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Full Length Research Paper

Immunomodulating polysaccharides from leaves of the Malian medicinal tree *Combretum glutinosum*; structural differences between small and large leaves can substantiate the preference for small leaves by some healers

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Medicinal plants are important for treating illnesses of different kinds all over the world. The leaves of the tree *Combretum glutinosum* Perr. ex DC. are amongst the most used remedies in West Africa. In Mali this tree can be found with two types of leaves on the same tree, large and small, growing on different branches. Some healers prefer to use the small leaves if they are available. The object of this paper was to evaluate if the water extract prepared from the two leaves had different immunological properties that could be related to differences in structures of the polysaccharides present in the leaves. Polysaccharides from large and small leaves of *Combretum glutinosum* were isolated and structurally characterized. Their bioactivities were tested in the human complement fixation assay, as well as their ability to produce nitric oxide from macrophages and to elicit cytokine release from B-cells and dendritic cells. Water extracts from the small leaves gave polysaccharides with a higher proportion of the xylogalacturonan region both for the 50 and 100C water extract than was found to be present in similar extracts from large leaves. The results of the different bioassays also show higher activities in the polymers from the small leaves, suggesting that the somewhat higher bioactivities in the small leaves can be linked to the xylogalacturonan region of their polymers. These results substantiate the preference that some healers have for the use of small leaves in their practice.

Key words: *Combretum glutinosum*, pectin, complement, cytokine, B-cell, dendritic cell.

INTRODUCTION

Combretum glutinosum Perr. ex DC (Combretaceae) is a tree growing in the Sahel region that can be up to 14 m high. It may also grow like a bush. The tree can have two types of leaves; large leaves that are thick and have leather like texture being sticky on the upper surface, and almost white and hairy beneath, and small leaves having a more narrow shape than the large leaves, and less sticky. The large once are app. 10 cm long and 5 cm wide, while the small leaves are app. 5 cm long and 2 cm wide, and these two types are not representatives of old and young leaves. In West Africa this tree is one of the most used medicinal remedies, and in some regions the tree is said to carry “the leaf that never disappoints”. The leaves are reported used traditionally as a diuretic, a cholagogue, against bronchitis, severe cough, cold, stomach spasms, and for treatment of wounds. They are also reported to be used against metroragi, blennoragi,

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and syncope (Neuwinger, 2000). Relatively little research has been performed on this tree despite its frequent use over whole West Africa. Water extracts of the leaves were shown to have antitussive effect when tested on guinea pigs. A dose of 1g/kg animal of the freeze-dried water extract gave similar effect as a dose of 100 mg codeine per kg animal (Ngaba et al., 1980). Methanol and hydromethanol extracts of leaves of *C. glutinosum* showed antimalarial activity against a chloroquine resistant *Plasmodium falciparum* strain *in vitro*. Young leaves had also effect on fever, icterus and liver problems (Quatarra et al., 2006).

One of the main objectives of the Department of Traditional Medicine (DMT), Bamako, Mali, is to produce Improved Traditional Medicines (ITMs). ITMs are medicinal products registered in Mali on the same line as conventional medicines. These products undergo chemical, biological and toxicological quality controls prior to the registration of the products. Previous ethno-pharmacological studies performed in different regions of Mali on the use of the leaves of the tree *Combretum glutinosum* showed that the use of these leaves against wounds and gastric ulcer were quite common (Inngjerdingen et al. 2004). In Bamako, one of the healers is selling a preparation called *"Tankamajafura"* which consists of powdered leaves of *C. glutinosum*. Some healers also said that the small leaves might be even more efficient than the large ones. As stated earlier *C. glutinosum* has a substantial use as a herbal remedy in Mali, and its leaves are also candidates for the development of new improved traditional medicines (ITM) at the Department of Traditional Medicine, Bamako, Mali, especially as a remedy against wounds.

Interestingly, some healers insist on using small leaves for certain ailments over the larger leaves. It was therefore of interest to perform surveys amongst healers in Mali on the use of the leaves of *C. glutinosum* and consequently to study potential differences in the polysaccharide contents and biological effects of the water extracts from large and small leaves of the tree. The reason for investigating the water extracts is that this is the type of preparation that most frequently is used by the healers.

Previous studies on other medicinal plants from Mali did show that water-soluble polysaccharides had interesting effects related to the immune system (Inngjerdingen et al., 2008; 2010; Togola et al., 2008; Ghildyal, 2010; Sutovska et al., 2010).

MATERIALS AND METHODS

Plant material

Large and small leaves from the tree *Combretum glutinosum* Perr. ex DC. (Combretaceae), were collected in the Dioila district, Mali, in March 2007. The material was identified by Professor Drissa Diallo, Department of Traditional Medicine (DMT), Bamako, Mali, and a voucher specimen is kept in the herbarium of the institute, no. DMT 0533.

Interviewing of traditional healers

Three villages in the Siby region west of Bamako, Mali, were visited for interviewing healers on their medicinal use of the leaves from the tree *C. glutinosum*. They were asked question on what ailments they used the leaves for, what type of leaves they were using, and also the mode of preparation and how the patient was using the remedy for treatment of the ailment.

Extraction and purification of polysaccharides

The small and large leaves were collected at the same time and treated in parallel. In order to remove low molecular weight and lipophilic compounds, both types of the leaves of *C. glutinosum* were pre-extracted by Soxhlet-extraction with dichloromethane (DCM), followed by methanol (MeOH). Subsequently, the dried plant material was extracted twice with 50°C water for 4 h, filtered through a fine nylon mesh and concentrated by rotary evaporation under diminished pressure at 40°C. To remove low molecular weight compounds the extract was dialysed in a Spectrapor dialysis tube with cut off 3500 Da. These dialysed extracts were called Cg50-S (small leaves) and Cg50-L (large leaves). After extraction with water of 50°C the residue was extracted using the same procedure as above apart from using100°C water extracts as well. These were called Cg100-S and Cg100-L. All dialysed extracts were further fractionated by anion-exchange chromatography on ANX Sepharose Fast Flow anion exchange column (5x30 cm) (Amersham Bioscience), with chloride as a counter ion. The column was coupled to a Perimax pump (Spetec). The extracts were filtrated (5 m) before application on the column. Neutral polysaccharides were eluted with distilled water (2 ml/min), while acidic polysaccharides were eluted with a NaCl gradient (0-2.0 M, 2 ml/min). Fractions of 10 ml were collected using a Superfrac fraction collector (GE Healthcare). The phenol-sulphuric acid assay was used to determine the carbohydrate elution profile (Dubois et al., 1956). In addition to the neutral fractions (which was not studied further as preliminary studies showed that they were devoid of bioactivity), each extract gave only one acidic polysaccharide peak. The relevant polysaccharide containing fractions from the ion exchange columns from all four extracts were pooled, dialysed and freeze dried. These fractions were denominated Cg50A and Cg100A respectively with an addition of S for small leaves and L for large leaves. Cg50A-S, Cg50A-L, Cg100A-S and Cg100A-L were further purified by gelfiltration on a HiLoad™ 26/60 Superdex™ 200 prep grade column (GE Healthcare), coupled to the FPLC system (GE Healthcare). 30 mg polymer was dissolved in 10 ml water, applied onto the column and eluted with 10 mM NaCl. Fractions of 5.1 ml were collected and the phenol-sulphuric acid assay was used to determine the carbohydrate elution profile. The high molecular weight fractions for each sample were pooled and freeze dried after dialysis. These samples were designated Cg50AP-S, Cg50AP-L, Cg100AP-S and Cg100AP-L, respectively. Appendix 1 gives the fractionation procedure. These four fractions were then studied further for comparison of the polysaccharides present in small and large leaves of *C. glutinosum*. The fractions were routinely tested for lipopolysaccharide (LPS) contamination.

Chemical analyses of the polysaccharides

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 (Barsett and Smestad, 1991;

Chambers and Clamp, 1971). 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) as described by Barsett and Smestad, 1991.

Determination of glycosidic linkage composition of the polysaccharides

Prior to methylation, the free uronic acids were reduced to their corresponding natural 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 Ciucanu and Kerek's method (1984) modified by McConville et al. (1990). The methylated polysaccharides were hydrolysed with TFA and the monomers reduced with 1 M $NaBD_4$ in 2 M NH_4OH. The monomers were acetylated to partially methylated alditol acetates, PMAA, by adding 200 µ l 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 in 2x1 ml dichloromethane (DCM, CH_2Cl_2).

The DCM-phase was extracted with 2x5 ml distilled water before the DCM was evaporated under N_2. The PMAA were dissolved in 100 µL MeOH prior to GC-MS analysis. The derived partially methylated alditol acetates were analyzed by GC-MS on Fisons GC 8065 using split injection and a Fisons fused silica column (30 m x 0.2 mm i.d.) with a film thickness of 0.20 mm as described by Inngjerdingen et al. (2006).

The compounds at each peak were characterized 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.

Precipitation with the Yariv-reagent

Precipitation with Yariv β-glucosyl reagent was performed on the samples as described by van Holst and Clarke (1985). The Yariv β-glucosyl reagent forms a red precipitate with compounds containing arabinogalactan II structures. A standard of Gum Arabic (1 µg/µl) was used as a positive control.

Polydispersity and molecular weight determination of the acidic polysaccharides were determined by gelfiltration on an analytical Superdex 200 10/30 GL (GE Healthcare) coupled to a RI-detector. For the gelfiltration 1 mg of the samples 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 times the column volume), collecting 1 mL fractions.

Bioactivity studies

Human complement fixating assay

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

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, University of Oslo 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 (FELASA) recommendations.

Cells and cell culture

Splenic mononuclear cells were obtained by crunching the spleen through a 70-µm cell strainer (Falcon; Becton Dickinson), 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 x 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, 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 isolated by passing the marrow through a 70-µm cell strainer. Red blood cells were lysed by NH_4Cl, and the remaining leukocyte suspension was cultured for 7 days in 100 mm Petri dishes (Nunc, Roskilde, Denmark) at 1 x 10^6 cells/ml in cRPMI containing 50 ng/ml GM-CSF (R&D Systems, Paisley, UK). Cells were fed every second day with fresh GM-CSF. The cells developed the phenotype of dendritic cells, 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 nitric oxide release

The mouse macrophage cell line Raw 264.7 was plated at a density of 5 x 10^5 cells/mL in 96-well flat bottomed plates (5 x 10^4 cells/well), and cultured with 10-fold dilutions of polysaccharides as indicated, 250 ng/ml LPS (derived from *P. aeruginosa* 10, Sigma-Aldrich, 2008), or medium alone in duplicates. Cells were incubated for 24 h and then centrifuged at 1300 rpm for 2 min. Cell-free supernatants were harvested, and the amount of nitrite (a stable breakdown product of nitric oxide) was determined using a colorimetric method with $NaNO_2$ as a standard (Green et al., 1982). 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.

Table 1. Monosaccharide composition and molecular weight of the purified polysaccharide fractions from small and large leaves of *C. glutinosum*.

	Cg50AP-S	Cg100AP-S	Cg50AP-L	Cg100AP-L
Arabinose	6.8	4.8	10.2	7.5
Rhamnose	3.3	3.5	5.0	3.1
Fucose	0.3	0.2	0.4	0.3
Xylose	14.2	7.2	4.5	3.2
Galactose	11.1	8.2	15.9	9.4
Glucose	7.4	5.4	6.2	4.9
Glucuronic acid	1.7	1.5	4.1	1.8
Galacturonic acid	55.2	69.2	53.8	69.9
MW kDa	204	168	232	290

Cytokine measurements

B cells or dendritic cells were plated in 96-well plates at $1x10^5$/well, and stimulated overnight in the presence of 100 µg/ml polysaccharide fractions, 250 ng/ml LPS, or medium alone as negative control. Cell-free 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, Millipore, Billerica, MA) 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 were 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, and represent the means from four independent experiments with duplicates unless stated otherwise. The statistical significance of differences between two mean values was evaluated by the two-tailed unpaired t-test, where values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Information obtained from the healers in the Siby district

In order to obtain more information on the use of the leaves *C. glutinosum* interviews of traditional healers were performed in the villages Dogoro, Gouena and Jisumala in the Siby region west of Bamako, Mali. 30 healers were interviewed, and 23 of these used the large leaves of *C. glutinosum* in their practice, while 14 used the small leaves, showing that some healers used both large and small leaves. In the Bambara language two names for the tree were used by the healers, *cangara* or *changara* and *damba*. It was noted that the majority of the women used the name *damba* for the tree; no obvious reason for this was given. Both types of leaves were used against various types of wounds, like gastric ulcer, stomach ache (abdominal pain), external wounds and otitis; the large leaves only, were used against malaria, fever, diarrhoea, and general pain in the body, while the small leaves only, were used against cerebral malaria, dysmenorrhoea, amenorrhoea and bilharzia. The healers preferred the small leaves for the last mentioned ailments, but if not available, the large ones could also be used.

All healers used hot water extracts for the treatments. The preference for the use of small leaves amongst some healers was the reason for the investigation of differences in structures and activities of polysaccharides from both types of leaves. Also, polysaccharides constitute the largest amount of water soluble compounds.

Isolation of the polysaccharides from small and large leaves of *C. glutinosum*

The polysaccharide materials from both small and large leaves of *C. glutinosum* were subjected to fractionation, isolation and purification into four polymers as shown in Appendix 1. Both 50 and 100°C temperatures were chosen as previous studies by our group have shown that successive extraction with these two temperatures will give a partial fractionation of the water soluble polysaccharides.

The small and large leaves and their extracts were treated in exactly the same way in order to have comparable materials. Initial testing of their biological effects in the complement fixating assay showed that the neutral fractions were devoid of activity. Based on these results, the acidic fractions from all four extracts after elution from the anion exchange column were purified further based on differences in molecular size by gelfiltration column. Their molecular weights were determined, and the results presented in Table 1. Polydispersity was observed for all samples. The four purified fractions thus obtained, Cg50AP-S, Cg100AP-S, Cg50AP-L, Cg100AP-L, were subjected to further chemical and biological

Table 2. Linkages present in the purified polysaccharide fractions from both small and large leaves of *C. Glutinosum*.

Type of linkage	Cg50AP-S	Cg100AP-S	Cg50AP-L	Cg100AP-L
Ara*f* T	5.1	2.2	7.1	4.7
1,5	0.8	1.3	2.2	1.5
1,3	0.4	0.2	0.4	0.4
1,3,5	0.4	1.1	0.5	1.0
Xyl T	14.2	7.2	4.5	3.2
Rha T	tr	-	1.9	0.7
1,2	2.4	2.0	2.7	2.1
1,2,4	0.9	1.3	0.4	0.3
Gal T	2.9	4.2	3.6	3.5
1,3	2.1	1.8	2.5	3.3
1,6	3.0	1.0	2.3	0.6
1,3,6	2.0	0.9	4.9	1.4
1,3,4,6	1.1	0.3	2.4	0.5
GlcA T	1.7	tr	2.0	1.8
1,4	tr	1.5	2.1	tr
GalA T	tr	tr	tr	2.1
1,4	32.4	61.2	48.3	67.3
1,3,4	22.8	8.0	5.5	0.5
Glc T	3.8	0.7	3.9	0.6
1,4	3.5	4.7	2.3	4.3

studies.

Monosaccharide composition and determination of their linkages

The monosaccharide composition of the four fractions was determined and is given in Table 1. From a quantitative point of view it is interesting to note that xylose is more abundant in the small leaves polysaccharides, galactose to a minor extent higher in the polysaccharides from the large leaves, and arabinose is more abundant in the polysaccharide from the large leaves. Similarities that can be seen is the presence of glucuronic acid in the 50°C extracts of both leaf types, which is almost not present in any of the 100°C extracts. The 100°C extracts from both leaf types contain more galacturonic acid than those from the 50°C extracts.

Linkage analyses of the four samples were also performed and shown in Table 2. Rhamnose linked 1,2 with some units having branches on position 4, concomitant with the galacturonic acid having 1,4 linkages suggests the presence of RG I (rhamnogalacturonan type I) regions in all polysaccharides studied (Schols et al., 2009). The interesting differences are the large amount of branch points on position 3 of the galacturonic acid units on Cg50AP-S, which also contains a large amount of terminal xylose units. The other three samples do also contain substitutions on position 3 of galacturonic acid in an amount fitting with the terminal xylose units present, but to a much smaller extent than in the Cg50AP-S fraction. Cg100AP-S has larger amounts of terminal xylose and substitutions on position 3 of galacturonic acid than what is seen from the large leaves. Based on these results it can be concluded that the polymers contain regions which can be called xylogalacturonans, which can be found in the galacturonan region of pectins as proposed by Perez et al. (2003). This region appears to be present in a larger part of the polysaccharide for especially Cg50AP-S, but also for Cg100AP-S, than for the polysaccharide fractions obtained from the large leaves.

The galactose units are present as terminal,1,3; 1,6; 1,3,6; and 1,3,4,6 linked units, and arabinose is basically present as terminal units in all samples with minor amounts of 1,3; 1,5 and 1,3,5 linked units. All these are typical linkages present in arabinogalactan type II polysaccharides (Schols et al., 2009). The precipitations with the Yariv reagent as shown in Figure 1 and the results from this experiment confirm the presence of AG II (arabinogalactan type II) polymers in all the four polysaccharides studied (van Holst and Clarke, 1984). Pectic polysaccharides containing both RG I regions and AG II structures have previously been found to be polysaccharides with interesting biological activities, especially related to the immune system. Several of these polysaccharides have been identified from plants traditionally used against gastric ulcer and against other wounds both in China, Japan, Mali and other countries (Yamada and Kiyohara, 2007).

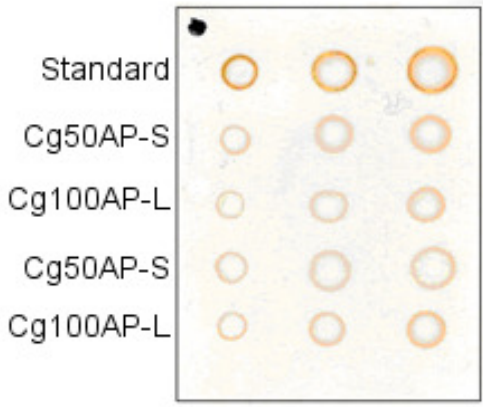

Figure 1. Yariv precipitation showing results of three different concentration of standard arabinogalactan type II polymer (Gum Arabic) top row, followed by Cg50AP-S, Cg100AP-S, Cg50AP-L and Cg100AP-L.

Human complement fixating activity

To test for putative differences in biological activities between the fractions, we first tested their effect on the complement system. The IC50 values relative to that of the polysaccharide standard PMII from *Plantago major* (Samuelsen et al., 1996) are given in Figure 2. PMII is a pectic type plant polysaccharide with a well documented activity in the complement assay and is a good indicator of effect in this assay (Michaelsen et al., 2000).

The experiments were repeated three times and those shown in Figure 2 are typical results obtained. Due to the nature of this bioassay, results from separate runs cannot be combined. It is interesting to note that the activities as represented with relative IC50 values of both extracts from the small leaves are more active than those from the large leaves, and that Cg100AP-S is the one with the highest activity in this system. Complement fixating activity has previously been shown to be a good indicator for a biological effect of polysaccharides from medicinal plants in the immune system (Inngjerdingen et al., 2006, 2007; Togola et al., 2008; Nergard et al., 2005).

Production of nitric oxide from macrophages

We next assessed the ability of the polysaccharide fractions from both types of leaves to activate macrophages. The production of nitric oxide (NO) was measured after treatment of the macrophages with 100, 10, or 1 µg/ ml of polysaccharides for 24 h. LPS (250 ng/ml), a bacterial-derived polysaccharide which potently stimulates cells of the monocytic lineage (Sweet and Hume, 1996) and the pectic polysaccharide PMII (100 µg/ml) were used as positive controls. From Figure 3 it is interesting to note that Cg50AP-S was the only active polysaccharide, with an activity comparable to that of the positive control PMII. The results from the other samples are on the level of the control and are thus not dose response related. The fraction Cg100AP-S, which showed highest activity in the complement fixation assay, as well as the polysaccharides isolated from large leaves, had only minor, if any, activity towards macrophages. Although there is a discrepancy in the identity of the active fractions, it is interesting to note that it is only polysaccharides from the small leaves that induced activity of macrophages. However, we have previously shown that there is a correlation between complement fixation and macrophage activation (Togola et al., 2008).

Cytokine release by B cells and dendritic cells

Production and secretion of cytokines is an important event following activation of leukocytes. To further test the leukocyte stimulatory properties of the polysaccharides from fraction Cg50AP-S, the production of cytokines was determined in dendritic cells and B cells, which next to macrophages are the other main antigen presenting cells in the immune system. The activity of Cg50AP-S was compared to that of Cg50AP-L, as a representative for one fraction from the large leaves with low activity in the other bioassays. The B-cells were stimulated for 24 h in the presence of either medium alone, 250 ng/mL LPS, or 100 µg/mL of Cg50AP-S or Cg50AP-L, while the dendritic cells were stimulated by 500 ng/mL LPS, or 100 µg/ml of PMII, Cg50AP-S or Cg50AP-L. Cell-free supernatants were tested using a multiplex cytokine assay, where we simultaneously measured the presence of IL-1α, IL-6, IL-10, IL-12p70, IL-18, TNF-α, MCP-1, MIP-1α and IP-10. The results showed that dendritic cells were induced to release IL-1α, IL-6, IL-10, and TNF-α (Figure 4A) in response to both Cg50AP-S and Cg50AP-L, indicating that polysaccharides from *C. glutinosum* are able to induce production of cytokines. We observed no preferential cytokine release in response to Cg50AP-S as compared to Cg50AP-L. The exception was IL-1α, where Cg50AP-S induced significantly higher amounts than Cg50AP-L ($p < 0.022$). We detected limited cytokine release from B cells in response to either Cg50AP-S or Cg50AP-L. Cg50Ap-L induced the release of the chemokine MIP-1α, while there was a tendency for higher release of IL-10 after stimulation with Cg50AP-S (Figure 4B). However, we can not based on these results, pinpoint cytokine release activity to either large or small leaves, as polysaccharides from both types of leaves seem to have the ability to stimulate cytokine production.

Conclusion

C. glutinosum is an unusual tree that appears to

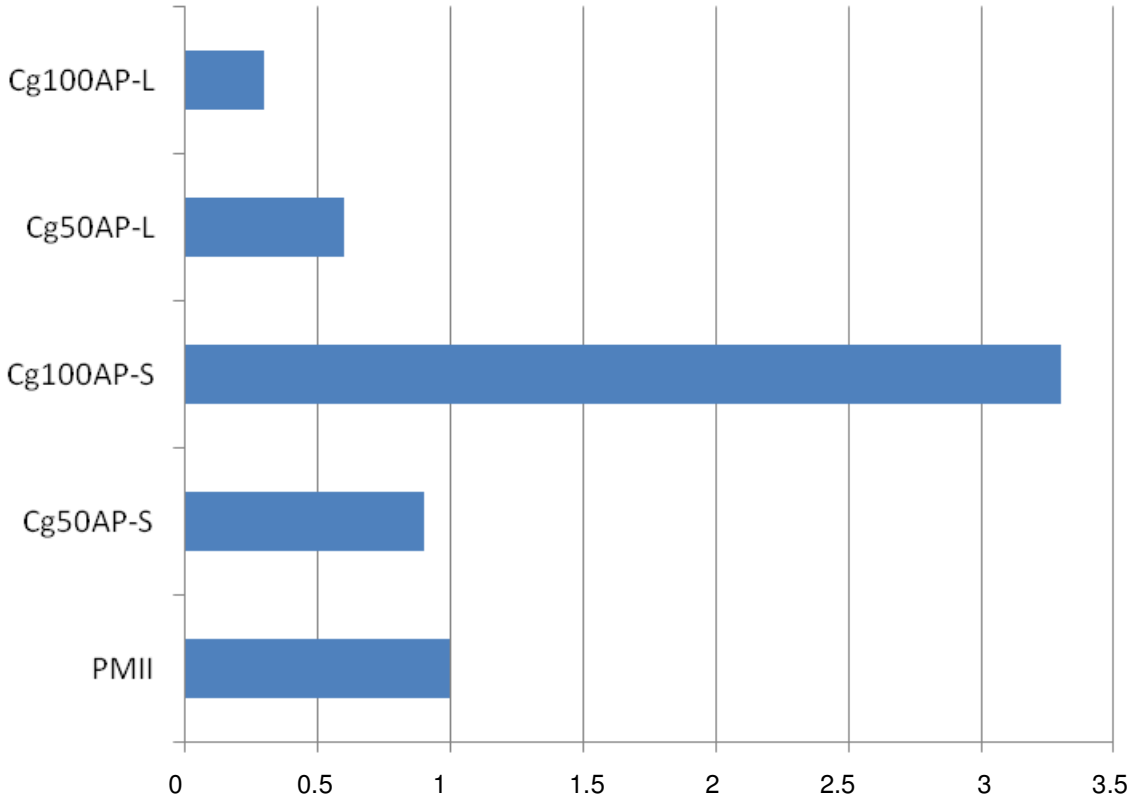

Figure 2. Effect on the complement system of the polysaccharide fractions isolated from small and large leaves relative to the standard PMII (IC50 PMII / IC 50 sample) from a typical experiment.

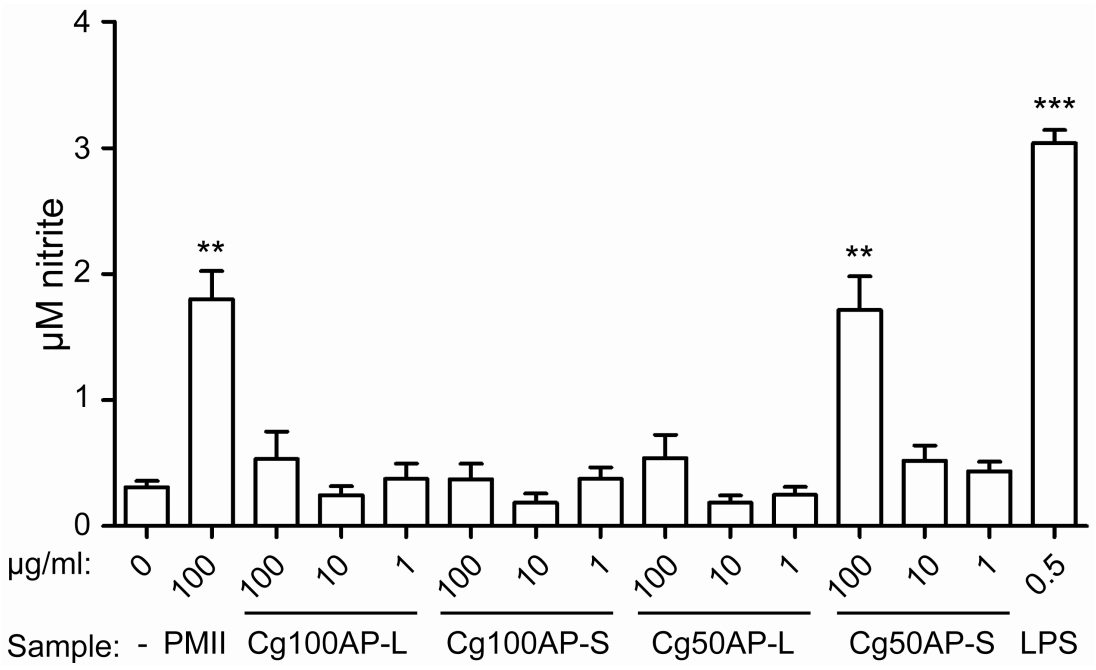

Figure 3. Leukocyte stimulatory activities of *C. glutinosum*. Measurement of nitric oxide (NO) release from mouse Raw 264.7 macrophages after overnight stimulation with different concentrations of *C. glutinosum* fractions, and 100 µg/ml of the pectic polysaccharide PMII and 250 ng/ml LPS as positive controls. Data are presented as the mean values from collected from four independent experiments ± SEM. ***, $p < 0.0001$; **, $p < 0.009$.

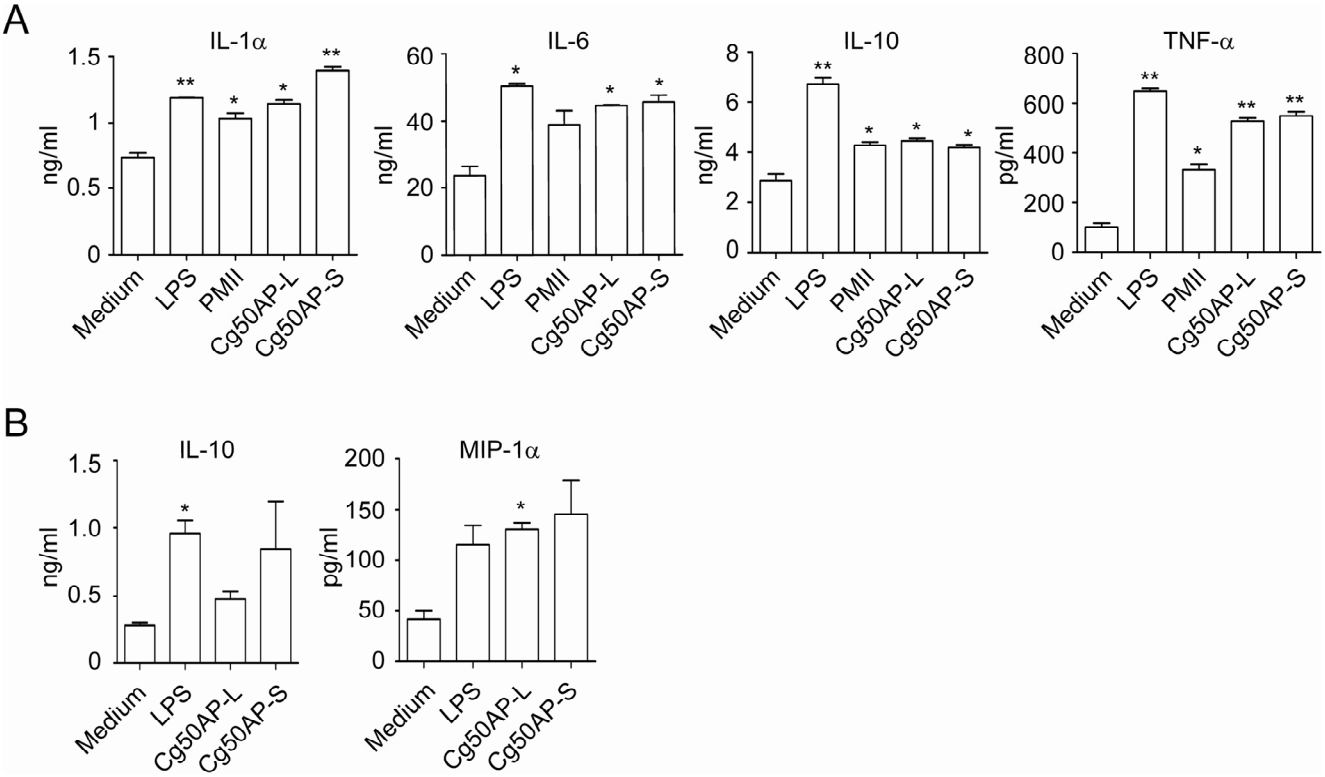

Figure 4. Induction of cytokine secretion from (a) dendritic cells and (b) B cells. Cells were stimulated in duplicates with Cg50AP-L or Cg50AP-S at 100 µg/ml, 250 ng/ml LPS and 100 µg/ml PMII as positive controls, or medium alone as negative control. The data are presented as the mean of duplicate values from one out of two experiments, *, $p < 0.05$; **, $p < 0.009$.

have the ability to produce two different types of leaves, large ones, that are the most common, and small ones. These can often be seen on different branches on the same tree and are quite different in size, shape and colour.

The results show an interesting difference in the structure between the polysaccharides present in small and large leaves. The presence of rather large proportions of terminal xylose units concomitant with a high amount of branched galacturonic acid residues (xylogalacturonan regions) in the polysaccharides from the small leaves compared with the large leaves appears to be the major reason for the difference in the biological effects. These results also substantiate the preference that some healers have for the use of small leaves in their practice. Other components may also be responsible for the differences seen and further studies will give information on this.

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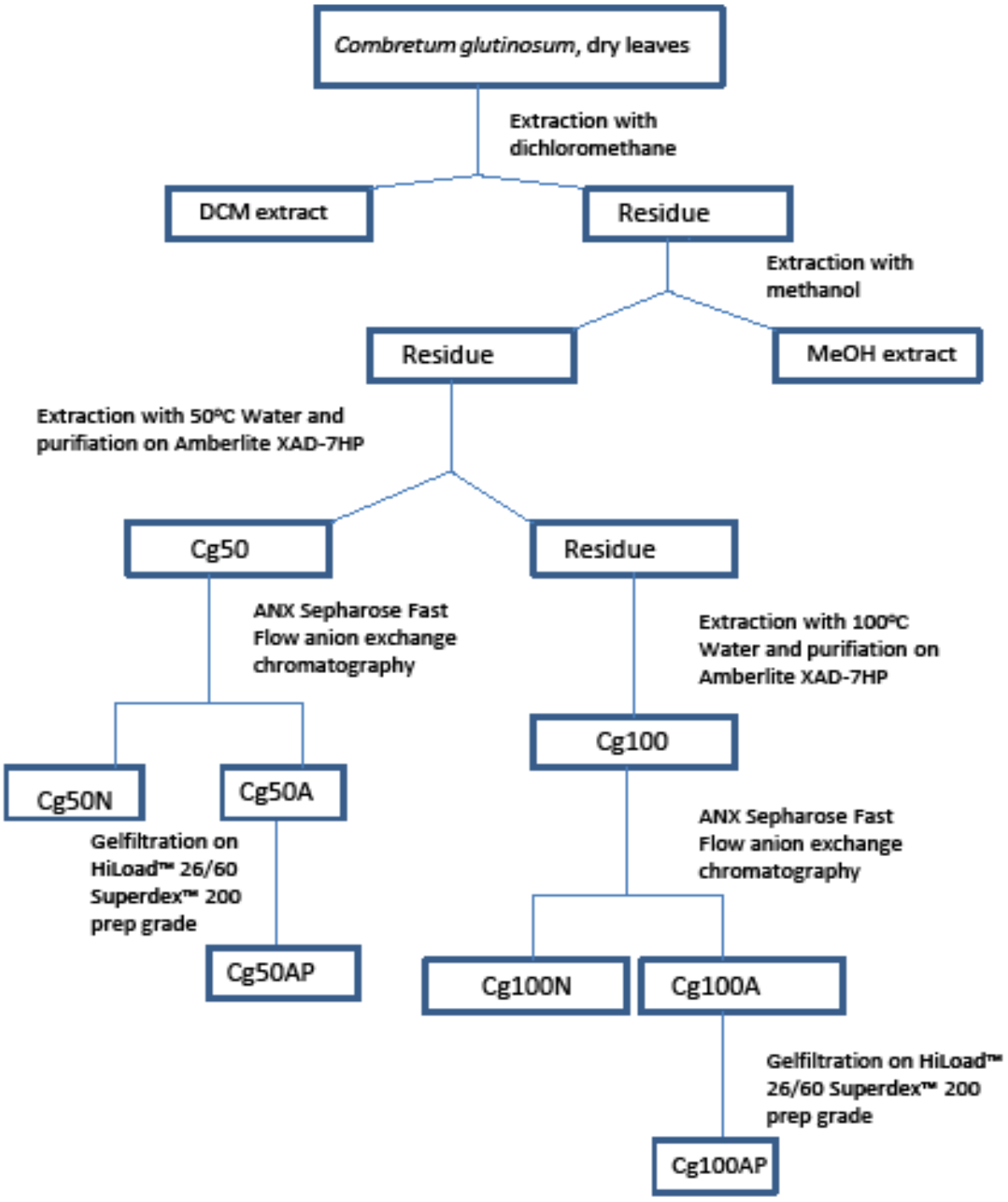
Appendix



Appendix 1: Extraction scheme leading to the products studies for both types of leaves. Fractions isolated from the small leaves are denominated Cg50AP-S and Cg100AP-S, and those from large leaves Cg50AP-L and Cg100AP-L.