, 1995).

In order to study whether GOA1 and GOA2 could affect macrophages, we generated macrophages from human monocytes, and found that both fractions attracted macrophages in a chemotaxis assay. Donor variations were experienced in the ability to chemoattract macrophages, where the most pronounced difference was the effect of GOA2. As both GOA1 and GOA2 are contaminated by small amounts of LPS, the effect of chemotaxis by LPS was tested. None of the concentrations of LPS that are found in the fractions induced macrophage chemotaxis (data not shown).

The ability of GOA1 and GOA2 to chemoattract human T cells and NK cells were tested and both fractions were shown to induce the chemotaxis of T cells and NK cells. These results suggest that GOA1 and GOA2 might function as chemoattractants to recruit leukocytes. A α-glucan from the rhizome of *Urtica dioica* L. (UPS I) has previously shown to stimulate leukocyte migration. Other glucans (laminarin, lentinan) have also showed stimulation of chemotaxis (Wagner and Kraus, 2000). Another glucan, PS-G from *Ganoderma lucidum* (G.) submitted to a chemotactic assay revealed the ability to increase neutrophil migration (Hsu et al., 2003). Regarding wounds, lymphocytes are not required for the initiation of wound healing, but an intact cellular immune response is essential for a normal outcome of tissue repair.

An inflammatory response implicates macrophages and neutrophils, which secrete a number of mediators responsible for initiation, progression and persistence of inflammation. Nitric oxide (NO) is amongst these mediators and is produced in macrophages by inducible nitric oxide synthase (iNOS). NO is responsible for vasodilatation, increase in vascular permeability and oedema formation at the site of inflammation (Kaur et al., 2004). The fact that GOA1 and GOA2 did not induce the release of NO from macrophages indicates that they are not acting as general macrophage activators.

There is no clear information obtained on how a polysaccharide has to be structurally designed in order to have an optimal inducing effect on certain immune cells. A hypothesis is that the immunological activity of a polysaccharide is dependent on a specific conformational feature and the presence of a certain number of anionic domains (Wagner et al., 1999), and that rather the three-dimensional structure of exposed, flexible side chains than a specific type of monosaccharide may be important for the activity (Alban et al., 2002).

Whether or not the complement fixating and chemotactic activities of the pectic polysaccharide containing fractions can be linked to the assumed effects of *Glinus oppositifolius* is an open question, but the polysaccharides may partly be responsible. The present study shows only partial structural characterisation of the isolated fractions, and more detailed work on the structure and activity are in progress. The ultimate goal is providing efficient and non-toxic medicines to the population in Mali, where medicinal plants play a vital role in the primary health care of the people.

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