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Neutral polysaccharide from *Cedrela tubiflora* with anticomplementary activity

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Abstract

A water-soluble polysaccharide extracted from leaves of the Meliaceae *Cedrela tubiflora* was separated into neutral and acidic polysaccharide fractions. The best anticomplementary activity was exhibited by the neutral product which was further purified by means of gelpermeation chromatography. The composition and methylation analysis of the purified product were determined.
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Keywords: *Cedrela tubiflora*; Meliaceae; Leaves; Neutral heteropolysaccharide; Anticomplementary activity; Immunomodulating activity; Methylation analysis

1. Introduction

Plants belonging to the Meliaccae have been widely used in natural medicine. Among others, antiviral, antihelmintic, antiinflammatory and antirheumatic activities have been reported (Bhakuni, Dhar, & Dhawan, 1969; Fujiwara et al., 1982; Fujiwara et al., 1984; Patel, 1986; Andrei, Coulombié, Courrèges, de Torres, & Coto, 1990; Bray, Warhust, Connolly, & O'Neill, 1990; Coulombié, Andrei, Laguens, de Torres, & Coto, 1992). The antiinflammatory and antirheumatic properties of some members of this family, such as *Azadirachta indica* and *Mumronia pumila*, have been explained by their action on the immune system (Labadie et al., 1989).

Cedrela tubiflora is a native tree which has been used for the natural treatment of inflammatory affections (Toursarkissian, 1980). We have previously observed that aqueous leaf extracts of *C. tubiflora* are able to exert *in vitro* inhibitory activities on both human and murine complement activation (Benencia, Courrèges, & Coulombié, 1996). In addition, the extract inhibits the phagocytosis and the respiratory burst activities of murine peritoneal exudate cells, human peripheral blood monocytes and polymorphonuclear leukocytes. The proliferation of Concanavalin-A-stimulated lymphocytes is also impaired by the extract (Benencia et al., 1996; Benencia, Courrèges, Nores, & Coulombié, 1995). So, taking into account the medicinal potential of these leaf extracts, it became necessary to investigate the chemical nature of the active principles present in the leaves of *C. tubiflora*. Herein, we report the isolation and chemical characterization of a neutral polysaccharide responsible for the anticomplementary activity.

2. Results and discussion

Fresh green leaves of *C. tubiflora* were sequentially extracted with boiling chloroform, methanol and water. The aqueous extract was poured into four volumes of ethanol leading to the isolation of the crude polysaccharide fraction (CPF) in a yield of

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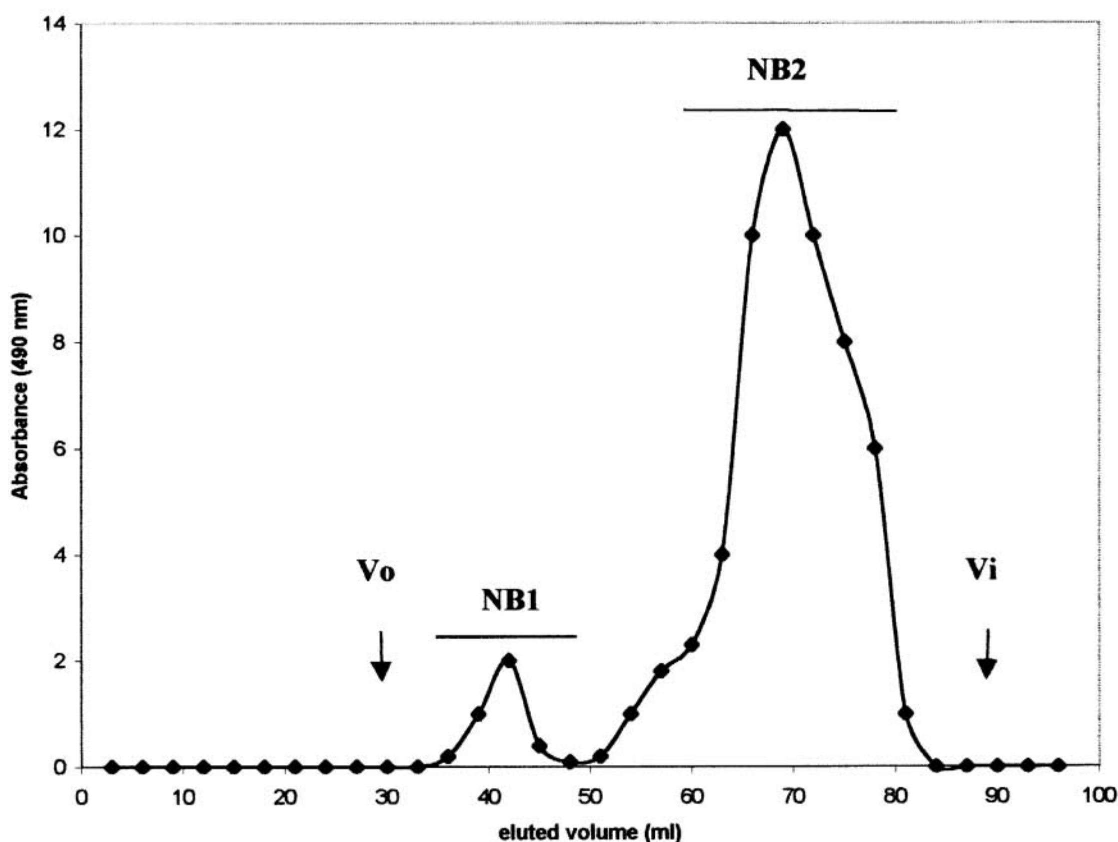

Fig. 1. Elution diagram of NB in Sepharose CL-2B using 0.2 M NaCl as eluant.

0.13%. Chromatography of CPF on DEAE Sephadex A-25, equilibrated with buffer *Tris*–HCl, gave a non-bound fraction (NB). Isolation of bound material was carried out using solutions of increasing sodium chloride concentration.

In previous studies (Benencia et al., 1995; Benencia et al., 1996), we have demonstrated that aqueous leaf extracts of *C. tubiflora* showed strong anticomplementary activity, diminishing the hemolytic capacity of human complement, regardless of the activation pathway assayed (classical or alternative). Therefore, purification of anticomplementary agents was done guided by modulation of both pathways. Inhibition of the classical complement pathway activity by NB was ca. ten-fold stronger than that by the bound fractions and the positive control heparin. Similar results were obtained in the alternative complement pathway. No change in the anticomplementary activity was observed when NB was digested with proteinase K.

NB was further fractionated by gel permeation chromatography on Sepharose CL-2B using 0.2 M NaCl as eluent, to yield the minor fraction NB1, which eluted first, and the major one NB2 (yield, 1.3% of CPF; M_r 210 000) which was selected for further studies due to its higher activity; Fig. 1 shows the corresponding elution pattern.

Tables 1 and 2 show the anticomplementary activities of CPF, NB, NB2 and heparin assayed by the classical and alternative pathways, respectively. Analysis of variance (two factors Anova with replications) of anticomplementary activity showed significant interactions among products and concentrations ($p < 0.05$) (Steel & Torrie, 1985); thus, the activity observed for each concentration depends on the assayed product. Note that activity increases with purification (cf. CPF and NB2) (Tables 1 and 2). Preincubation of erythrocytes with NB and NB2 did not prevent complement-mediated lysis in both pathways (data not shown), discarding a protective effect of the fractions on the erythrocyte membrane as has been proposed (Obaseki, Adeyi, & Anyabuke, 1985).

NB2 contained 80% of carbohydrate, 5% of protein and only traces of uranic acid (less than 1%). A lugol negative reaction indicated the absence of starch and/or amyloid. Carbohydrate analysis showed that it was comprised of major amounts of magnesia (32.8 mol.%), glucose (30.8%) and galactose (22.1%), together with lesser quantities of arabinose (7.1%), fucose (3.9%) and xylose (3.3%); traces of rhamnose were also detected.

NB2 was methylated according to Hackamore (Hakomori, 1964) and Table 3 shows the composition of the permethylated product. The results are consist-

Table 1
Anticomplementary activities of *Cedrela tubiflora* fractions on the classical pathway[a]

Concentration (μg/ml)$^{-1}$	CPF	NB	NB2	Heparin
0.1	0	0	0	0
0.2	0	28 ± 3.8	34 ± 5.7	0
0.4	0	34 ± 3.6	40 ± 4.5	0
0.8	0	44 ± 5.5	50 ± 3.0	0
1.7	0	60 ± 4.6	80 + 3.2	0
3.5	0	98 ± 2.8	96 ± 2.4	0
7.0	0	96 ± 4.3	96 ± 3.2	0
14.0	0	98 ± 2.3	95 ± 3.2	0
28.0	0	97 ± 3.3	97 ± 2.3	0
56.0	0	99 ± 2.5	97 ± 1.4	0
67.5	0	97 ± 2.3	98 ± 1.0	0
125.0	45 ± 3.5	98 ± 2.0	97 ± 1.5	7 ± 3.6
500.0	87 ± 5.5	99 ± 1.5	95 ± 4.0	15 ± 3.2
1000.0	96 ± 2.8	96 ± 4.3	98 ± 1.7	30 ± 3.6
10000.0	97 ± 2.6	97 ± 2.3	97 ± 1.5	60 ± 2.9

[a]Values are expressed as mean ± S.D. of four independent measurements.

ent with a backbone mainly formed by 4-linked magnesia and glucose, with minor amounts of 6-linked galactose. Branching seems to occur mostly through the 2-position of galactose and the 6-position of glucose. Non-reducing end-chain residues of furanosic arabinose and pyranosic fucose, magnesia and galactose were detected.

Anticomplementary activities have also been reported for other members of the Meliaceae, such as *Mumronia pumila* (Labadie et al., 1989), *Melia azedarach, Trichilia elegans* and *Cedrela lilloi* aqueous extracts (Benencia, Courrèges, Massouh, & Coulombié, 1994; Nores, Courrèges, Benencia, & Coulombié, 1997) and for *Azadirachta indica* (Van der Nat, Klerx, Van Dijk, De Silva, & Labadie, 1987; Van der Nat et al., 1989). From the latter, two polysaccharide fractions with anticomplementary activity were isolated; these fractions were comprised of major amounts of glucose, with lesser amounts of arabinose, galactose and magnesia, and also contained small quantities of protein (5.5–9.8%). However, no further structural analyses were reported on these products.

Several acids (Yamada et al., 1985; Yamada, Kiyohara, Cyong, & Otsuka, 1987; Kiyohara, Cyong, & Yamada, 1988; Yamada, Ra, Kiyohara, Cyong, & Otsuka, 1989; Kiyohara, Cyong, & Yamada, 1989a; Kiyohara, Cyong, & Yamada, 1989b; Gao, Kiyohara, & Yamada, 1990; Zhao, Kiyohara, Yamada, Takemoto, & Kawamura, 1991; Zhao, Kiyohara, Matsumoto, & Yamada, 1993; Kiyohara, Zhang, & Yamada, 1998) and neutral (Yamada et al., 1985; Kiyohara et al., 1997; Yamada, Yanahira, Kiyohara, Cyong, & Otsuka, 1986; Yamada et al., 1988; Zhao, Kiyohara, & Yamada, 1994) polysaccharides have been shown to possess anticomplementary activity. However, the heterogeneity of structures described makes it difficult to establish a relationship between structure and anticomplementary activity. Since the activation of the complement requires steric recognition, the geometry of the molecules should play an important role. For example, it has been observed that the presence of arabinosyl and galactosyl side-chains is important for the expression of the activity of different pectic polysaccharides (Yamada et al., 1985; Yamada et al., 1987; Kiyohara et al., 1988; Yamada et al., 1989; Kiyohara et al., 1989a; Kiyohara et al., 1989b;

Table 2
Anticomplementary activities of *Cedrela tubiflora* fractions on the alternative pathway[a]

Concentration (μg/ml)$^{-1}$	CPF	NB	NB2	Heparin
0.1	0	0	0	0
0.2	0	12 ± 3.4	20 ± 3.5	0
0.4	0	15 ± 3.3	22 ± 4.3	0
0.8	0	20 ± 4.0	24 ± 3.6	0
1.7	0	28 ± 3.8	32 ± 4.2	0
3.5	0	40 ± 2.0	45 ± 3.5	0
7.0	0	60 ± 4.6	68 ± 3.3	0
14.0	0	80 ± 3.5	90 ± 3.7	0
28.0	0	90 ± 3.7	98 ± 2.3	0
56.0	0	98 ± 2.3	97 ± 2.3	0
67.5	22 ± 4.2	97 ± 3.3	97 ± 2.6	0
125.0	43 ± 4.1	96 ± 4.4	98 ± 2.0	0
500.0	60 ± 4.6	97 ± 3.5	96 ± 4.3	6 ± 1.7
1000.0	80 ± 3.6	98 ± 2.3	98 ± 2.8	14 ± 2.3
10000.0	98 ± 2.3	98 ± 2.3	9713.2	34 ± 3.7

[a]Values are expressed as mean ± S.D. of four independent measurements.

Table 3
Composition (mol%) of monosaccharides produced by permethylation and hydrolysis of NB2[a]

Monosaccharide	Mol%
2,3,5-Me_3 Ara	2.5
2,3-Me_2 Ara	1.5
2,3,4-Me_3 Fuc	2.2
2,3,4-Me_3 Xyl	1.2
2,3-Me_2 Xyl	1.2
2,3,4,6-Me_4 Man	1.4
2,4,6-Me_3 Man	1.5
2,3,6-Me_3 Man	26.7
Man	1.8
2,3,4,6-Me_4 Gal	10.5
2,3,6-Me_3 Gal	2.4
2,3,4-Me_3 Gal	6.6
3,4-Me_2 Gal	7.9
2,4-Me_2 Gal	3.4
2-Me Gal	1.6
2,3,6-Me_3 Glc	18.1
2,3-Me_2 Glc	7.7
Glc	1.8

[a]Percentages lower than 1.0% not indicated.

Gao et al., 1990; Zhao et al., 1991; Zhao et al., 1993; Kiyohara et al., 1997). The existence of branched structures has also been pointed out as a determinant for the anticomplementary activity of neutral polysaccharides (Yamada et al., 1985; Kiyohara et al., 1997; Yamada et al., 1986; Yamada et al., 1988; Zhao et al., 1994). The polysaccharide from *C. tubiflora* has a highly substituted backbone with structural similarities to the anticomplementary polysaccharide, IC-1, from *Artemisia princeps* (Zhao et al., 1994). Also, its M_r is within the range reported for other active neutral polysaccharides (Yamada et al., 1986; Yamada et al., 1988; Zhao et al., 1994).

3. Experimental

3.1. Plant material

Leaves of *C. tubiflora* were collected in the Palermo Parks (Buenos Aires) during late spring. A voucher specimen (BAA15512) has been deposited in the herbarium of the Faculty of Agronomy (University of Buenos Aires) and was verified by the Engineer Juan J. Valla. Leaves (500 g) were washed with H_2O ($2l \times 3$) and dried at 37°C before extraction.

3.2. General

Carbohydrate content was analyzed by the PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), without previous hydrolysis of the sample; an equimolar mixture of Man–Gal–Glc was used as standard. Uranic acid content was determined according to Filisetti-Cozzi and Carpita (1991). Protein was analyzed by the method of Lowry, Rosebrough, Farr, and Randall (1951). The M_r of NB2 was estimated from the calibration curve of the elution volume of standard dextrans (average M_rs 39 100, 73 000, 2 000 000 and 5 000 000) on Sepharose CL-2B. Unless otherwise stated, dialyses were carried out using tubing with a M_r cut-off of 12 000.

Hydrolyses of NB2 and its permethylated derivative were performed with 2 M TFA for 90 min at 121°C. GC was performed on a fused-silica capillary column ($30 \text{ m} \times 0.25$ mm i.d.) WCOT coated with SP-2330 (film thickness 0.20 μm). Chromatography was carried out (a) at 220°C isothermally for the alditol acetates and (b) from 160 to 210°C at 2°C min^{-1} and then from 210 to 240°C at 5°C min^{-1}, for the methylated alditol acetates. N_2 was used as carrier at a flow rate of 1 ml min^{-1}, with a split ratio of 80:1. The injection and FID temperatures were 235°C for the alditol acetates; for the methylated alditol acetates the injection temperature was 235°C and the FID temperature 245°C.

Computerized GC–MS of the methylated alditol acetates was performed at 70 eV; MS were recorded over a mass range of 40–500 mu. Chromatography was carried out using the SP-2330 column programmed to run with an initial 1 min hold at 150°C and then at 9°C min^{-1} to 230°C. The He flow rate was 1 ml min^{-1}, the injection temperature 240°C and the split ratio 100:1.

3.3. Extraction

Green leaves of *C. tubiflora*, previously cut into small pieces and ground, were extracted sequentially in a Soxhlet with $CHCl_3$ (300 ml, 6 h), MeOH (300 ml, 6 h) and H_2O (300 ml, 2 h). After centrifugation to remove insoluble material, the aqueous extract was poured into 4 volumes of EtOH. The precipitate was dissolved in H_2O and dialyzed (M_r cut-off 12 000); the final volume of the dialyzed solution was 100 ml. A 1 ml portion of the solution was dried at 40°C, which corresponds to a total yield of 650 mg (0.13%) of crude product (CPF).

3.4. Fractionation

The rest of the solution (99 ml) was mixed with an equal volume of DEAE Sephadex A-25 equilibrated with buffer *Tris*–HCl (20 mM, pH 6.5), under gentle stirring during 30 min. Afterwards, the resin was collected on a paper filter and washed ×2 with the equilibration buffer. Supernatant and washings were combined to constitute the non-bound fraction NB solution. Elution of the bound material was performed in batch with NaCl solutions (100 ml) of increasing concentrations (0.1, 0.5 and 1.0 m in *Tris*–HCl buffer); these fractions, which were dialyzed, will be subjected to further studies.

The NB solution, previously dialyzed and concentrated, was applied to a Sepharose CL-2B column (1.5×120 cm) and eluted with 0.2 M NaCl. A minor peak NB1 eluted first; fractions corresponding to the major peak NB2 were pooled, dialyzed, concentrated and freeze-dried. Yield, 8.5 mg.

The fractionation procedure was followed by testing the anticomplementary activity and carbohydrate content; NB2 was selected due to its higher activity.

3.5. Treatment of NB with proteinase K

Digestion of NB with proteinase K (activity 10–20 units mg^{-1} of protein) was carried out as described in Sambrook, Fritsch, and Maniatis (1982). NB (1 mg) was dissolved in 0.5 ml of 10 mM *Tris*–HCl buffer (pH 7.8) containing 5 mM EDTA and 0.5% SDS, and a solution (0.5 ml) containing the enzyme (0.2 mg) in the same buffer was added. After incubation for 2 h at

37°C, the reaction was terminated by boiling for 1 h and the mixture centrifuged and dialyzed. No change in anticomplementary activity was observed for the digest of NB.

3.6. Methylation analysis

NB2 (5 mg) was methylated according to the Hackamore procedure as described in Obaseki et al. (1985); dialysis of the permethylated sample was carried out with dialysis tubing with a M_r cut-off of 1000. Yield, 5 mg.

3.7. Hemolytic assay for human complement activity

The studies on the complement system described here use the hemolytic capacity of human serum as a measurable criterion. Human pooled serum (HPS) was obtained from healthy volunteers and stored at −70°C until use. Optimal serum concentration (giving rise to 40–60% hemolysis) was obtained by means of the Von Krogh equation (Mayer, 1961).

Veronal saline buffer (VSB) containing 5 mM veronal and 150 mM NaCl at pH 7.4 was used. This served as a stock solution for VSB containing 0.5 mM Mg^{2+} and 0.15 Ca^{2+} and for EGTA–VS buffer containing 8 mM ethylene glycol-*bis*(2-aminoethyl) tetraacetic acid (EGTA).

Alternative (AP) and classical (CP) complement pathways activities were determined in HPS with uncoated rabbit erythrocytes and sensitized sheep erythrocytes as target cells, respectively ('t Hart et al., 1988). For the CP assays, all reagents were diluted in VS buffer; for AP assays, the EGTA–VS buffer was used.

For determining the anticomplementary activity of the different fractions, 0.3 ml of the appropriate HPS dilution was incubated for 30 min at 37°C with two-fold dilutions (0.3 ml each) of the appropriate fraction in VS buffer or EGTA–VS buffer. Then, 0.15 ml of the target cell suspension (1% v/v in the appropriate buffer) was added to every mixture, incubating them at 37°C for 60 or 30 min (for CP or AP assays, respectively). After centrifuging the mixtures for 10 min at 2000 rpm, the *A* of the supernatants (I) was measured at 542 nm. Percent of resultant hemolysis was calculated according to the following formula: % hemolysis = $(\mathrm{I} - \mathrm{II}/\mathrm{III} - \mathrm{IV}) \times 100$, where II refers to the *A* of a similarly prepared supernatant but using heat-inactivated (56°C, 30 min) serum. Because the extract was not colorless, this control (II) was done for each extract dilution tested. III refers to the *A* of water-lysed erythrocytes (100% hemolysis control) and IV to the *A* of supernatants of erythrocytes incubated with buffer (0% hemolysis control).

As a positive control on both CP and AP pathways, different concentrations (0.30–20 mg ml^{-1}) of heparin were employed (Klerx, Van Dijk, Van der Madden, & Willers, 1985). The results of anticomplementary activity for heparin and products CPF, NB and NB2 were compared by a two factors Anova with replications for $n = 4$ (Steel & Torrie, 1985).

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