

Antibacterial, antifungal, antiviral, biological and immunomodulatory chemical characterization of leaves polysaccharides from *Plantago palmata* Hook plant

RB Singh¹, Ravi Prakash²

¹ Department of Zoology, Dr. Bhimrao Ambedkar University, Khandari Campus, Agra, Uttar Pradesh India

² Department of Chemistry, B.S.A. College, Mathura, Uttar Pradesh, India

Abstract

A previous work on *Plantago palmata* Hook polysaccharides (PS) attributed immunomodulatory properties of leaves to a polysaccharide fraction (PS50) that stimulated NO and TNF- α production by interferon gamma- (IFN- γ -) activated macrophages. The present work aims to elucidate the chemical structure of these antibacterial, antifungal, antiviral, biological and immunomodulatory polysaccharides. Size exclusion chromatography showed that the active polymers present an active fraction with a very high molecular weight (1200 kDa). These polysaccharides are pectic in nature, with a predominantly unbranched galacturonan domain and with a domain bearing side chains that consist of highly branched arabinan, galactan, and or arabinogalactan. Comparatively to the well-known *Plantago major* biologically active PS, *Plantago palmata* PS50 contained less arabinogalactan-proteins (AGPs) and had a different composition in glucose, galactose, and galacturonic acid. DNA contamination of the polysaccharide was estimated at about 0.04%, a concentration much lower than those reported immunomodulatory in hyaluronic acid preparations (3 - 15%). Therefore, the eventuality of a contaminating DNA-mediated biological activity could be ruled out.

Keywords: Antibacterial, antifungal, antiviral, biological, immunomodulatory, Polysaccharides, *Plantago palmata* leaves)

Introduction

Plantago palmata Hook grows in the humid Himalayan regions of Northern India at 1800 - 3000 m height and is widely used in traditional system of medicine. Its leaves are often used as herbal remedies in the treatment of a number of diseases such as wounds, hepatitis, parasitic or infectious diseases, pregnancy and lactation troubles. They are also used for the improvement of health after disease [1]. A survey of the literature indicates a certain convergence between these *Plantago palmata* Hook Plants are medically uses in antibacterial, antifungal, antiviral, biological and immunomodulatory chemical characterization and those of other *Plantago* species [2, 3, 4, 5, 6, 7]. This led us to investigate three groups of *Plantago* metabolites possibly related to these traditional uses and biological activities i.e. iridoids, phenylethanoid glycosides and polysaccharides [8]. We previously showed that *Plantago palmata* leaves contains:

1. Appreciable amounts of iridoids and phenylethanoid glycosides, with aucubin and acteoside as major compounds [1].
2. Present immunomodulatory properties, which could be attributed to polysaccharides (PS): The effects of crude extracts and fractions obtained by high performance liquid chromatography/size exclusion chromatography (SEC) were investigated by the measurement of nitric oxide (NO), tumor necrosis factor- α (TNF- α), and interleukin-10 (IL-10) production using interferon gamma- (IFN- γ -) activated macrophages and nonactivated macrophages as control. IFN- γ -activation of J774 cell line macrophages synergized with a raw 50°C aqueous extract from leaves (PS50) and one of its fractions obtained by SEC (fraction F2) to produce both NO and TNF- α , but not IL-10. Eventual contamination of polysaccharides extracts and fractions by lipopolysaccharide (LPS) was ruled out by showing the

same pattern of response on peritoneal macrophages harvested from C3H/HeJ mice, a strain known to be a very low responder to LPS.

Due to their potential biological activities, such as antioxidant, antiulcer, hepatoprotective, antitumor, and immunostimulatory effects, polysaccharides from natural sources have attracted more attention in recent years [9,10,11]. To the best of our knowledge, primary structures, spatial shapes, and functions of polysaccharides are intimately related. Therefore, as a prerequisite to better understand immunomodulatory activities of *Plantago palmata* polysaccharides, but also to develop parameters for quality control, the present work aims to investigate their primary chemical structures.

Materials and Methods

Plant material and polysaccharides (PS) extraction:

Plantago palmata Hook seeds were harvested in the Department of Zoology, School of life Sciences, Dr. Bhimrao Ambedkar University, Khandari Campus, Agra, U.P. (India). After three months of culture, fresh leaves were collected, immediately immersed into acetone and stored in this solvent for a maximum of three months. The material was washed three times with acetone and the extraction of polysaccharides was performed with some modifications. Briefly, dried and crushed leaves (17.6 g) were extracted by water (1 \times 1000 mL) at 50°C for three hours and filtered. The extract was concentrated to 20 mL at 30°C under vacuum. The residual crushed leaves were further incubated in water at 100°C (1 \times 1000 mL) and similarly treated. Both extracts were dialyzed against distilled water for 48 h at 4°C and lyophilized to yield extracts designated as PS50 and PS100, respectively; filtration on a Millipore cellulose filter (0.22 μ m) yielded

extracts PS50F and PS100F. These extracts were maintained at -18°C . Biological testing of both extracts, before filtration, showed a synergistic effect between IFN- γ and PS50 only; this effect was abolished by the filtration step. For structure comparison purposes, the same extraction was performed on *Plantago major* leaves.

Fractionation of the PS50 crude extract: The polysaccharides crude extract (2mg) were dissolved by swelling in 1 mL of 5 mM NaCl (aq) (24 h), sonicating (2 min) and vortexing (1 min) to obtain an homogeneous solution; 25 μL of this solution were injected in a HPLC Agilent 1100 system equipped with a Agilent 1100 RID detector, and two polyhydroxymethacrylate gel permeation columns (SHODEX OH-pack SB-804-HQ followed by a OH-pack SB-806 M-HQ, 300×8 mm i.d.; exclusion limits, 1000 and 20000 kDa; connected in series with a PWXL 12 μm , 40×6 mm. i.d. guard column. The mobile phase was 5 mM NaCl (aq) at a flow rate of 0.8 mL/min. Three fractions and six subfractions were collected according to the elution volume. After four injections, the collected fractions were pooled and dialysed at 4°C against distilled water for 3 days, renewing the dialysis bath every 24 hours, and dialysates were lyophilized. This size exclusion chromatography yielded a fraction significantly more active than PS50, fraction F2, but the subsequent subfractionations were unsuccessful to concentrate the activity.

Estimation of the molecular weights: After a 24 hours equilibration of the size exclusion chromatography (SEC) system with the mobile phase, a series of calibration standards were injected (5 mg/mL solutions of pullulan P5, P20, P-100, P-200, P-400, P-800 in the mobile phase; 25 μL injection; 2 injections per calibrator), covering a 5.8 to 853 kDa Mw range. The ChemStation GPC add on A.02.01 (Agilent) was run on recorded chromatograms to establish a calibration curve "log Mmax versus Elution volume" (fitting with a quadratic function) and the molecular weights of the polymers were estimated as number average molecular weights (Mn), weight average molecular weights (Mw), Z average molecular weights (Mz), and peak molecular weights (Mp).

Reaction with the Yariv β -glucosyl antigen: An agarose (3.5 ml) suspension (1% in 0.15 M NaCl and 0.02% w/v Na₃N (aq)) were added with 10 $\mu\text{g}/\text{mL}$ (aq) of the Yariv reagent and cast on glass plates to form a 1-mm gel. After solidification, 1.2mm i.d. wells were dug into the gel and filled with 0.8 μL of tested polysaccharides solutions. (0.1 to 1 $\mu\text{g}/\text{well}$). The plates were incubated overnight in a humidified atmosphere and red halos were compared with those obtained from an Arabic gum suspension applied at the same concentrations.

Determination of the monosaccharides composition by HPLC: HPLC was performed upon prechromatographic derivatization with 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP)^[13]. Each PS (1 mg) or fraction (200 μg) was hydrolyzed in 1 mL 2 M trifluoroacetic acid (aq) (100°C , 1 hour), dried under vacuum (SpeedVac) and taken up in 3 portions of 500 μL methanol (30 sec sonication). The combined methanolic solutions were added or not with 10 μL of the internal standard (I.S., talose, 1 mg/mL (aq)), dried, taken up in 150 μL water, and filtered.

Individual solutions of monosaccharides (arabinose, fucose, glucose, galactose, mannose, rhamnose, xylose, glucuronic acid, and galacturonic acid) were dissolved in methanol (1 mg/mL), mixed in different proportions to prepare calibrators (2 to 40 μg of each one), evaporated to dryness and dissolved in 150 μL water.

Hydrolyzed polymers or calibrators were added with 20 μL of a 0.5 M methanolic solution of 3-methyl-1-phenyl-2-pyrazolin-5-one and 20 μL of 0.3 M NaOH (aq), incubated 2 h at 70°C , neutralized by 20 μL 0.3 M HCl (aq) and extracted 3 times with 500 μL of butyl ether (10 s vortex, 5 min centrifugation at 2000 rpm). The aqueous phase was then dried under vacuum (SpeedVac) and redissolved in 150 μL water; 10 to 25 μL of this solution were injected in a HPLC Agilent 1100 system equipped with a Agilent 1100 DAD detector set at 254 nm and a 250×4.6 mm i.d. Atlantis dC18 column. The mobile phases A (triethylamine: acetonitrile: water, 0.4: 10: 90, v/v) and B (acetonitrile: water, 60: 40, v/v) were pumped at 1 mL/min with the following gradient: 0 min, 10% B; 22 min, 28% B; 55 min, 28% B; 56 min, 10% B; 65 min, 10% B.

Determination of the monosaccharide composition by TLC: Hydrolyzed polysaccharides (5 $\mu\text{g}/\text{spot}$) and standards (0.5 $\mu\text{g}/\text{spot}$) were applied on silica gel 60 F254 plates (Merck, Germany). The plates were eluted with acetonitrile: water (80: 20) (2 developments over 9 cm with mobile phase renewing), dried, sprayed with a mixture 0.2% w/v naphtoresorcinol in ethanol: H₂SO₄ (96: 4, v: v) and heated at 105°C for 15 min. 2.

Linkage analysis: Prior to linkage analysis by methylation and GC-MS^[14], the uronic acid groups of the polysaccharides were reduced. PS50 (1 mg) or its fractions (200 μg) were treated by sodium borodeuteride (NaBD₄) after activation with carbodiimide^[15]; the use of borodeuteride allows to differentiate the masses of the reduced uronic acids from the corresponding neutral monosaccharides. The reduced polysaccharides were then methylated by methyl iodide and hydrolyzed in 2.5 M TFA (aq) (100°C , 1 hour). After reduction by NaBD₄ and acetylation with acetic anhydride, the resulting partially methylated alditol acetates were analyzed by GC-MS (I.S. inositol) on a Hewlett-Packard methyl silicone capillary column (12 m \times 0.22 mm i.d., 0.33 μm film) in the E.I. ionization mode, as previously described^[16]. Methanolysis was performed^[17] with modifications as previously described^[12], using 4 M HCl in anhydrous methanol for 24 h at 80°C ; the methyl glycosides were then silylated and analyzed by GC^[12].

Measurement of DNA contamination: The crude polysaccharides extract of *Plantago major* or *Plantago palmata* Hook were dissolved by swelling overnight at 4°C in 8 mL buffer (10 mM Tris, 0.2 M NaCl, 1 mM EDTA (aq), pH 7.4). A series of dilutions of this solution were analyzed with the kit DNA quantitation Hoechst 33258 bisbenzimidazole following the manufacturer's protocol.

Results and Discussion

Harvesting and extraction: Preliminary extraction trials on *Plantago palmata* Hook leaves that were dried upon harvesting resulted in a brownish material, heavily contaminated with chlorophyll. Colorless material could not

be separated, which may indicate an enzymatic activity during the drying step, probably leading to some covalent bonding. Postharvesting treatment with acetone overcame the problem, removing chlorophyll and other colored low molecular weight lipophilic material and inactivating enzymes, so that white polysaccharides fractions could be obtained.

Fractionation of the polysaccharides fraction (PS50) crude extract: Typical SEC chromatogram obtained for *Plantago palmata* Hook (PS50), indicating the collected fractions; biological data indicate that is the most active one. Referring to a calibration curve obtained with pullulan standards, the molecular weights of PS50 and PS50F were estimated (Table-1). Three peaks could be discerned from the chromatogram of the active PS50, a very high molecular weight (Mw about 1200 kDa), and 2 lower weight (Mw about 270 kDa and 12 kDa, resp.) polymers. As there is no obvious difference between the active PS50 and inactive PS50F, these data do not allow explaining the detrimental effect of the filtration step on biological activity. As SEC determinations are not dependent on the molecular mass but on the hydrodynamic volume of the solute [18], these mass

values however need confirming by an absolute method based on multiangle laser light scattering. Indeed arabinogalactan-proteins (AGPs) are known for globular structures which may strongly influence the retention on SEC columns [19].

Reaction with the yariv β -glucosyl antigen: As arabinogalactans are important immunomodulatory substances, their presence within the polysaccharides has been investigated [20]. The Yariv β -glucosyl reagent precipitates some AGPs of vegetal cell walls, especially those with a high proportion of 1,3,6-linked galactose residues [21]. As this reagent presents a still unexplained specificity for polymers with bioactive structures, the formation of a red precipitate is a recognized indication for the presence of bioactive AGPs. As AGPs with immunomodulatory (anticomplementary) activity have been shown in *Plantago major* [18, 22], we compared its AGPs content with *Plantago palmata* Hook. Although the method is only semiquantitative (rapid saturation of the halo with increasing concentrations), it is clear that both *Plantago* contain AGPs, but with a significantly lower amount in *Plantago palmata* Hook.

Table 1: Molecular weights of polymers obtained from *Plantago palmate* Hook leaves by aqueous extraction (50°C, 3 hrs),

Polymer	Elution volume (mL)	M _n (g/mol)	M _w (g/mol)	M _z (g/mol)	M _p (g/mol)	Polydispersity (M _w /M _n)
PS50	11.8 to 13.8 mL ^a	1129867 (\pm 39000)	1192500 (\pm 51000)	125567 (\pm 65000)	1190167 (\pm 37000)	1.05
	13.8 to 16.8 mL ^b	222537 (\pm 16000)	271913 (\pm 12000)	322567 (\pm 8000)	303167 (\pm 12000)	1.22
	16.8 to 19.6 mL ^c	39485 (\pm 650)	11818 (\pm 720)	14856 (\pm 760)	9895 (\pm 550)	1.24
PS59F	11.8 to 13.8 mL	1189650 (\pm 9000)	1278550 (\pm 14000)	136950 (\pm 20000)	1262000 (\pm 13000)	1.07
	13.8 to 16.8 mL	230515 (\pm 1000)	277390 (\pm 620)	325485 (\pm 605)	304600 (\pm 6000)	1.20
	16.8 to 19.6 mL	12860 (\pm 720)	12860 (\pm 720)	16068 (\pm 960)	10597 (\pm 3300)	1.24

M_n = Number average molecular weight; M_w = Weight average molecular weight; M_z = Z average molecular weight;

Table 2: Monosaccharides constitutive of the polysaccharides extracted from *Plantago palmata* Hook and *Plantago major* of total carbohydrates (%).

	PS50 from <i>plantago major</i> ^a	Sample PS50 from <i>Plantago palmata</i> ^a	Fraction F2 from <i>Plantago palmata</i> ^b
Man	5.7% (5.8%-5.5%)	5.4% (4.9%-6.0%)	27.7%
Ara/ Xyl ^c	27.87% (28.8%-26.8%)	26.8% (25.5%-28.0%)	17.7%
Fuc	Traces	Traces	n.d.
Glc	20.7% (21.0%-20.3%)	4.8% (4.7%-4.9%)	16.9%
Gal	30.0% (30.8%-28.7%)	39.2% (37.4%-41.0%)	13.3%
Rha	7.6% (5.6%-9.6%)	9.6% (10.9%-8.3%)	12.5%
GlcA	Traces	n.d.	n.d.
GalA	8.5% (8.1%-8.8%)	14.2% (16.5%-11.9%)	11.8%

^a Data from 2 experiments in duplicate; in brackets, the values from the 2 experiments.

^b Data from 1 experiment in duplicate. CTLC shows that the ratio Ara/Xyl is approximately 9: 1 in both *Plantago palmata* Hook and *Plantago major* plants.

Determination of the monosaccharide's composition: The composition in monomers was determined by HPLC with 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP) prechromatographic derivatization (Table-2). The method was found linear in the investigated range (2 to 40 μ g monomers derivatized, typical R₂ > 0.990 with or without I.S.), allowing the determination of neutral monosaccharides and uronic acids in a single step, without the need for acid groups reduction. The resolution was however lower than in GC and we could separate neither rhamnose from the intended internal standard, talose, nor arabinose from

xylose. As data computed with or without I.S. were very similar, talose was omitted from the analysis. Both pentoses could be readily resolved by TLC and GC after methanolysis which both showed that the ratio ara/xyl was approximately 9: 1 in the PS50 from *Plantago major* and *Plantago palmata* Hook. Both PS50 had a roughly similar composition, compatible with pectins; there were however some differences and *Plantago palmata* PS50 showed a different content in glucose and, to a lesser extent, in galactose and galacturonic acid.

Linkage analysis: The major linkage is 1,4-GalA followed by 1,3,6-Gal. Three major pectic polysaccharides are known to exist in all cell walls,

- an unbranched homogalacturonan domain (compatible with the observed high ratio of 1,4-GalA in PS50);
- A highly diversified branched rhamnogalacturonan-I domain with arabinan/galactan/arabinogalactan lateral chains; and
- A highly conserved rhamnogalacturonan-II domain. The linkages found (Table -3) are typical for homogalacturonan regions (1,4-GalA) and hairy regions of pectins [22].

The rhamnose residues are part of the pectin backbone whereas neutral side chains composed of arabinogalactans, galactans and arabinans are linked to position 4 of the 1,2-linked rhamnose residues. The galactose residues are 1,3-, 1,6-, and 1,3,6-linked while the majority of the arabinose residues are terminally linked indicating the presence of arabinogalactan. The presence of such structures was confirmed by the positive reaction with Yariv antigen. Most of the linkages found in PS50 were also detected in PS50

fraction 2 but could not be quantified due to the small sample size available for analysis. From previous studies, immunomodulatory activities of pectin polysaccharides seem to depend on the presence of arabinogalactan side chains [23,24]. This might also be the case in the present study, but it remains to confirm this by isolation of pectin hairy regions from *Plantago palmata* PS50. The fragmentation by size exclusion chromatography allows to partly resolve the complex and high molecular weight PS fraction but yields only small amounts of polymers, insufficient for complete characterization. The elucidation of structure probably requires additional methods, based on anion exchange chromatography, enzymatic degradation of the polymer and mass spectroscopy of fragments. Such a glycomic approach however still requires major developments, comparatively to genomic and proteomic. Indeed, the many linkages/branching possibilities and the similar M/Z of many of the monomers limit analytical possibilities; moreover, the available glycosidases still either lack specificity or are significantly contaminated with undesired activities.

Table 3: Products of the polysaccharides extracted from *Plantago palmata* Hook (% mole) after reduction, methylation, hydrolysis, and acetylation, followed by GC-MS.

Monosaccharide	Linkage	PS50 from <i>Plantago palmata</i>	Fraction 2 from <i>Plantago palmata</i> PS50 ^a
Ara f	Terminal	7.3	X
	1,2	1.3	X
	1,3	1.3	X
	1,3	0.6	x
Total		10.5	
Fuc p	Terminal	3.4	
Gal	Terminal	3.8	X
	1,3	2.1	X
	1,6	5.0	X
	1,3,6	13.8	X
Total		24.7	
Glc	Terminal	1.4	X
	1,4	6.3	x
Total		7.7	
Rha p	Terminal	1.5	
	1,2	1.0	
	1,3	0.4	
	1,2,4	1.1	x
Total		4.0	
Xyl p	Terminal	0.8	
	1,4	38.7	x
Total		1.9	
Gal A	Terminal	0.8	X
	1,4	38.7	xx
	1,3,4	0.5	
	1,2,4	0.4	
Total		40.4	
Glc A	Terminal	1.9	x

% linkage not quantifiable due to the minute number of polysaccharides analyzed; the x indicates that the linkage was effectively detected.

Polysaccharides Contamination by DNA: DNA contaminating hyaluronic acid preparations has been shown to be responsible for the induction of proinflammatory cytokines IL-12 and TNF- α in human monocytes [25]. The DNA contents of *Plantago palmata* and *Plantago major* PS50 were $0.04 \pm 0.01\%$ and $0.08 \pm 0.01\%$, respectively. These concentrations are much lower than those reported active in hyaluronic acid preparations (3 to 15%) [25]. This most probably rules out the eventuality of a contaminating DNA mediated biological activity on J774 cells.

Conclusion

The new polysaccharide with antibacterial, antifungal, antiviral, biological and immunomodulatory activities isolated from *Plantago palmata* Hook has a very high molecular weight (1200 kDa) and is pectic in nature, with a predominantly unbranched galacturonan domain and with a domain bearing side chains that consist of highly branched arabinan, galactan, and or arabinogalactan. Comparatively to the wellknown *Plantago major* biologically active polysaccharide (PS), *Plantago palmata* PS50 contain less

arabinogalactanproteins and has different contents of glucose, galactose, and galacturonic acid. It would be interesting to compare the biological activities of both *Plantago palmata* Hook and *Plantago major* species.

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