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Structural characterization of an immunomodulating (1→6)-β-D-glucan isolated from the leaves of *Catharanthus roseus* (Nayan thara)

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Abstract

A water-soluble glucan was isolated from hot aqueous extract of the leaves of *Catharanthus rosea*. This polysaccharide was found to consist of D-glucose only. On the basis of total hydrolysis, methylation analysis and NMR studies (^1H , ^{13}C , TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC) the repeating unit of the polysaccharide is established as



This polysaccharide showed the macrophage, splenocyte, and thymocyte activation.

Keywords: *Catharanthus rosea*, glucan; structure; NMR, immuno activation

1. Introduction

Catharanthus rosea ^[1], family *Apocynaceae* originates from Madagascar but now spreads throughout the tropics and subtropics region of the world. The other names of this plant are periwinkle, madagascar periwinkle, and sadabahar. Plant extract of different parts of *Catharanthus rosea* possesses the antibacterial ^[2], antifungal ^[3], antiviral ^[4], and antioxidant⁵ properties. The alkaloids of *Catharanthus rosea* are famous for anticancer activities ^[6-8]. Several animal studies showed that ethanolic ^[9-11] as well as aqueous ^[12] extract of leaves of *Catharanthus rosea* lowered the glucose level of blood exhibiting antidiabetic properties. It has been reported that the leaf juice of *Catharanthus rosea* produced a significant decrease in serum total cholesterol, triglyceride, LDL-cholesterol, and VLDL-cholesterol ^[13] of rats. From these observation a detailed structural characterization and immunomodulating properties of a polysaccharide isolated from the leaves of *Catharanthus rosea* were carried out.

2. Results and Discussion

2.1 Isolation, purification and Structural characterization of the polysaccharide

The polysaccharide was isolated from the leaves of *Catharanthus rosea* (500 g) by hot water extraction followed by alcohol precipitation, dialysis, centrifugation and freeze drying; yield 450 mg of crude polysaccharide (PS). 25 mg of PS on fractionation through Sepharose 6B in aqueous medium yielded two fractions PS-I (8 mg) and PS-II (4 mg). We are reporting herein the structural characterization of PS-II only. The total carbohydrate of this fraction was estimated to be 98.5% using phenol-sulfuric acid method¹⁴. The pure polysaccharide has a specific rotation of $[\alpha]_{\text{D}}^{25} -31.6$ (c 0.6, water) and the molecular weight of the polysaccharide was estimated $\sim 1.8 \times 10^5$ Da from a calibration curve prepared with a standard dextrans ^[15]. The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) and the alditol acetate on analysis through GLC using columns A (3% ECNSS-M) and B (1% OV-225) indicate the presence of only glucose. The absolute configuration of glucose was determined as D by the method of Gerwig. ^[16] The absorption at 900 cm^{-1} in the IR spectrum indicate that the polysaccharide has β -glucopyranosidic linkages ^[17]. Now to find the mode of linkage the glucan was methylated using the method of Ciucanu and Kerek ^[18] and then Purdie and Irvine ^[19] followed by

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Hydrolysis and conversion into alditol acetates. The alditol acetate was analyzed by GLC using columns A and B and GLC-MS using an HP-5 fused silica capillary column and found to contain 1, 5, 6-tri-*O*-acetyl-2, 3, 4-tri-*O*-methyl-D-glucitol only. This indicates the presence of only (1→6)-linked D-glucopyranosyl moiety in the glucan.

The 500 MHz ^1H NMR spectrum (Fig. 1) of PS at 27 °C showed one anomeric signal at 4.50 ppm and the coupling constant values ($J_{\text{H-1,H-2}} \sim 8.5$ Hz and $J_{\text{H-1,C-1}} \sim 160$ Hz) suggested that it is β -linked. The proton chemical shifts (Table 1) from H-1 to H-6 were assigned from the DQF-COSY and TOCSY spectra.

The 125 MHz ^{13}C spectrum (Fig. 1) of PS at 27 °C exhibits one anomeric carbon signals at 103.4 ppm and this anomeric carbon signal was assigned for the β -linked residue and this assignment was also corroborated by HMQC experiment. All the carbon signals (Table 1) of the glucopyranoside residue were assigned with the help of HMQC spectrum. The carbon signals at 73.5, 76.0, 69.9, 75.3 and 69.2 ppm correspond to the C-2, C-3, C-4, C-5 and C-6 respectively of the glucopyranoside residue. The C-6 signal of the glucopyranoside residue at 69.2 ppm is shifted to 7.4 ppm downfield compared to the standard methyl glycosides $^{[28, 29]}$ due to the α -glycosylation effect.

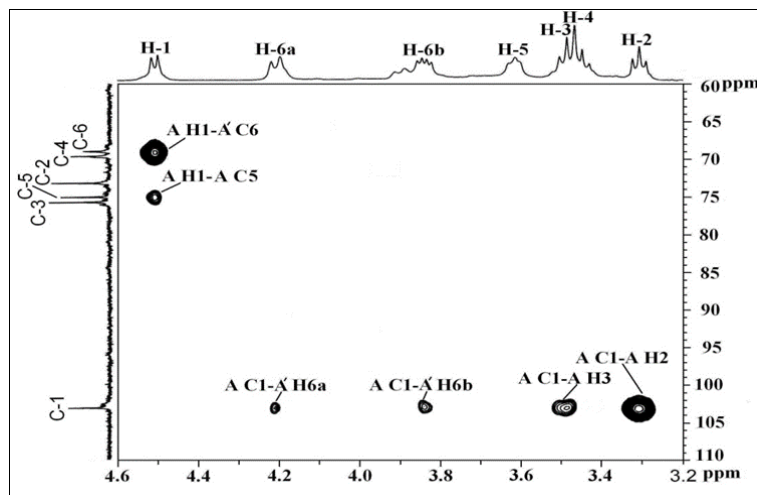


Fig 1: HMBC spectrum of the polysaccharide isolated from the leaves of *Catharanthus rosea* recorded with delay time of 80 ms.

The sequence of glycosyl residues of the polysaccharide was confirmed from HMBC experiment. Long-range ^{13}C - ^1H correlations were obtained from the HMBC spectrum (Fig. 1). The cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined and both inter and intra residual connectivities were observed from the HMBC experiment (Table 2). For explanation of HMBC experiment

two units of glucose (A and A') are considered. Cross peaks were found between H-1 (4.50 ppm) of residue A and C-6 (103.4 ppm) of residue A' (A H-1, A' C-6) and vice versa and C-1 (103.4 ppm) of residue A and H-6a (4.20 ppm) and H-6b (3.84 ppm) of residue A' (A C-1, A' H-6a, A C-1, A' H-6b) and vice versa.

Table 1: ^1H NMR^a and ^{13}C NMR^b chemical shifts for the polysaccharide Isolated from the leaves of *Catharanthus rosea* in D_2O at 27 °.

Glycosyl Residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a, H-6b / C-6
→ 6)- β -D-Glcp-(1→	4.50	3.31	3.49	3.47	3.61	4.20 ^c , 3.84 ^d
	103.4	73.5	76.0	69.9	75.3	69.2

Table 2: The significant $^3\text{J}_{\text{H,C}}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *Catharanthus roseus*.

Residue	Sugar linkage	H-1/C-1 $\delta_{\text{H}}/\delta_{\text{C}}$	Observed connectivities	
			$\delta_{\text{H}}/\delta_{\text{C}}$	Residue Atom
A	→ 6)- β -D-Glcp-(1→	4.50	69.2	A' C-6
			75.3	A C-5
			4.20	A' H-6a
		103.4	3.84	A' H-6
			3.48	A H-3
			3.31	A H-2

Thus the appearance of these cross peaks clearly supports the presence of the following repeating unit in the polysaccharide isolated from *Catharanthus rosea*.



2.2 Assay for macrophage activity by no

Some biological studies were carried out with this

polysaccharide. Macrophage activation of the polysaccharide was observed *in vitro*. On treatment with different concentrations of the polysaccharide an enhanced production of NO was observed in a dose dependent manner with optimum production of 20.5 μM NO per 5×10^5 macrophages at 100 $\mu\text{g}/\text{mL}$ of the polysaccharide (Fig. 2a). The various types of polysaccharides like lentinan inhibits tumor growth by stimulating the immune system²⁰ through activation of macrophages, T-helper, NK, and other cells.

2.3 Splenocyte and thymocyte proliferation assay

Proliferation of splenocyte and thymocyte is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide and assayed by the MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method $^{[21]}$. The polysaccharide was tested to proliferate splenocyte and thymocyte as shown in Fig. 2 (b) and 2 (c), respectively, and the asterisks on the columns indicated the statistically significant differences compared to PBS

(Phosphate Buffer Solution) control. At 50 $\mu\text{g/mL}$ of the polysaccharide, splenocyte proliferation index was observed maximum as compared to other concentrations. Hence, 50 $\mu\text{g/mL}$ of the polysaccharide can be considered as efficient splenocyte proliferators. Again 50 $\mu\text{g/mL}$ of that sample showed maximum effect on thymocyte proliferation. The

splenocyte proliferation index (SPI) as compared to PBS control was close to 1 or below indicated low stimulatory effect on immune system. Some mushroom [22-26] polysaccharides had also shown similar type of splenocyte activation as well as phagocytic response of macrophages as reported earlier by our group.

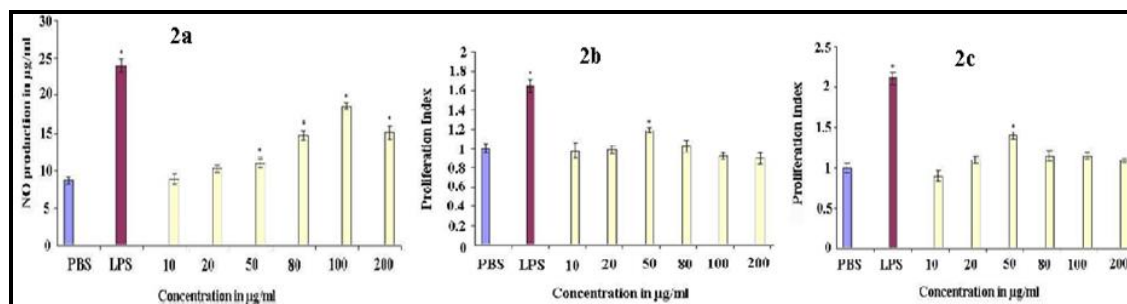


Fig 2: (a) *In vitro* activation of peritoneal macrophage stimulated with different concentration of the polysaccharide in terms of NO production. Effect of different concentration of the polysaccharide on (b) splenocyte, and (c) thymocyte proliferation (asterisks indicate the statistically significant compared to PBS control).

3. Materials and Methods

3.1 Isolation, fractionation, and purification of the crude polysaccharide

The fresh leaves (500 g) of *Catharanthus rosea* were collected from local forest and washed properly with water. Its leaves were cut into fine pieces and washed with distilled water, then boiled with distilled water at 100 °C for 6 h. The whole mixture was kept over night at 4 °C and filtered through a linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 30 min at 4 °C. The supernatant was collected and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material (polysaccharide) was washed with ethanol for five times and then freeze-dried. The freeze-dried material was dissolved in 40 mL of distilled water and dialyzed through cellulose membrane (Sigma-Aldrich, retaining > M.W. 12 kDa) against distilled water for 10 h to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried. Thus crude polysaccharide was obtained (450 mg). The crude polysaccharide (25 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose-6B in water as eluent (0.4 mL min⁻¹) using a Redifrac fraction collector. 95 test tubes (2 mL each) were collected and assayed aliquots of the fractions using the phenol-sulphuric colorimetric assay method [14] and the absorbance was recorded at 490 nm by UV-vis spectrophotometer. Two homogeneous peaks, PS-I (test tubes 16-34, yield 8 mg) and PS-II (test tubes 44-64, yield 4 mg) were obtained. They were collected and freeze dried. This purification process was carried out in ten lots and each individual fraction was again purified and collected; PS-I (yield, 60 mg), PS-II (yield, 30 mg).

3.2 Monosaccharide analysis

The polysaccharide sample (2.5 mg) was hydrolyzed with 2 M CF₃COOH (2 mL) and the monosaccharide analysis was carried out in the same fashion as described in our earlier publications [17-19].

3.3 Methylation analysis

The polysaccharide was methylated using the procedure described by Ciucanu and Kerek²⁶ as described previous [17-19].

3.4 Absolute configuration of monosaccharides

The method used was based on Gerwig. [16] as reported earlier [17-19].

3.5 Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25 °C.

3.6 Determination of molecular weight

The molecular weight of polysaccharide was determined by the method as described in our previous publications [15].

3.7 NMR studies

The NMR experiment was carried out as reported earlier [27-29].

3.8 GLC-MS experiments

All the GLC-MS experiments were carried out in a Hewlett-Packard 5970 MSD instrument using HP-5 fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min⁻¹ up to a final temperature of 200 °C.

3.9 Test for macrophage activity by Nitric oxide assay

Peritoneal macrophages (5 × 10⁵ cells mL⁻¹) after harvesting were cultured in complete RPMI (Rose well Park Memorial Institute) media in 96-well plate [21, 31]. The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the wells in different concentrations. The cells were cultured for 24 h at 37 °C in a humidified 5% CO₂ incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction [32]. Equal volumes of Greiss reagent (1:1 of 0.1% in 1-naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was observed at 550 nm.

3.10 Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus were prepared from the normal mice under aseptic conditions by frosted slides in Phosphate Buffer Solution (PBS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After washing two

times in PBS the cells were resuspended in complete RPMI (Rose well Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μ L) were plated in 96-well flat-bottom plates and incubated with 20 μ L of various concentrations (10 μ g/mL to 200 μ g/mL) of the polysaccharide with lipopolysaccharide (LPS, which is positive control) of 4 μ g/mL. Cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO₂. Proliferation was checked by MTT assay method^[21]. Data were reported as the mean standard deviation of six different observations and compared against PBS control^[31, 33].

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