Medicinal use of *Cochlospermum tinctorium* in Mali

Anti-ulcer-, radical scavenging- and immunomodulating activities of polymers in the aqueous extract of the roots

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**Abstract**

*Cochlospermum tinctorium* A. Rich. (Cochlospermaceae) is a widely used medicinal plant in the West African country, Mali. An ethnopharmacological survey was conducted and 106 traditional practitioners interviewed. The roots were the part of the plant reported to be the most frequently used for medicinal purposes. The main indications were to treat jaundice (41), gastrointestinal diseases or ailments (28), malaria (12), schistosomiasis (10) and dysurea (6). A high-molecular weight water extract (25, 50 and 100 mg/kg, body weight) significantly inhibited HCl/ethanol-induced gastric lesions in mice. The extract showed DPPH-radical scavenging- and immunomodulating activities in vitro. The main components of the extract were identified as polysaccharides (59.3%) and polyphenols (9.3%). The polysaccharides were purified and characterised as highly complex pectic arabinogalactans type II. As parts of the polyphenol compounds gallotannins and ferulic acids were identified. This study shows that the polysaccharides are partly responsible for the bioactivities observed in vitro. Both polysaccharides and polyphenols may be responsible for the anti-ulcer activities observed.

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**Keywords**: *Cochlospermum tinctorium*; Cochlospermaceae; Anti-ulcer; Radical scavenging; Immunomodulation; Polyphenols; Bioactive polysaccharides; Pectic arabinogalactan; Mali

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**1. Introduction**

*Cochlospermum tinctorium* A. Rich. is a plant of widespread occurrence in the savannah and scrub land throughout the drier parts of the West African Region. The plant is reported to be used as traditional medicine in Ivory Coast, Ghana, Cameroon, Nigeria, Gambia, Guinea, Senegal (Burkill, 1985) and Burkina Faso (Ballin et al., 2002). The roots are the most frequently used part of the plant and are used in the treatment of many illnesses. The plant has been widely used in the treatment of malaria and jaundice or liverish fevers and the research on the roots has so far been focusing on these diseases. Antiplasmodial effects of extracts have been investigated (Benoit et al., 1995; Benoit-Vical et al., 1995; Benoit-Vical et al., 1995).
The powdered roots (413 g) of Cochlospermum tinctorium were extracted with ethanol (96%, v/v) using a soxhlet apparatus in order to remove low molecular weight constituents. When no more coloured material could be observed in the ethanol extract the procedure was ceased. The residue was extracted twice with water (4 l) at 50 °C for 2 h, filtered through gauze and Whatman GF/A glass fibre filter, concentrated at 40 °C in vacuum and dialysed at cut-off 3500 Da to give a 50 °C crude extract (yield 6.3 g, 1.5%). The extract was kept at −18 °C or lyophilized.

2.4. Ion-exchange chromatography of the crude extract (C₅₀)

The crude extract was filtered through Acro 50A 0.45 μm filter and applied to a DEAE-Sepharose fast flow column. To obtain a neutral fraction, the column was first eluted with water. The acidic fractions were obtained by elution of a linear NaCl gradient (0–1.4 M) in water. The carbohydrate elution profile was determined using the phenol-sulphuric acid method (Dubois et al., 1956) modified to be read by a microplate spectrophotometer (BioRad™ 3550). Finally, two column volumes of a 2 M sodium chloride solution in water were eluted to obtain the most acidic polysaccharide fraction. The relevant fractions based on the carbohydrate profile were pooled, dialysed and lyophilized.

2.5. Carbohydrates

2.5.1. Quantitative determination of carbohydrate composition and content by methanolysis and gas chromatography (GC)

Samples were methanolysed by 4 M HCl in anhydrous methanol at 80 °C for 24 h followed by trimethylsilylation as described by Chambers and Clamp (1971), with the modifications described by Samuelsen et al. (1995). Mannitol as an internal standard was included throughout the total procedure.

2.5.2. Precipitation of type II arabinogalactans by the Yariv (β-glucosyl) reagent

The presence of type II arabinogalactans was detected by single radial diffusion in an agarose gel, containing β-glucosyl-Yariv reagent, which specifically interacts with and precipitates type II arabinogalactans as described by van Holst and Clarke (1985). A positive reaction was identified by a reddish circle (halo) around the well. Yariv β-glucosyl reagent
jector temperature was 250 °C (30 mFisons GC 8065 on a SPB-1 fused silica capillary column). Partially methylated alditol acetates were analysed by GC–MS acetylated as described by Kim and Carpita (1992). The glycosidic linkages were hydrolysed and the partially methylated carbohydrates were de-protonated and methylated, then used. In the methylation procedure, free hydroxyl groups in the glycosyl residues in GC–MS, sodium borodeuteride was used as methylating agent at each peak was characterized by an interpretation of the characteristic mass spectra and retention times in relation to the standard sugar derivatives.

2.5.3. Determination of molecular weights

The approximate sizes of the polysaccharide fractions isolated were determined by gel filtration on a Superose 6 column coupled to a FPLC system (Pharmacia, Sweden), by elution with 10 mM NaCl solution at a flow rate of 30 ml/h. Fractions of 0.5 ml were collected by a Frac-100 fraction collector (Pharmacia, Sweden) and the eluent was monitored with a Shimadzu Refractive Index detector 10A (Shimadzu Corporation, Japan). The phenol–sulphuric acid test was also used to determine the elution profile of the polysaccharides. Dextran polymers (Pharmacia) 2000, 475, 223, 98, 16, 12 and 6 kDa were used as calibration standards.

2.5.4. Determination of the linkages present in the polysaccharides by gas-chromatography mass-spectrometry (GC–MS)

Prior to methylation, samples, containing uronic acids, were reduced to primary alcohols on the polymer level. To distinguish between reduced uronic acids and the corresponding neutral sugars in GC–MS, sodium borodeuteride was used. In the methylation procedure, free hydroxyl groups in the carbohydrates were de-protonated and methylated, then the glycosidic linkages were hydrolysed and the partially methylated monosaccharides were isolated to alditols and acetylated as described by Kim and Carptia (1992). The partially methylated alditol acetates were analysed by GC–MS Fisons GC 8065 on 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 was 300 °C and the column temperature was 80 °C when injected, then increased with 30 °C/min–170 °C, followed by 0.5 °C/min–200 °C and then 30 °C/min–300 °C. The compound at each peak was characterized by an interpretation of the characteristic mass spectra and retention times in relation to the standard sugar derivatives.

2.6. Polyphenols

2.6.1. Quantitative determination of total amounts of phenolic compounds

2.6.1.1. The Folin–Ciocalteu assay. The amount of total phenolics was determined according to the Folin–Ciocalteu assay (Singleton and Rossi, 1965). Four hundred microliters of the lyophilized samples dissolved in water (three replicates) was added the same amount of Folin–Ciocalteu’s phenol reagent (1:2 in water, Merck,Rehovot), mixed and left for 3 min at room temperature. Four hundred microliters of 1 M Na2CO3 was added, the tubes were mixed and allowed to stand for 1 h. The absorbance was measured at 750 nm in a 4049 NovaSpec spectrophotometer (LKB Biochrom). The standard curve was plotted using ferulic acid. The total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

2.6.2. Identification of the phenolic components (tannins and ferulic acid)

2.6.2.1. Tannins. The ability of tannins to precipitate water-soluble proteins is the main basal activity by which various naturally occurring polyphenols are defined as tannins (Okuda et al., 1985). One ml extract (5 mg/ml) was filtered (5 μm, PVDF Durapore, Millipore®-SV), added a few drops of 1% gelatine—10% sodium chloride reagent and precipitation was taken as an indication for the presence of tannins. Hot water extracts from Quercus robur cortex and Areca catechu semen were used as positive controls. One ml extract without addition of the gelatine-salt reagent was used as control. The presence of tannins was confirmed by the black colour produced with ferric chloride solution (FeCl3, 2%) (Persin and Quinn, 1967).

2.6.2.2. Condensed tannins. Oxidative depolymerisation of the sample (Ct50) was carried out as described by Porter et al. (1986), using HCl in n-butanol (5:95), heated at 100 °C for 60 min. After depolymerisation, the sample was assayed for absorbance at 550 nm, which was used for the estimation of the proanthocyanidin content (Mathisen et al., 2002).

2.6.2.3. Hydrolysable tannins (gallic acid). O-1-Galloyl-β-glucopyranosyl bonds may be selectively cleaved by trifluoroacetic acid (TFA) (Okuda et al., 1982). The sample (Ct50) was hydrolysed using 2.5 M TFA, heated at 100 °C for 2 h. Gallic acid was identified by TLC (Si gel 60 F254, 0.2 mm thickness, Merck) using ethyl acetate-formic acid-acetic acid–water (100:11:11:27) as eluent. Spots were visualised by UV irradiation (254 and 366 nm) or and by spraying with a methanolic DPPH solution of a concentration sufficient to give a violet colour to the plate (approx. 0.4 mg/ml). Radical scavengers were visible as yellow spots (Glavind and Hølmer, 1967). Identification of gallic acid was confirmed by two-dimensional TLC (Polygram Cel 300 UV254, 0.1 mm Celulose MN300, Macherey-Nagel Co.) using glacial acetic acid–water (2:98, solvent 1) and butan-1-ol-glacial acetic acid–water (60:15:25, solvent 2) as eluents. Spots were visualised by spraying with a saturated solution of potassium iodate in distilled water (Makkar, 2003). Gallic acid was used as reference.

2.6.2.4. Ferulic acid. Ferulic acid is the most commonly covalently linked insoluble phenolic compound, which may be esterified to arabinose or galactose in pectins in plant cell walls. Ferulic acid may have an important role in the cross-linking of pectins and also forms sites for lignification (Brett and Waldron, 1996). The sample (Ct50) was saponified with minor modifications, by 1 M NaOH at room temperature under argon over night in the dark, followed by neutralisation with 2 M HCl. Phenolic compounds were extracted with ether. The ether phase was evaporated at 40 °C and the phenolics were dissolved in methanol and identified by TLC (Si gel 60 F254, 0.2 mm thickness, Merck) using chloroform–ethyl


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acetate–formic acid (50:50:1) as eluent. The spots were detected by UV irradiation (254 and 366 nm) and spraying with 1% ferric chloride in methanol (Sharma et al., 1998). Ferulic acid was used as reference.

2.7. Proteins

2.7.1. Quantitative determination of protein content: modified Lowry assay

As phenols are known to interfere with the Lowry protein determination, the modified assay described by Bensadoun and Weinstein (1976) was employed. The proteins were separated from the interfering materials by precipitation using sodium deoxycholate (2%) and trichloroacetic acid (24%). The protein levels were then estimated quantitatively by the standard Lowry procedure employing the Folin–Cöocalteu's phenol reagent (Merck) (Lowry et al., 1951). Bovine serum albumin (BSA) was used as reference.

2.8. Radical scavenging activity

2.8.1. Qualitative determination of radical scavenging activity on TLC plates

The samples (0.2 mg in 10 μl) were applied on a TLC plate (Sil gel 60 F 254, 0.2 mm thickness, Merck) and sprayed with 1% ferric chloride in methanol (Sharma et al., 1998). Ferulic acid was used as mobile phase.

2.8.2. Quantitative determination of radical scavenging activity (Malterud et al., 1993)

To a solution of DPPH (1,1-diphenyl-2-picryl-hydrazyl) (DPPH) solution of a concentration sufficient to give a violet colour to the plate (approx. 0.4 mg/ml). Radical scavengers were visible as yellow spots (Glavind and Hølmer, 1967). For elution ethyl acetate–formic acid–water (50:50:1) was used as mobile phase.

2.8.3. Quantitative determination of radical scavenging activity (modified assay)

As phenols are known to interfere with the Lowry protein determination, the modified assay described by Bensadoun and Weinstein (1976) was employed. The proteins were separated from the interfering materials by precipitation using sodium deoxycholate (2%) and trichloroacetic acid (24%). The protein levels were then estimated quantitatively by the standard Lowry procedure employing the Folin–Cöocalteu's phenol reagent (Merck) (Lowry et al., 1951). Bovine serum albumin (BSA) was used as reference.

2.9. Pharmacological evaluation

2.9.1. Animals

Specific-pathogen-free C3H/HeJ female mice (LPS low-sensitive, for in vitro assays) and male ICR mice (for in vivo assays) were purchased from SLC (Shizouka, Japan) and used at 6–8 weeks of age. The mice were maintained under specific pathogen-free conditions and given free access to standard laboratory chow (CE-2, CLEA Inc., Japan) and water.

For the in vivo assay the animals were used for the experiment after 18–20 h of fasting, but with free access to water. The procedure from the Prime Minister’s Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals was followed. The experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation of the Kitasato Institute.

2.9.2. Induction of ulcerogenesis in mice (Mizui and Doteuchi, 1983; Yamada et al., 1991)

After fasting, the mice were administered test drugs (Cs 50 or 16,16-dimethyl prostaglandin E 2, 16-dmPGE 2 purchased from Caymen Chemical Co., MI) or water orally. One hour later, each mouse received 0.24 ml 0.3 M HCl-60% ethanol orally. Each animal was killed by cervical vertebral dislocation 1 h after administration of the necrotizing agent. The stomachs were excised and inflated by injection of saline (2 ml) and fixed in 5% formalin for 30 min. After opening along the greater curvature, the lengths (mm) of the gastric lesions were measured and the lesion index was expressed as the sum of the length of all lesions in each mouse.

2.9.3. Preparation of immune cells from spleen

The C3H/HeJ mice were sacrificed by cervical amputation under light anaesthesia and spleens were removed using aseptic techniques. The spleens were passed through a sterilized stainless sieve (150 mesh) to obtain a single-cell suspension. Erythrocytes were destroyed by 0.85% NH₄Cl/20 mM Tris–HCl (pH 7.5) and the remaining cells were washed twice with 2% fetal bovine serum (FBS, Cell Culture Laboratories, USA) in Dulbecco’s phosphate buffered saline (PBS, Sigma, USA). The cells were resuspended in RPMI 1640 medium supplemented with 5% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5 × 10⁻⁵ M 2-mercaptoethanol (RPMI 1640-FBS) at a density of 2.0 × 10⁶ cells/ml.

2.9.4. Measurement of the proliferation of the cells

2.9.4.1. Cell growth was measured by means of a fluorometric assay, the Alamar Blue™ reduction assay (Page et al., 1993; Sakurai et al., 1999). Spleen cells (2 × 10⁶ cells/ml) were cultured with samples (10 or 100 μg/ml), concanavalin A (ConA, 5 μg/ml, Sigma, USA), bupleuran 2Î²c (BR2Î²c, 100 μg/ml, Yamada et al., 1989; Sun et al., 1991) or anti-IgM F(ab)2 (10 μg/ml, Jackson ImmunoResearch Laboratories Inc., USA) in 96-well plates (Falcon 3072, Becton Dickinson, USA) for 3 days at 37°C under humidified atmosphere of 5% CO₂-95% air in a CO₂ incubator. Four hours before culture termination 20 μl Alamar Blue™ (Alamar Bio-Science Inc., USA) was added to each well. The fluorescence intensity was measured by Fluoroscan II (Labosystems Oy, Finland), at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The delta soft II (Version 4.13 FL, BioMetallics Inc., USA) was used for data management.
2.9.4.2. Selective measurement of B cell proliferation in the spleen cell suspension was measured by the alkaline phosphatase assay (APase assay) (Hashimoto and Zubler, 1986; Feldbush and Lafrenz, 1991). Spleen cells from C3H/HeJ mice (2.0 × 10⁶ cells/ml) were cultured with samples (10 or 100 µg/ml) or bupleuran 2IIc (BR2IIc, 100 µg/ml, Yamada et al., 1989; Sun et al., 1991) in 96-well plates for 3 days at 37°C under humidified atmosphere of 5% CO₂-95% air in a CO₂ incubator. Cell cultures were terminated by spinning the plates at 400 × g for 5 min. The culture medium was removed by aspiration. Thereafter, 50 µl 1% Triton X-100 and 150 µl 1 mg/ml p-nitrophenyl phosphate disodium salt in 0.1 M diethanolamine buffer (pH 9.5) was added to each well. The reaction was terminated by adding 50 µl 1 M NaOH to each well and the optical density at 405 nm was measured using a microplate reader (BioRad™, Model 250, Nippon BioRad™, Japan).

2.9.5. Interaction with human complement: complement fixation assay

The complement fixation test is an in vitro test for the ability of the samples to interact with the complement cascade reaction. The method is described by Michaelsen et al. (1991). Briefly, serum with human complement is incubated with the samples (dissolved in veronal buffer pH 7.2, containing 2 mg/ml BSA and 0.02% sodium azide, VB/BSA) for 30 min before sensitised sheep erythrocytes are added. After another incubation for 30 min the remaining erythrocytes were removed by aspiration. Thereafter, 50 µl 1 mg/ml p-nitrophenyl phosphate disodium salt in 0.1 M diethanolamine buffer (pH 9.5) was added to each well. The reaction was terminated by adding 50 µl 1 M NaOH to each well and the optical density at 405 nm was measured using a microplate reader (BioRad™, Model 250, Nippon BioRad™, Japan).

3. Results

3.1. Ethnomedical information

In our study, 106 traditional healers were interviewed about their use of Cochlospermum tinctorium in traditional medicine in Mali (Fig. 1). Of the 62 healers interviewed in Dogonland, 23 did not use the plant medicinally, while the corresponding number for the southern region was only three healers. The healers reported 39 different indications for the use of the plant, but only the most commonly used indications are reported here. Of the healers using the plant medicinally 46% (Dogonland) and 56% (southern region) used it in the treatment of jaundice, which was the main indication.

In the southern part of the study areas, the following local names of the plant, in Bambara language, were given by the healers: Nitirab, Nidiibara, Tiilba or Tiilibara. In Dogonland, there are many local dialects in the communities, and the following local names that were reported there are given with the dialect(s) in brackets: Yansinginuun (Tingso), Yaanseggere (Dono soo), Yaansenjer (Tenju-kan), Yensent (Tomoso), Yensentjinrin (Tenju soo) and Solu anji (Doguloso, Dogolonso, Dongoso, Tommo soo, Donno soo, Toro soo). The roots were the parts of the plant most frequently used (95%), while leaves were used by a minority of the healers for the treatment of malaria (3), ulcer (1) and flatulence (1). Flowers were used by one healer in the treatment of constipation.

After collection the roots are washed and the outer part is removed. The remaining of the roots is normally cut into small pieces, dried and finally, ground in a mortar into a very fine powder. The powder is usually stored in a plastic vessel. The most common preparation of the drug is a decoction in water administered per orally. In the treatment of jaundice a decoction is also often prepared for washing/bathing the patient. The powder may in some cases also be added to meals, e.g. porridge.

3.2. Extraction and fractionation by anion-exchange chromatography

The yield of polymeric material (Ct50), obtained after extraction with water at 50°C was 1.5%, based on dried, powdered roots of Cochlospermum tinctorium. Anion-exchange
chromatography of the crude extract gave four fractions: a neutral fraction (Ct50N, 0.01%) and three acidic fractions (Ct50A1, 0.24%; Ct50A2, 0.09%; and Ct50A3, 0.07%). The yields given in brackets are based on the dried, powdered roots. The elution pattern of the acidic polymers is given in Fig. 2.

3.3. Carbohydrates

3.3.1. Carbohydrate composition and content

Methanolysis and gas chromatography revealed relatively high amounts of polysaccharides in the fractions isolated (69.5–101.3%). The neutral fraction (Ct50N) consisted
Table 1
Characterisation of the crude extract, Ct50 and the polysaccharide fractions, Ct50N, Ct50A1, Ct50A2 and Ct50A3, obtained after ion-exchange chromatography of Ct50

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ct50</th>
<th>Ct50N</th>
<th>Ct50A1</th>
<th>Ct50A2</th>
<th>Ct50A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Carbohydrate (%)</td>
<td>59.3</td>
<td>76.8</td>
<td>101.3</td>
<td>76.6</td>
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</tr>
<tr>
<td>Phenols (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.3</td>
<td>n.d.</td>
<td>trace</td>
<td>trace</td>
<td>2.2</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>0.9</td>
<td>n.d.</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
</tbody>
</table>

| Monosaccharide composition (mol%) | | | | |
| Arabinose | 4.7 | 12.6 | 6.7 | 4.4 | 3.7 |
| Rhamnose | 19.2 | 3.1 | 16.4 | 28.9 | 29.0 |
| Xylose | trace | 1.2 | 1.1 | 0.6 | trace |
| Mannose | trace | 0.9 | trace | trace | trace |
| Galactose | 24.7 | 32.5 | 53.7 | 25.5 | 22.4 |
| Glucuronic acid | 9.5 | -- | 12.7 | 8.3 | 9.1 |
| Galacturonic acid | 21.5 | -- | 8.3 | 30.7 | 31.2 |

| Presence of AGII<sup>b</sup> | + | + | + | + | + |

<sup>a</sup> The total phenolic content is expressed as ferulic acid equivalents (FA/Sample) × 100%.

<sup>b</sup> The presence of arabinogalactans type II (AGII) was identified by precipitation with the <sup>/H9252</sup>-glycosyl Yariv reagent.

Table 1 shows the characterisation of the crude extract and the polysaccharide fractions obtained after ion-exchange chromatography. The main carbohydrates in Ct50 are glucose (49.7%), galactose (32.5%), and arabinose (12.6%). The acidic fractions (Ct50A1, Ct50A2, and Ct50A3) contain high amounts of uronic acids (both galacturonic acid and glucuronic acid), rhamnose, and galactose. The monosaccharide composition and total carbohydrate content of the crude polymer extract and the fractions obtained after ion-exchange chromatography are presented in Table 1.

### 3.3.2. Precipitation of type II arabinogalactans by the Yariv β-glucosyl reagent

In all fractions isolated (Ct50N, Ct50A1, Ct50A2, and Ct50A3) the occurrence of type II arabinogalactans (AGII) were identified by the Yariv-test (Table 1).

### 3.3.3. Molecular weights

The approximate molecular weights of the acidic fractions were determined by comparing their elution patterns with that of dextrans of known molecular weights, using a Superose 6 column (Fig. 3). All fractions were relatively polydisperse regarding molecular weights, but the least acidic fraction (Ct50A1) contained mainly polysaccharides of lower molecular weights (in the range of 16–12 kDa) compared with those present in the more acidic fractions (Ct50A2 and Ct50A3). The molecular weight profiles of the two most acidic fractions (Ct50A2 and Ct50A3) were quite similar, with a major amount of polymers eluted in the void volume, representing a MW over 475 kDa.

![Fig. 3. Elution profiles of the polysaccharides of Ct50N, Ct50A1, Ct50A2 and Ct50A3 in gelfiltration on Superose 6 (Pharmacia, Sweden). The approximate sizes were determined by comparing with the elution profiles of dextran polymers of known molecular weight (2000, 475, 233, 98, 16, 12 and 6 kDa, Pharmacia, Sweden). Vo, void volume; Vt, total volume.](image-url)

Fig. 3. Elution profiles of the polysaccharides of Ct50N, Ct50A1, Ct50A2 and Ct50A3 in gelfiltration on Superose 6 (Pharmacia, Sweden). The approximate sizes were determined by comparing with the elution profiles of dextran polymers of known molecular weight (2000, 475, 233, 98, 16, 12 and 6 kDa, Pharmacia, Sweden). Vo, void volume; Vt, total volume.

### 3.3.4. Linkage analysis of the polysaccharides

The linkage analysis revealed the polysaccharides to be of a highly complex nature (Table 2). The neutral fraction (Ct50N) contained mainly 1,4-linked Glc (43.4%), but also terminally linked (4.2%) and 1,4,6-branched Glc (2.1%). Ct50N also contained large amounts of Gal with 1,6-(22.4%) linkages as the major type of linkage, in addition to 1,3,6-(4.6%), 1,3-(2.5%) and 1,3,4-(0.6%) linkages. Terminal Ara in both furanose (4.5%) and pyranose (1.4%) form was present, while the interlinkage present in the chain, mainly 1,5-linked Ara (4.7%), were in the furanose form. Smaller amounts of Rha (terminally-linked), Fuc (terminally linked), Xyl (terminally- and 1,2-linked) and Man were also present in the neutral fraction.

Ct50A1 was a Gal rich polymer with 1,6-linkage as the predominant linkage (29.0%) followed by 1,3,6-(16.1%) linkages.

![Diagram](image-url)
Table 2

<table>
<thead>
<tr>
<th>Glycosyl residue</th>
<th>Deduced linkage</th>
<th>Cu50N (mol%)</th>
<th>Cu50A1 (mol%)</th>
<th>Cu50A2 (mol%)</th>
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The total mol% of each monosaccharide was determined by methanolysis and GC.

* The linkage was not deduced.

3.4. Polyphenols

3.4.1. Quantitative determination of total phenolic compounds

The crude polysaccharide extract contained 9.3% ± 0.2 phenolic compounds expressed as ferulic acid equivalents. Fractionation and purification of the polysaccharides by anion exchange resulted in purified polysaccharide fractions with lower amount of phenolics than in the crude extract. The least acidic fractions, Cu50A1 and Cu50A2, contained trace amounts of phenolics only, while Cu50A3, which was a slightly coloured fraction, contained 2.2% ± 0.1 (Table 1).

3.4.2. Identification of phenolic components in the crude extract (Cu50)

No precipitation of tannins could be observed by treatment of the crude extract with the gelatine-salt reagent, but the extract did reduce FeCl₃ to give a black/brownish colour.

An UV-scan of the extract in water showed an absorbance at 244–300 nm, which confirmed the presence of phenolic substances in the extract. After oxidative depolymerisation of the extract, no absorbance at 550 nm, characteristic for condensed tannins (proanthocyanidins), could be observed. Gallic acid, a typical component of hydrolysable tannins, was identified in the extract after acid hydrolysis with TFA by separation on TLC and detection by spraying with a DPPH-solution. The result was confirmed by separation on two-dimensional TLC, which was sprayed with a saturated KIO₃-solution.

The extract was saponified and ferulic acid was identified by spots on the TLC by UV-irradiation and by spraying with a ferric chloride-solution.

After TLC, a brownish spot (acid- and base-stable polymer) could still be observed on baseline for both the acid- and base-hydrolysed sample.

3.5. Proteins

3.5.1. Quantitative determination of protein content

According to the modified Lowry method, the crude extract (Cu50) contained 0.9% ± 0.1 protein, while the purified polysaccharide fractions contained trace amounts (<0.2%) only (Table 1).

3.6. Radical scavenging activity

The crude extract (Cu50) and the most acidic fraction (Cu50A3) clearly showed radical scavenging activities demonstrated on the TLC-plate sprayed with DPPH-solution as yellow spots on the violet background. Paler spots, indicating lower radical scavenging activity, could be observed for the fractions Cu50A2 and Cu50A1. No spots were observed above baseline after the elution was terminated.

Quantitative determination of radical scavenging activities was performed and the crude extract showed dose-dependent radical scavenging activity (Fig. 4). In order to
obtain detectable radical scavenging activities for the acidic fractions, high concentrations of the samples had to be used. Ct50A1 showed low radical scavenging activity (4.1%) even at the highest concentration tested (340 μg/ml). Ct50A2 also showed low activity although it seemed to be dose-dependent, while Ct50A3 clearly demonstrated the highest radical scavenging activity of the acidic fractions, which also seemed to be dose-dependent (Table 3). The positive control quercetin showed 50% radical scavenging activity at 10.8 ± 0.4 μM.

3.7. Pharmacological evaluation

3.7.1. Anti-ulcer activity of the crude polysaccharide extract in mice

The oral administration with 25, 50 and 100 mg/kg of the crude extract (Ct50) 1 h before the HCl/ethanol treatment significantly reduced the occurrence of mucosal gastric lesions in a dose-dependent manner (Table 4).

3.7.2. Mitogenic activity on mice spleen cells in vitro

At the highest concentrations tested (100 μg/ml), both the crude extract and all the fractions isolated showed modest, compared with positive control, but significant mitogenic activity on mice spleen cells in vitro (Fig. 5). The increase in alkaline phosphatase activity indicated the fractions to stimulate the proliferation of B cells (Fig. 6). The acidic polysaccharide fractions showed a dose-dependent activity, whereas, the increase in dose did not increase the mitogenic activity of the crude extract.

3.7.3. Effect on human complement in vitro

The crude extract showed relatively high and dose-dependent complement fixation activity, whereas, although dose-dependent, the activities of the purified polysaccharide fractions were modest compared with the positive control PMII (Fig. 7).

4. Discussion

The ethnopharmacological survey was conducted in two very distinct areas in Mali (Fig. 1), and the use of the plant varied with respect to them. There was a large discrepancy in the share of healers using the plant medicinally in Dogonland and the southern part. Several of the healers in Dogonland explained the lack of use of Cochlospermum tinctorium by the...
Fig. 5. Mitogenic activity of Ct50, Ct50N, Ct50A1, Ct50A2 and Ct50A3 on spleen cells. Spleen cells (2 × 10^6 cells/ml) from C3H/HeJ mice were cultured with the samples (10 or 100 μg/ml) for 3 days in an incubator with humidified 5% CO_2–95% air. 4 h prior to culture termination, Alamar Blue™ solution was added to each well. The relative fluorescence intensities were measured with an excitation wavelength at 544 nm and emission wavelength at 590 nm as described in Section 2. Concanavalin A (ConA, 5 μg/ml), anti-IgM F(ab′)2 (anti-IgM, 10 μg/ml) and bupleuran 2IIc (BR2IIc, 100 μg/ml) were used as positive controls. Each value is presented as mean ± S.D. ANOVA followed by Fisher’s PLSD. Asterisks indicate significancy (*P < 0.001, **P < 0.05).

According to our ethnopharmacological study, the use of Cochlospermum tinctorium in the treatment of gastrointestinal diseases and ailments was equally distributed in the two areas. The roots have, previously been reported to be used against stomach pains and for indigestion in Ivory Coast and Burkina Faso (Burkill, 1985), but to the authors’ knowledge no investigations have been performed in order to evaluate a possible beneficial effect of Cochlospermum tinctorium related to gastrointestinal diseases or ailments. In our study, the polysaccharide enriched extract (Ct50) from the roots of Cochlospermum tinctorium significantly reduced the formation of gastric ulcer induced by HCl/ethanol in mice (Table 4). A dose-dependent response on the intensity of gastric ulceration was noted. The HCl/ethanol-induced ulcer model is commonly used for screening of anti-ulcer agents, but although extensive studies on the pathophysiology of the acute gastric mucosal lesions have been carried out, the pathogenesis of the mucosal lesions is not fully understood (Yoshikawa et al., 1990). Possible mechanisms for anti-ulcer effects suggested are anti-secretory activity on pepsin and acid, mucosal protection by increased mucus synthesis, prostaglandin level (PGE-2), protective coating and radical scavenging (Matsumoto et al., 1995; Yamada, 1994). The crude extract showed dose-dependent radical scavenging activity (Fig. 4), which may at least in part be responsible for the anti-ulcer activity of the

poor accessibility of the plant in the area. Before the plant was abundant in this region, and some healers explained the eradication by the lack of rain and/or by domestic animals eating the root as a nutrient. In this study, the majority of healers (51%) used the roots from Cochlospermum tinctorium for the treatment of jaundice, which is the indication together with the treatment of malaria, which have been the most frequently cited and investigated in the literature (Diallo et al., 1987, 1992; Benoit et al., 1995; Benoit-Vical et al., 1999, 2001; Ballin et al., 2002). The reason(s) why no healers in Dogonland reported the plant to be used against malaria, and why the treatment of schistosomiasis by this plant is almost exclusively located in Dogonland is not known, but an explanation may be the existence of other local plants efficiently used for the treatment of the respective diseases. Previously, the use of the roots in the treatment of schistosomiasis has been reported in Ivory Coast and Burkina Faso (Burkill, 1985). The reported use against dysuria may also be related to schistosomiasis, as urinary obstruction is a common feature in patients suffering from this disease, which often occurs in Sub-Saharan Africa (WHO/TDR, 2002). Schistosomiasis ranks second behind malaria in terms of socio-economic and public health importance in tropical and subtropic areas (WHO, 1996), and further investigation of Cochlospermum tinctorium for such activities may therefore, be of interest.
Fig. 6. Mitogenic activity of Ct50, Ct50N, Ct50A1, Ct50A2 and Ct50A3 on B cells. Spleen cells (2 × 10^6 cells/ml) from C3H/HeJ mice were cultured with the samples (10 or 100 μg/ml) for 3 days. Mitogenic activity of B cells was measured using alkaline phosphatase assay as described in Section 2. Bupleuran 28c (BR28c, 100 μg/ml) was used as positive control. Each value is presented as mean ± S.D. ANOVA followed by Fisher’s PLSD. Asterisks indicate significance (*) P < 0.001 compared to control (water).

Fig. 7. The ability of the samples to consume human complement was measured by the inhibition of sheep red blood cell lysis (% inhibition of lysis). A pectin fraction, PMII, from the leaves of Plantago major L. (Samuelsen et al., 1995) was used as a positive control.


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Phenols (Table 1), greatly decreased the DPPH-radical scavenging activity (Table 3). Tannins are high-molecular weight polyphenolics that are present in many plant foods (Chung et al., 1998), but although, a black colour was produced in the extract by adding a ferric chloride solution, no precipitation of protein could be observed when a gelatine-salt reagent was added to the extract. The precipitation of proteins is the main basal activity by which naturally occurring polyphenolics are defined as tannins (Okuda et al., 1985), however, due to the high content of pectin-like polysaccharides in the extract the precipitation of proteins may have been inhibited by the formation of a strong tannin-pectin complex. This phenomenon has been reported previously (Taira et al., 1997). The formation of this complex is favoured when tannins are of higher molecular weights (MW > 3000 Da), and may be formed in vivo or during the course of extraction (Swain, 1965). The formation of such complexes may explain the low yield of polysaccharides obtained after purification by the ion-exchange chromatography (approx. 40%). In higher plants, tannins consist of two major groups of metabolites: the hydrolysable and condensed tannins or proanthocyanidins. Both the hydrolysable and condensed tannins are widely distributed in nature. In many species, both classes are present together, but generally one or the other predominates, at least in any given part of the plant (Swain, 1965). A lack of absorbance at 550 nm after oxidative depolymerisation of Ct50 did not indicate the presence of condensed tannins. However, after TFA-hydrolysis, selective cleavage of O-1-galloyl bounds, gallic acid could be identified after separation by TLC, indicating the presence of hydrolysable tannins (gallo-tannins) in Ct50. The gallo-tannins are polymers of a sugar or related polyhydric alcohol and gallic acids, and an increase in the degree of condensation by self-esterification between gallic acid units may occur. In complex hydrolysable tannins, one or more oxidation/hydration steps may occur to form molecules of large molecular weights, which seems to be present in the Ct50 extract. Recently, purified tannins from Quercus suber have been reported to have antioxidant activity (Khennouf et al., 2003). The ferulic acids, which were also the phenolic acids identified in the crude extract, may be esterified to arabinose and galactose in pectic-polysaccharides (Brett and Waldron, 1996), and may thereby, constitute in the purified acidic polysaccharide fractions. Ferulic acid showed DPPH-scavenging activity, and may therefore, be responsible part of the molecules for the scavenging activity noted in the acidic fractions. However, polysaccharides may also to some extent possess radical scavenging activities, such as the anti-ulcer pestic polysaccharide, bupleuran 2IIc, which has been reported to scavenge hydroxyl radicals effectively (Matsumoto et al., 1993). The polysaccharides of the most acidic fractions, C50A2 and C50A3, were very similar in monosaccharide composition, structural features (Table 2) and molecular weight profiles (Fig. 3), indicating the presence of almost identical polysaccharides, and the fact that the radical scavenging activity of C50A3 seems much higher than that of C50A2 (Table 3) indicate that the polysaccharides are not the main compounds responsible for this activity.

Another possible mechanism for the anti-ulcer activity of the Ct50 extract may be the polysaccharides' ability to bind to the surface mucosa and function as a protective coating. Diseases of the gastrointestinal system are often related with irritations and pathological changes in mucus membranes, and polysaccharide-containing plants are widely used in traditional therapy of such diseases. Recently, rhamnogalacturonans have been found to adhere significantly to colon mucous membranes, which may lead to a protective coating and shielding of the epithelia, subsequent rehydration and accelerated healing (Schmidgall and Hensel, 2002). The protective coating was also considered to be one of the mechanisms for the anti-ulcer pestic polysaccharide, bupleuran 2IIc (Yamada, 1994).

Anti-ulcer polysaccharides have been isolated from several plants, including Asparagus racemosus (Sairam et al., 2003), Panax ginseng (Sun et al., 1992), Angelica sinensis (Ye et al., 2003) and Bupleurum falcatum (Matsumoto et al., 2002). The most extensively investigated is probably bupleuran 2IIc (BR2IIc) from Bupleurum falcatum. This pectic-polysaccharide has several interesting immunomodulating activities, e.g. immune complex clearance, complement activation (Yamada et al., 1989), selective proliferation, maturation (Sakurai et al., 1999) and enhancement of IL-6 secretion from B cells (Gao et al., 2000). The connection between gastroprotection and the immunomodulating activity of polysaccharides is not known but as dietary fibre is implicated in reducing the number of gastrointestinal and metabolic diseases, their potential immunoregulatory activity may have some bearing on this (Brett and Waldron, 1996). Gastric ulcers may arise due to various factors. The theory that the main factor in ulcer development is chronic gastritis due to long-standing colonization of the mucosa by the Helicobacter pylori is supported by epidemiological studies, but also the ulcerogenic effect of many frequently used medications, such as, e.g. salicylates, is a serious problem, where a net imbalance in the mucosal offensive and defensive factors will occur (Weiss and Fintelmann, 2000).

Since, an infection is the main factor in ulcer development, components with immunomodulating activities may be beneficial for an anti-ulcer treatment. We investigated for this reason the crude extract and the purified polysaccharides from Cochlioporum tinctoriun for mitogenic activity and complement fixation ability. The polysaccharides possessed both mitogenic (Figs. 5 and 6) and complement fixation (Fig. 7) activities, and may therefore, at least partly be responsible for...
the immunomodulating activities of the crude extract. All samples seemed to stimulate alkaline-phosphatase (Fig. 6), which is a selective marker for B cell activation. The samples may either stimulate the B cells directly or indirectly through the stimulation of other immune cells, e.g. T cells or macrophages, in the spleen cell suspension. In contrast to the purified polysaccharide fractions, the crude extract did not show dose-dependent mitogenic activity. The absence of a dose-effect response may be due to the complex composition of the crude extract, and that other compounds (e.g. tannins) may antagonise the effect of the polysaccharides when their dose is increased above a threshold.

The neutral polysaccharide fraction (Ct50N) showed low but significant mitogenic activity only at the highest concentration tested (100 μg/ml). The linkage analysis and a positive reaction with the Yariv reagent, suggest that Ct50N contains arabinogalactan type II. In the literature, arabinogalactans have been reported for their immunomodulating activities (Samuelsen et al., 1998; Yamada and Kiyohara, 1999; Yamada, 2000; Yu et al., 2001), although the most frequently found belong to the pectic arabinogalactans, where the arabinogalactans of type II are linked as side chains to rhamnose units of rhamnogalacturonans (Yamada and Kiyohara, 1999; Paulsen, 2002). In addition to the content of AGII, the monosaccharide analysis revealed Ct50N to be composed of 50% glucose (Table 1). The positive reaction with iodine and the linkage analysis revealing the glucose to be 1,4-linked with a branching in position six for every 20th glucose residue (Table 2), confirmed the glucose to originate from starch. The presence of approx. 50% inactive starch in Ct50N may explain the low-mitogenic activity. Since, the amount of Ct50N obtained was very low compared with the other fractions, removal of the starch for further investigations of Ct50N was not performed.

All the acidic polysaccharide fractions showed moderate but significant and dose-dependent mitogenic activity (Figs. 5 and 6). A very similar immunomodulating capacity, monosaccharide composition (Table 1) and linkages (Table 2) and a quite similar molecular weight profile (Fig. 3) indicated the polysaccharides in Ct50A2 and Ct50A3 to be almost identical. The linkage analysis revealed the polysaccharides to be of a very complex nature possibly with a highly branched rhamnogalacturonan I core with both arabinogalactan type I (1,4-linked Gal) and type II (1,3,6-linked Gal) side chains. The relatively high amount of glucuronic acid, which is both terminally- and 1,4-linked, is a quite unusual feature for pectin-like polysaccharides, and partial degradation and further analysis will be necessary in order to obtain the exact structural features for these highly complex polymers. The trace amounts of proteins assigned may be associated with the arabinogalactan structures of the polysaccharides.

The least acidic fraction, Ct50A1, differs from Ct50A2 and Ct50A3 in both composition and linkages of the monosaccharides (Table 2) in addition to having a profile of lower molecular weights (Fig. 3). Ct50A1 also possesses some mitogenic activity, although it seems lower than that of Ct50A2 and Ct50A3 (Figs. 5 and 6). The dominant monosaccharide in Ct50A1 is galactose, with a high amount 1,6- and 1,3,6-linked, which is typical for arabinogalactan type II structures. The fact that these polymers were eluted in the acidic fractions indicates that the AGII structures also are linked to the acidic polymer. The high amount of terminal rhamnose relative to the 1,2- and 1,2,4-linked is a quite special pattern for Ct50A1 compared to Ct50A2 and Ct50A3, and so is also the high amount of glucuronic acid relative to galacturonic acid.

The complement system is essential for the operation of the innate as well as the adaptive immune defence, and is involved in many biological activities related to inflammatory responses (Yamada and Kiyohara, 1999). The complement fixation assay gives an indication for the interaction of the samples with the complement system, but does not discriminate between activation and inhibition. In the complement fixation test the crude extract showed a higher activity and a clear dose-dependent response compared with the purified polysaccharides (Fig. 7). The activity may therefore, also be dependent on other compound in the extract. The activity of the crude extract may also be false positive due to the tannin content as tannins in high concentrations can interfere with this assay (Wagner et al., 1999). All the polysaccharide fractions possessed modest but dose-dependent complement fixation activity, and are therefore, at least partly responsible for the complement fixation activity of the extract. To avoid false-positive results the LPS content of Ct50A1, Ct50A2 and Ct50A3 was determined and found to be less than 0.03, 0.01 and 0.01%, respectively. At this low level the effect of LPS is negligible.

In this study, we investigated a carbohydrate enriched, high polymeric extract from Cochlospermum tinctorium, which possessed significant, dose-dependent anti-ulcer activity in mice. The radical scavenging activity, which mainly seems to be due to polyphenols, may be partly responsible for the anti-ulcer effect. Highly complex pectin-like polysaccharides with arabinogalactan type II structures with in vitro immunomodulating activities have been isolated and partially characterised. The immunomodulating polysaccharides may be involved in the gastroprotective activity of the extract, and further investigations are now ongoing for structure and structure-activity studies of the polysaccharides isolated.

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References


