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A polysaccharide with 40% mono-*O*-methylated monosaccharides from the bark of *Cola cordifolia* (Sterculiaceae), a medicinal tree from Mali (West Africa)

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Abstract

A novel type polysaccharide was isolated from the bark of *Cola cordifolia* (Cav.) R. Br. (Sterculiaceae), a plant used in traditional medicine in Mali (West Africa). The polysaccharide was isolated from the water extract by ion exchange chromatography. Structural studies showed that this was a highly complex new type polysaccharide containing 20% of 2,3- and 2,4-linked rhamnose, 24% of galacturonic acid mostly 4-linked, 15% of terminal, 3- and 4-linked galactose, 20% of terminal and 3-linked 2-*O*-methyl galactose, 18% of 4-*O*-methyl glucuronic acid which was also terminally linked, and 2% of terminal 2-*O*-methyl fucose. This paper reports in addition to structural features, physical property and complement fixating activity using human serum as target of this novel polysaccharide. This is a first report of a plant polysaccharide containing such a diverse composition and quantity of natively methylated monosaccharides.

Keywords: *Cola cordifolia*; Polysaccharide; Native methylated monosaccharides; Structure

1. Introduction

The medicinal tree, *Cola cordifolia* (Cav.) R Br., Sterculiaceae, locally known as "N′tabanokò", is a large tree, 15–25 m high, with a short buttressed trunk and low degree of branching with a dense crown growing on the savannah from Senegal to Mali (West Africa). Its heavy crown gives ample shade, thus the tree is an admirable "palaver" tree in Africa, (Palaver trees are those which leaves are tightly packed, consequently, give a condense shadow that is use as refreshment area). The mature fruit is edible and is the primary harvested plant part. It resembles that of the true Kola, *Cola nitida*, and the seeds are surrounded by a salmon-pink seed-coat which is sweet and pulpy (Burkill, 2000). All parts of the tree, such as the leaves, roots, bark and seeds are used in traditional medicine. Burkill (2000) reports that the bark of the tree is used against constipation in Gambia, and against chest-affections and dysentery in Senegal, and the leaves are used as a remedy for eye-treatment. Despite the many therapeutic uses of this tree in traditional medicine, no report was found in the literature on the identification of biologically active or isolated compounds from *C. cordifolia*.

In our search for immunomodulatory compounds in plants used in traditional medicine in Mali, a survey was performed to identify the plants used in wounds healing. 123 species, belonging to 50 families, were reported in that study. The fifteen species that were the most frequently cited by the traditional healers were subjected to chemical and biological studies and *C. cordifolia* was among those (Diallo et al., 2002). Water extracts of different plant parts

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were subjected to screening for effects on the human complement system *in vitro*. The polysaccharide containing fraction from *C. cordifolia* was shown to have complement fixing activity, although not as active as other plant-extracts tested in the study. The polymers were extremely difficult to dissolve in water, and when dissolved, they gave a very viscous solution. In the current study the traditional use, the chemical structure and physical properties of the polysaccharide fraction from the water extract of the bark from *C. cordifolia* is studied, and the effect of the polysaccharide on the complement system is investigated.

2. Material and methods

2.1. Traditional uses

In 2003 a field work was performed in two different parts of Mali: Dioila situated in the Southern part and Dogonland situated in the Northern part of Mali. The aim was to collect information on all the uses of *C. cordifolia* in traditional medicine in these regions. The interviews with the traditional healers was performed by a multidisciplinary team composed of an agro-forester, a medical doctor (or nurse) and a pharmacist. The traditional healers were asked about the diseases against which the plant was used, the local name of the plant, and the preparation and administration of the remedies.

2.2. Material

The bark of *C. cordifolia* was collected in March 1999 at Kinieroba (Mali). The plant material was identified at the Department of Traditional Medicine; a specimen is deposited at this department under the number 1331 DMT. The plant material was air dried and pulverised to fine powder by a mechanical grinder.

2.3. Extraction

The powdered bark (41.6 g) was extracted with chloroform using a soxhlet, followed by maceration in ethanol overnight in order to remove coloured and low molecular weight compounds. The residue was extracted with water (1 L) at 50 °C to give the crude extract Cc50.

2.4. Isolation of polysaccharide Cc50-I by anion exchange chromatography

The crude extract Cc50 was centrifuged in a Hermle Z 382 K centrifuge, at a speed giving 20,000*g* for 5 min. The supernatant was filtered through a 5 μm diameter Acro 50A devise with Versapor Membrane (Gelman Laboratory). The viscous solution (500 mL) was diluted with 300 mL of distilled water prior to application on the column (5 × 50 cm) filled with DEAE Sepharose fast flow (Pharmacia) using a peristaltic pump P-3 (Pharmacia) at a flow of 1 mL/min. The column was connected to a LKB-Super Frac™ (Pharmacia) fractions collector. The neutral material from the crude extract was eluted from the column using distilled water (2 vol), followed by elution with a NaCl gradient (0–1.5 M) in water for elution of acidic polymers. The elution profile of the fractions collected was monitored by the phenol sulphuric acid test (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). One peak was obtained and the relevant fraction (Cc50-1) was pooled, dialysed in a Spectra MWCO diameter 3.500 mm dialysis tube (Spectrum laboratories) and freeze dried.

2.5. Determination of the monosaccharide composition

2.5.1. Methanolysis and gas chromatography (GC)

The monosaccharide composition was determined by GC analysis of the trimethylsilyl (TMS) derivatives of the methyl-glycosides obtained after methanolysis. 1 mg of Cc50-1 was subjected to methanolysis with 1 mL of 4 M hydrochloric acid in anhydrous methanol for 24 h at 80 °C. 100 μg of mannitol was added as an internal standard (Barsett & Paulsen, 1985). The TMS derivatives were analysed using a Carlo Erba 6000 Vega Series gas chromatograph with flame ionisation detector (FID) having helium as the mobile phase at a flow of 1.8 mL/min. The injection temperature was 260 °C and the detection temperature was 310 °C. The monosaccharides were identified by comparison of their retention times with that of pure standards analysed under the same conditions. This method allows the identification and quantitative determination of both neutral and acidic monomers present in the native Cc50-1.

2.5.2. Determination of native methylated sugars

The GC traces mentioned above indicated the presence of mono-methylated monosaccharides in Cc50-1. A combination of methods was used to identify each of these sugars. 5 mg of sample were hydrolysed with 500 μL of 2.5 M trifluoroacetic acid (TFA) for 2 h at 100 °C. TFA was removed by a stream of nitrogen. After drying, one half was reduced and converted into alditol acetates and subjected to GC–MS as described under Section 2.9.1. The other half was used for the demethylation process described below.

2.5.2.1. Identification of 2-O-Me-Gal. The GC–MS results obtained after hydrolysis, reduction and conversion to alditol acetates showed that Cc50-1 contained a large amount of 2-*O*-methyl-hexose. The retention time and electron impact mass spectrum of the latter were compared with the data of 2-*O*-Me-Glc, a component present in polysaccharides from Cyanobacteria (Hu, Liu, Paulsen, Petersen, & Klaveness, 2003) and a partly methylated galactitol acetate standards prepared in our laboratory (McConville, Homans, Thomas-Oates, Dell, & Bacic, 1990). The 2-*O*-Me hexose present in Cc50-1 was, based on these comparisons, identified as 2-*O*-Me-Gal. To confirm this result, hydrolysed Cc50-1 was treated with borotrichloride

(BCl_3) (method described below) and analysed after methanolysis and TMS-derivatisation by GC. The result showed the absence of a major peak, concomitant with an increase in the galactose-peaks.

2.5.2.2. Identification of 2-O-Me-Fuc. GC–MS results of the alditols acetates obtained after hydrolysis, reduction and acetylation of Cc50-1 showed the presence of a 2-*O*-Me-desoxyhexose, which could be either 2-*O*-Me-Rha or 2-*O*-Me-Fuc. These data were compared with that of 2-*O*-Me-Rha obtained from Cyanobacteria (Hu et al., 2003), and no match was found between the retention times. Partly methylated fucitol acetate standards were prepared in our laboratory (McConville et al., 1990). The retention time of 2-*O* methylated fucitol acetate was compared with that of the unknown 2-*O*-methyl derivative in our sample, and this was found to be the same as for 2-*O*-Me-Fuc. An increase in fucose compared with the native chromatogram as discussed above for 2-*O*-methyl galactose was also obtained after removal of the methyl group by BCl_3 treatment, thus confirming the presence of the fucose derivative.

2.5.2.3. Identification of 4-O-Me-GlcA. Hydrolysed Cc50-1 and 4-*O*-Methyl glucuronic acid (4-*O*-Me-GlcA) standard were run separately in the HPAEC-PAD system. A monomer fraction was collected at the same retention time as the standard and both were analysed by methanolysis and GC. 4-*O*-Me GlcA was then identified in Cc50-1 by comparison with the standard obtained by methanolysis and TMS-derivatisation of the product "Aldouronic acid mixture (product number O-AMXR)" obtained from Megazyme, Ireland. After BCl_3 treatment, as described below, no 4-*O*-Me-GlcA was present, and instead glucuronic acid was identified. This monosaccharide was not present in the original sample.

2.5.2.4. Demethylation with BCl_3. The other half portion after TFA hydrolysis described above was thoroughly dried under nitrogen at 40 °C. The residue was dissolved in 1 mL of ice cold (−80 °C) dichloromethane and 1 mL of BCl_3 (−80 °C) was added. The mixture was kept at −80 °C for 30 min, followed by room temperature for 16 h, then dried under nitrogen and washed twice with 3 mL of methanol (Fieser & Fieser, 1967). The residue was dissolved in 1 mL of distilled water, freeze dried, and subjected to methanolysis and analysis by gas chromatography as describe above.

2.6. Determination of the molecular weights

The relative molecular weight of the isolated polymer was determined by gel filtration on a Superose™ 6 10/300 GL column (Amersham Biosciences) connected to a Fast Protein Liquid Chromatography system (Amersham Biosciences), by elution with 10 mM NaCl solution at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and monitored with a Refractive Index Detector (Shimadzu RID 6-A). The phenol sulphuric acid test was used to determine the elution profile of the polysaccharides. Dextran polymers of 2000, 233, 98.4, 16.4 and 5.6 kDa (Pharmacia) were used as calibration standards.

2.7. Determination of total phenolics content

Sample (0.4 mg) was dissolved in distilled water at a concentration of 1 mg/mL and added to 0.4 mL of the Folin-Ciocalteu reagent. After 3 min, 0.4 mL of Na_2CO_3 was added, the mixture was left to stand for 1 h, and the absorbance was read at 750 nm (Swain & Hillis, 1959) in a LKB-Biochrom Novaspec spectrophotometer. A standard curve of ferulic acid (from 4 to 100 μg/mL) was used to determine the amount of phenolics present as ferulic acid.

2.8. Determination of the protein content

The polysaccharide was dissolved in distilled water at a concentration of 0.2 mg/mL. Bovine Serum Albumin (BSA) was used as standard and dissolved at the same concentration in a Micro BCA™ protein assay reagent (Pierce Biotechnology). The samples were incubated at 60 °C for 1 h, cooled at room temperature and the absorbance was measure at 562 nm using a LKB-Biochrom Novaspec 4049 spectrophotometer.

2.9. Linkage analysis

2.9.1. Linkage determination by methylation or ethylation and GC–MS analysis

As the polysaccharide contains hexuronic acids, the carboxyl groups were first subjected to reduction as described by Kim and Carpita (1992). The sample was then divided into two parts, half of the material was methylated as describe by McConville et al. (1990) using methyl iodide for determination of the linkages present totally in the polysaccharide, and the second half was ethylated using ethyl iodide as ethylation agent for detection of the linkages of the natively methylated sugars present in the polymer.

Both samples were hydrolysed using TFA and reduced with sodium borodeuteride to label C-1. The partly methylated/ethylated alditols were then acetylated using ethyl acetate and acetic anhydride with perchloric acid as catalyst (Harris, Henry, Blakeney, & Stone, 1984). The resulting partly methylated and partly ethylated alditol acetates were identified by GC–MS. E.I. mass spectra were obtained using a Hewlett–Packard (Palo Alto, CA, USA) Mass Selective Detector 5970 with a Hewlett–Packard GC. The injector temperature was 250 °C, the detector temperature 300 °C and the column temperature 80 °C when injected, then increased with 30 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then 30 °C/min to 300 °C. Data was processed with Fisons Masslab software (version 1.3). The compound at each peak was character-

ized by an interpretation of the characteristic mass spectra and retention times in relation to standard sugar derivatives.

2.9.2. Periodate oxidation

Sample (20 mg) was dissolved in 0.2 M acetate buffer pH 3.6. Sodium metaperiodate (100 mg) was added, and the reaction was carried out in the dark at room temperature. After 24 h the reaction was stopped by the addition of an excess of ethylene glycol. This was allowed to stand for 2 h. After dialysis, the sample was reduced to the respective polyalcohol by sodium borohydride (20 mg). After 10 h, excess borohydride was destroyed by adjusting the pH to 7.0 by addition of acetic acid, and the solution was dialysed overnight and then lyophilized. The resulting products were analysed by gas chromatography after methanolysis, TMS-derivatisation as describe above and the presence of threonic acid was verified by comparison with threonic and erythronic acid standards.

2.10. Intrinsic viscosity measurement

The polysaccharide was dissolved in distilled water at a concentration of 0.5% (w/v); the sample was filtered prior to viscosity measurement. A serial dilution (0.25%; 0.125%; 0.062%; 0.031%) was made from this original solution. A glass capillary viscometer equipped in a viscometer bath (Schott-Geräte, Germany) was used to measure the passage time of the polysaccharide solution and dilutions flowing through the capillary which was filled with 2 ml of sample and equilibrated at 25 ± 1 °C for 5 min. All the measurements were done four times and the average values are reported. The viscosities measured in this way were converted to specific viscosity (η_{sp}) using Eq. (1), then to reduce viscosity (η_{red}) using Eq. (2). The intrinsic viscosity $[\eta]$ was estimated using the plot of reduced viscosity versus concentration as indicated in Eq. (3). The Huggins' constant (K') was estimated through the empirical relation (4) that represents the concentration dependence of the reduce viscosity known as Huggins equation. (η_c, viscosity of the solution; η_s, viscosity of the solvent; C, concentration.)

$$\eta_{sp} = (\eta_c - \eta_s)/\eta_s \quad (1)$$

$$\eta_{red} = \eta_{sp}/C \quad (2)$$

$$[\eta] = \lim_{C \to 0} \eta_{sp}/C \quad (3)$$

$$\eta_{red} = [\eta] + k'[\eta]^2 C \quad (4)$$

2.11. Complement fixating test

The activity of the samples was tested in the complement system. This test is based on inhibition of antibody-sensitized sheep red blood cells by human sera as described by Michaelsen, Gilje, Samuelsen, Hogasen, and Paulsen (2000). The pectin fraction PMII from the leaves of *Plantago major* L. (Samuelsen, Cohen, Paulsen, & Wold, 1996) was used as positive control.

3. Results

3.1. Ethnopharmacological information

The collected ethnopharmacological uses of *C. cordifolia* are presented in Table 1. The plant was used against a variety of illnesses among which, abdominal pain, wounds, fever and headache were the most cited ones.

3.2. Isolation and molecular weight of Cc50-1

After anion exchange chromatography fraction Cc50-1 was obtained. The approximate molecular weight of the polymer was determined using a Superose 6 column by comparing the elution pattern with that of dextrans of know molecular weights. Cc50-1 was relatively polydisperse and had a high molecular weight ranging between 1000 and 2000 kDa.

3.3. Monosaccharide composition and their linkages

The results of the monosaccharide composition and linkages analysis of Cc50-1 are shown in Table 2. 4-*O*-Methylglucuronic acid was identified by comparison with a standard as described under Section 2.5.2. The neutral mono-methylated sugars were identified by comparison as alditol acetates both by retention times and mass spectras with corresponding standards prepared as described in Section 2.5.2. The amounts of the mono-methylated sugars were determined by the following procedure: methanolysis of the native polysaccharide gave a ratio of the common monosaccharides present in the polymer. In addition, various other compounds of unknown compounds were present. After demethylation by borotrichloride, followed by methanolysis, TMS-derivatisation and GC analysis, the increase in the amounts of fucose, galactose and glucuronic acid compared to the chromatogram of the native polymer, gave the ratio of the monosaccharides that originally were mono-methylated. That is, the increase was due to 2-*O*-methyl galactose, 4-*O*-methyl-glucuronic acid and 2-*O*-methyl-fucose and gave the amount of these sugars present in the native polysaccharide. Rhamnose, galacturonic acid and galactose were then found as the major monosaccharides in the polysaccharide in the ratio 1–1.25–0.75. Arabinose, xylose and glucose were present in trace amount only. 2-*O*-Me-Gal, 4-*O*-Me-GlcA and 2-*O*-Me-Fuc were identified at a ratio of 1–0.9–0.1. Based on these results the ratio between the monosaccharides present in the native polysaccharides is as given in Table 2.

As the polysaccharide contained native methylated monosaccarides, the linkages of these had to be determined by ethylation of the polysaccharide instead of methylation which is the normal method of derivatisation. This method was successfully used for investigation of linkages in the

Table 1
Traditional uses of *Cola cordifolia*

Plant part	Ailments	Preparation and administration
Leaves	Stitch	Use a decoction as steam bath of the body
	Oedema	Use a decoction to wash the oedema area
	Head ache	Mix the leaf powder with that and the gum of *Burkea africana*, burn the mixture and inhale the smoke Use the hot decoction as steam bath of the head
	Gastritis	Drink the macerate in water of the mixture of the leaf powder and that of *Xylopia aethiopica* and honey
	Malaria	Drink and use as bath the macerate of the fresh crushed leaves
	Malnutrition	Use as bath the decoction and drink
	Birth induction	Drink and use as body wash the decoction
	Stomach pain	Drink and use as bath the decoction
	Chronic diseases	Drink and use as bath the decoction
	Coma due to epilepsy and fever	Use the decoction as steam bath and body wash
	Fever	Wash the body with the decoction and drink
	Body pain and weakness	Drink the decoction
Stem bark	Legs oedema and oedema	Use the decoction as steam bath and washing of the legs and drink
	Internal wounds	Drink the decoction
	Eye diseases	Wash the eyes with a decoction
	Over weight	Drink the decoction tree time a day
	Constipation	Drink a decoction of a powder fresh bark
	Hypertension	Drink the decoction
	Malaria	Drink and use as bath the decoction
	River blindness	Wash the eyes with the decoction and drink
	Amenorrhea	Eat the powder of mixture of the bark and the red cola nut Drink the decoction
	Wounds	Pound the bark, add the butter of *Butyrospermum parkii* and apply the ointment on the wound Wash the wound with the decoction and apply the powder of a carbonized bark
	Migraine and headache	Use the decoction to wash the head whit a traditional soap
	Hot legs	Mix a burned bark with the butter of *Butyrospermum parkii* and apply under the foot; or a decoction as a steam bath of the foot
	Heart disease	Pound the bark, add some water, the flower of maize, and leaves of *Annona senegalensis*, cook the mixture and eat
	Tiredness and body pain	Wash the body with a decoction
	Parkinsons	Drink a decoction
	Kneepains	Applied a decoction on to the knees
	Asthma	Eat a mixture of the bark powder and salt
	Stomach pains	Add the bark powder to the water and drink
	Chest pain	Use the decoction as body wash
	Fever	Drink and use as steam bath the decoction
	Dysentery	Drink the decoction
	Jaundice	Drink the decoction
Root	Constipation	Drink before breakfast some water containing the powder of the dried root bark
	Haemorrhoid	Drink a decoction in the morning
	Malaria	Drink and use as body bath the decoction
	Dysentery	Drink the decoction
	Increase weight	Eat a meal containing a mixture of the powder from the root and the seeds of *Xylopia aethiopica* twice a day
	Fever	Drink the decoction and use it as steam bath
	Diarrhoea	Mix the powder with warm water and drink or with food and eat
	Stomach pain	Add the powder to water and drink Drink the decoction
	Head ache	Use the decoction as steam bath of the head
	Inflammation of the skin	Burn the root and mix the ash with the butter of *Butyrospermum parkii* and apply to the skin
	Dysuria	Drink a decoction of the root and that of *Grewia bicolor* and *Gardenia ternifolia*
	Internal wounds	Drink a decoction of the root and that of *Waltheria americana*
Loranthus	Depression	Mix the powder in the water use for bath
	Induce labour	Drink some water containing the powder
	Anxiety	Use the decoction as body wash Burn the powder and use the smoke for fumigation
	Oedema	Wash the oedema area with water contain the powder and apply the powder with butter as ointment
	Chest pain	Use the decoction as body wash and steam bath
Seed	Wounds	Apply an ointment of the powder of burned seed and the butter of *Butyrospermum parkii*

Table 2
Monosaccharide composition (%) and their linkages (mol%) of the bark polysaccharide Cc50-1 isolated from *Cola cordifolia*

Monosaccharides	Primary fragments by GC–MS of ethylated alditol acetates	Deduced linkages	Cc50-1
Ara Total			0.9
	59, 132, 189	Araf Terminal	0.3
	59, 189, 204	Araf 2-	Trace
	59, 132, 261	Araf 3-	Trace
	132, 203	Araf 5-	0.6
Gal Total			14.8
	59, 132, 189, 190, 247	Gal Terminal	4.5
	59, 132, 189, 262	Gal 3-	4.9
	59, 132, 190, 261	Gal 4-	3.9
	132, 190, 203, 261	Gal 6-	0.9
	132, 275	Gal 4,6-	0.5
	132	Gal 3,4,6-	0.1
2-*O*-Me-Gal Total			19.5
	59, 118, 176, 189, 247	2-*O*-Me-Gal Terminal	9.0
	59, 118, 189, 248	2-*O*-Me-Gal 3-	7.3
	118, 203	2-*O*-Me-Gal 3,6-	1.3
	118	2-*O*-Me-Gal 3,4,6-	1.9
Rha Total			20.4
	145, 204	Rha 2-	Trace
	145, 276	Rha 2,3-	9.9
	204, 217	Rha 2,4-	10.6
2-*O*-Me-Fuc Total			2.4
	118, 145, 176, 203	2-*O*-Me-Fuc Terminal	2.4
4-*O*-Me Glc A Total			17.6
	47, 132, 175, 190, 233	4-*O*-Me Glc A Terminal	17.6
Gal A Total			24.3
		Gal A 4-[a]	≈16
		Branch point GalA[a]	≈8
Total carbohydrate content			88.2
Proteins			1.1
Phenols			1.3

Total carbohydrate, phenol and protein contents are also given.
[a] Approximate values determined after periodate oxidation.

polysaccharide from the bark of *Ulmus glabra* containing mono-methylated Galactose (Barsett & Paulsen, 1992). Galactose and 2-*O*-methyl galactose are responsible for approximately 35% of the total carbohydrates in the polymer. The galactose units are present in almost equal amounts as terminal, 3- and 4-linked units, while the 2-*O*-methyl galactose units basically are terminal and 3-linked with smaller amounts of branch points as determined after ethylation.

Rhamnose, approximately 20% of the carbohydrate present, is responsible for the major part of the branch points in the molecule. The 2,4- and 2,3-linked units are present in almost equal amounts. 4-*O*-methylglucuronic acid is only present as terminal units. The other monomers, 2-*O*-methylfucose, arabinose, xylose and glucose are present in minor amounts. Arabinose was mostly terminal and 5-linked, and 2-*O*-Me-Fuc only terminal linked. Cc50-1 contained trace amount of 4-linked xylose.

Periodate oxidation was performed to identify the linkages of the galacturonic acid. The presence of threonic acid was confirmed by comparison with threonic acid standard, and this confirmation also showed that galacturonic acid basically was 4-linked. Galacturonic acid is also responsible for some branch points as free galacturonic acid also was identified. Rhamnose was not degraded by periodate oxidation which confirms that rhamnose is present as branch points only as found by the linkage analysis after the methylation process.

3.4. Determination of the presence of ester groups by Infrared (IR) spectroscopy

Cc50-1 was subjected to IR spectroscopy to identify esterified hexuronic acids in Cc50-1. The absorbance of the sample was measured before and after treatment with sodium hydroxide, no presence of ester group was found.

3.5. Determination of phenolic and protein content

Cc50-1 contained relatively small amount of phenols as seen in Table 2, and based on the Micro-BCA protein assay, Cc50-1 contained 1.1% protein. Compounds like lipids, reduced sugars and phenols, that might have been present in the sample, might also slightly interfere with the measurement.

3.6. Viscosity measurement

The intrinsic viscosity was estimated through reduced viscosity versus concentration plots. As shown in Fig. 1, the relationship gave no linear extrapolation to infinite dilution but an approximate linear intercept ($[\eta]$) at $C = 0$ was used to estimate the Huggins' constant $K' = 1.2$. Such result implied that when water was used as solvent, molecular aggregations were likely occurred to some extent.

3.7. Complement fixating activity

Cola cordifolia polysaccharides did show a low complement fixation activity. The measured ICH_{50} (the lowest concentration of sample needed to give 50% inhibition of haemolysis of antibody-sensitized SRBC) was 100 μg/mL for the crude extract Cc50, 480 μg/mL for Cc50-1 and 130 μg/mL for PMII, the positive control. These results show that the polysaccharides present in the crude extract has only a moderate immunostimulating activity that may influence the wound healing processes. Other products in the crude extract appear to be more responsible for the bioactive response of the extract.

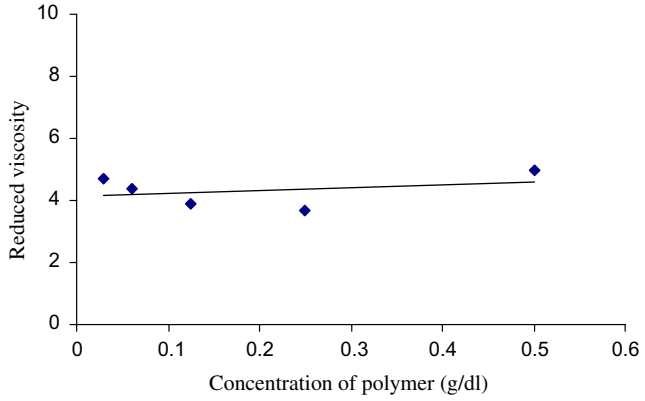

Fig. 1. Estimation of intrinsic viscosity of Cc50 in distilled water (25 °C) using the plot of reduced viscosity versus concentration.

4. Discussion

A highly complex polysaccharide (Cc50-1) was isolated from the 50 °C water extract of the bark of *C. cordifolia* used to cure several illnesses in traditional medicine in Mali. The polymer was purified by ion exchange chromatography. Cc50-1 has an average molecular weight between 1000 and 2000 kDa, i.e., quite polydisperse, The polymer contains approximately 40% of natively methylated monomers identified as 2-*O*-Me-Gal, 4-*O*-Me-GlcA and traces of 2-*O*-Me-Fuc, all these mostly terminally linked. It also contains 20% rhamnose being responsible for the main part of the branch points in the molecule and 24% galacturonic acid basically 4-linked, but also responsible for some branch points. The latter linkage deduction is based in the results after periodate oxidation, after which threonic acid was identified. The polymer forms a viscous solution, and with the high content of hydrophobic monosaccharides, almost 40% of the total carbohydrate having a methyl group attached, this is an unusual type of polysaccharide. In addition to the high amount of hydrophobic sugars the polymer contains around 40% hexuronic acids which by IR was shown to not be esterified. Lack of good solubility as well as high viscosity can be caused by the high degree of hydrophobic monosaccharides present in the polymer. The low solubility made it impossible to perform NMR analysis of the polysaccharide.

The linkages identified in Cc50-1, and the fact that it was impossible to reduce the galacturonic acid present, lead to the conclusion that this polysaccharide is of a complex structure. The backbone may be composed of alternately 4-linked galacturonic acid and 2-linked rhamnose, the latter branched at position 4 (≈51%) and position 3 (≈49%) with side chains composed mainly of galactose and 2-*O*-methylgalactose. The side chains are mainly terminated by 2-*O*-methylgalactose and 4-*O*-methylglucuronic acid. Galacturonic and 4-*O*-methylglucuronic acid could not be reduced by the carbodiimide method. We believe that this was due to the presence of the compact three-dimensional structure of the polymer due to steric hindrance by the side chains on the rhamnose units, in addition to the presence of a high amount of native methylated monomers which are of hydrophobic nature making the carboxyl groups not accessible to the carbodiimide group.

Periodate oxidation followed by reduction, methanolysis, TMS-derivatisation and GC analysis as described above showed that rhamnose was intact after this procedure. This confirms that the monomer is responsible for most of the branch points in the molecule. The only monosaccharides remaining after this treatment were rhamnose, galactose and galacturonic acid. In the native polymer they were present in the ratio rha:gal:galA of 1:0,7:1,2, while after periodate oxidation the ratio was 1:0,3:0,4. These results are consistent with the methylation results showing that galactose have approximately 1/3 of the units as 3-linked and as branch points, thus not being susceptible to periodate oxidation. Only 1/3 of galacturonic acid remains after this treatment indicating that galacturonic acid also is carrying side chains. In addition to these monomers, threonic acid was identified after the analysis procedure mentioned above. This is a proof for galacturonic acid being 4-linked in this polysaccharide.

When performing permethylation with methyl iodide naturally methylated and naturally non-methylated sugars cannot be distinguished. Therefore, an additional ethylation analysis was performed using ethyl iodide instead of methyl iodide as ethylation reagent. The resulting mass spectra allowed the identification of the type of linkages for 2-*O*-Me-Fuc, 2-*O*-Me-Gal and 4-*O*-Me-GlcA. The primary fragments leading to this identification are given in Table 2.

Barret and Northcote (1964) reported a polysaccharide with a galacturonosyl-(1,2)-rhamnose backbone containing 2-*O*-methyl fucose from Apple fruit, this seemed to be a RGI type but the authors did not specify it as so. Terminal 4-*O*-Me GlcA has previously been identified as component of pectic polymers. Inngjerdingen et al. (2005) identified for example 2% of this monosaccharides in a pectic polysaccharide isolated from *Glinus oppositifolius*. The presence of 4-*O*-Me-GlcA as high as 17% of the total amount of sugar present in a polysaccharide has not been reported before in this type of polymers from plant. 4-*O*-methylglucuronoxylans are the only other type of polysaccharides that have a high content of this monomer. In polysaccharides from *Mahonia aquifolium,* 13% (Kardosova et al., 2004) and *Castanea sativa* 14% (Moine et al., 2007) of the same monomer have been reported. No reports can be found in the literature about 2-*O*-Me-Gal as part of a polymer from plants. High amounts of mono-methylated sugars like 2-*O*-methyl glucose and 2-*O*-methyl rhamnose were previously isolated as parts of algae polysaccharides in our laboratory (Brüll et al. 2000; Hu et al. 2003), but these are not present in the polymer discussed in this paper.

Polysaccharides having such a high content of natively methylated monosaccharides seem to be quite uncommon as no other polysaccharide similar to Cc50-1 from *C. cordifolia* has been reported.

Tomoda and Satoh (1977) have reported a structure from *Hydrangea paniculata* called Paniculatan also containing 4-*O*-methyl glucuronic acid as most of the terminal units present. This is also a highly branched polysaccharide containing mainly rhamnose, galactose and galacturonic acid in addition to the mentioned 4-*O*-Me-GlcA. Based on their isolation of oligosaccharide units, they confirmed that the backbone was composed of 2-linked rhamnose residues having branches at position 4 and 4-linked galacturonic acid residues having branches at position 3 in the approximate molar ratio of 2:1. The structure of Paniculatan can then be an RGI type, but other possibilities also exist.

Future studies in our laboratory will reveal the real structure of Cc50-1

4.1. Viscosity and structure

Cc50-1 was poorly soluble in water. An improvement of dissolution was observed by using heat and sonication, but a fully dissolution could not be obtained. This could be explained by the removal of some solubilising compounds during the purification process knowing that Cc50-1 is originated from a water extract. Although viscosity of anionic compound is usually estimated in salt containing solution, the intrinsic viscosity of Cc50 was estimated in water since most of the performed experiment used this solvent. The value of the Huggins' coefficient was 1.2. This value should lie between 0.3 and 0.7 for polymers in good solvent condition. Values higher than one generally implies that aggregation likely occur. Inter molecular association of hydrophobic groups lead to additional interactions between macromolecules which bring about an important increase of viscosity (Durand, 2007; Lai & Yang, 2007). Since Cc50-1 is a highly branched macromolecule with hydrophobic groups, the same interaction might have happened, explaining the observed poor solubility in water like solvent.

4.2. Biological activity

The complement fixing activity was measured both for the crude water extract, Cc50 and for the isolated polysaccharide Cc50-1. The results showed that the crude extract was more active than Cc50-1. This is an opposite results of other studies performed in our laboratory (Nergard et al., 2004). Usually, purified polymers express higher activity upon the complement system relative to crude extracts. The explanation to that could be due to the poor solubility of Cc50-1, since the result of viscosity studies showed that aggregates are likely to form when hydrophilic solvent are used, this phenomenon might have happened during measurement of the biological activity since the test used freeze dried samples which needed to be dissolved in a water based buffer. It may also be that the purification on the anion exchange column to purify Cc50-1 has removed other compounds with positive influence on the complement fixing assay.

5. Conclusion

Ethnopharmacological studies of the use of the bark from the tree *C. cordifolia* reveals several uses in traditional medicine in Mali. The results indicated that it would be worth while studying the properties and possible bioactivity of the bark polysaccharide from this tree. This study reveals that the stem bark of the Malian tree *C. cordifolia* contains a type of polysaccharide that not has been reported before. The presence of both 20% 2-*O*-methyl galactose and 18% 4-*O*-methyl glucuronic acid in the same polymer is new, as well as such a high degree of branching on what appears to be the backbone of the molecule. A slight effect on the complement system was found indicating an effect of the polysaccharide on the immune system.

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