Carbohydrate Polymers 81 (2010) 584–591

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural characterization of an immunoenhancing heteropolysaccharide isolated from hot water extract of the fresh leaves of *Catharanthus rosea*

Sukesh Patra[a], Kankan K. Maity[a], Sanjoy K. Bhunia[a], Biswajit Dey[a], Debsankar Das[a], Soumitra Mondal[a], Bibhas Bhunia[b], Tapas K. Maiti[b], Syed. S. Islam[a,*]

[a] Department of Chemistry and Chemical Technology, Vidyasagar University, V.U. Road, Rangamati, Midnapore 721102, West Bengal, India
[b] Department of Biotechnology, Indian Institute of Technology (IIT) Kharagpur, Kharagpur 721302, West Bengal, India

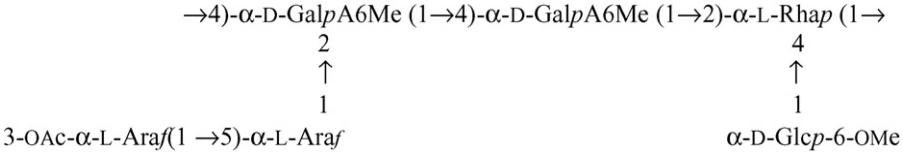
ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 30 January 2010
Accepted 8 March 2010
Available online 16 March 2010

Keywords:
Catharanthus rosea
Polysaccharide
Structure
NMR spectroscopy
Immunoactivation

ABSTRACT

An immunoenhancing polysaccharide (M.W. ~ 2.0×10^2 kDa) isolated from the aqueous extract of the leaves of *Catharanthus rosea* was found to consist of 6-*O*-methyl-glucose, arabinose, rhamnose, and methyl galacturonate with a molar ratio of nearly 1:2:1:2. On the basis of acid hydrolysis, methylation analysis, periodate oxidation, NMR experiments (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of the polysaccharide was established as



This polysaccharide showed optimum activation of macrophages at 100 μg/mL and both splenocyte and thymocyte at 50 μg/mL, respectively.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Catharanthus rosea (Raja, Nasir, Abbas, & Naqvi, 2009), family *Apocynaceae* originates from Madagaskar but now spreads throughout the tropics and subtropics region of the world. The other names of this plant are periwinkle, madagaskar periwinkle, and sadabahar. Plant extract of different parts of *C. rosea* possesses the antibacterial (Careew & Patterson, 1970), antifungal (Jaleel et al., 2007), antiviral (Fornsworth, Svoboda, & Blomster, 1968), and antioxidant (Zheng & Wang, 2001) properties. The alkaloids of *C. rosea* are famous for anticancer activities (El-Sayed & Cordell, 1981; El-Sayed et al., 1983; Ueda et al., 2002). Several animal studies showed that ethanolic (Chattopadhyay et al., 1991, 1992; Ghosh & Gupta, 1980) as well as aqueous (Singh et al., 2001) extract of leaves of *C. rosea* lowered the glucose level of blood exhibiting antidiabetic properties. It has been reported (Antia & Okokon, 2005) that the leaf juice of *C. rosea* produced a significant decrease in serum total cholesterol, triglyceride, LDL-cholesterol, and VLDL-cholesterol of rats. From these observation a detailed structural characterization and immunoenhancing properties of a hetero polysaccharide isolated from the leaves of *C. rosea* were carried out and reported herein since no work regarding this is available in literature.

2. Materials and methods

2.1. Isolation, fractionation, and purification of the crude polysaccharide

The fresh leaves (500 g) of *C. 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 polysac-

* Corresponding author. Tel.: +91 03222 276558x437; fax: +91 03222 275329.
E-mail address: sirajul_1999@yahoo.com (Syed.S. Islam).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.03.019

charide (25 mg) was purified by gel permeation chromatography on column (90 cm × 2.1 cm) of Sepharose-6B in water as eluent (0.4 mL/min) 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 (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) 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). Characterization of only PS-I is reported herein.

2.2. Monosaccharide analysis

2.2.1. Alditol acetate analysis

PS-I (3 mg) was hydrolyzed with 2.0 M CF_3COOH (2 mL) at 100 °C for 16 h in a boiling water bath and excess acid was completely removed by repeated co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GC and GC–MS. Quantization was carried out from the peak area, using response factors from standard monosaccharides using inositol as standard.

2.2.2. Preparation of carboxy methyl reduced polysaccharide

The polysaccharide (3.5 mg) was dissolved in 1.0 M imidazole–hydrochloric acid buffer, pH 7.0 (200 μL/mg) and cooled on ice. Sodium borohydride (40 mg) was then added and reacted on ice for at least 1 h. The excess borohydride was destroyed by adding glacial acetic acid (100 μL/40 mg borohydride) slowly to the cooled sample. An equal volume of distilled water was then added, and the reduced polysaccharide was precipitated by adding 3–4 times of volume of 95% (v/v) ethanol. The sample was reprecipitated two more times with 95% ethanol and the residue was freeze-dried. The carboxyl reduced (Maness, Ryan, & Mort, 1990) polysaccharide was divided into two portions. One portion was hydrolyzed with 2.0 M CF_3COOH for 16 h at 100 °C. After removing excess acid, the hydrolyzed product was reduced with $NaBH_4$ and finally acetylated with 1:1 pyridine–Ac_2O to yield alditol acetate and analyzed by GC. Another portion was subjected to periodate oxidation experiment.

2.3. Methylation analysis

PS-I (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek (1984) where distilled DMSO and finely grounded NaOH were used. The methylated product was isolated by making partition between $CHCl_3$ and water (5:2, v/v). The organic layer-containing product was washed with water for several times and dried. The methylated polysaccharide was hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$ and acetylated with pyridine and Ac_2O. The alditol acetates of the methylated sugars were analyzed by GC (using columns A and B) and GC–MS (using HP-5 fused silica capillary column). A portion of the methylated PS-I (2.0 mg) was dissolved in dry THF (2 mL), refluxed with $LiAlH_4$ (Abdel-Akher & Smith, 1950) (40 mg) for 5 h and kept overnight at room temperature. The excess of the reductant was decomposed by drop wise addition of EtOAc and aqueous THF. The inorganic materials were filtered off. The carboxyl-reduced methylated material was hydrolyzed with formic acid as before. The alditol acetate of the carboxyl-reduced, methylated sugar was prepared in usual way and analyzed by GC–MS.

2.4. Periodate oxidation

PS-I (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark for 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate-oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) material was hydrolyzed with 2 M CF_3COOH at 100 °C for 18 h, and this hydrolyzed material was used for paper chromatographic examination as well as alditol acetate preparation as usual for the GC analysis.

2.5. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatman nos. 1 and 3 mm sheets. Solvent systems used were: (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution (Hoffman, Lindberg, & Svensson, 1972).

2.6. Absolute configuration of monosaccharides

The method used was based on Gerwig, Kamarling, and Vliegenthart (1978) PS-I (1.0 mg) was hydrolyzed with CF_3COOH and then the excess acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in R-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with BSTFA. The products were analyzed by GC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.7. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25.6 °C.

2.8. Colorimetric estimations

Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer, model 1601.

2.9. Determination of molecular weight

The molecular weight of PS-I was determined by gel-chromatographic technique. Standard dextrans (Hara, Kiho, Tanaka, & Ukai, 1982) T-200, T-70, and T-40 were passed through a Sepharose-6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of PS-I was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.10. GC experiments

All gas liquid chromatography experiments were performed on a Hewlett-Packard Model 5730. A gas chromatograph having a flame ionization detector and glass columns (1.8 m × 6 mm) packed

with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GC analyses were performed at 170 °C.

2.11. GC–MS experiments

All the GC–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 up to a final temperature of 200 °C.

2.12. NMR studies

The ^{1}H and ^{13}C NMR experiments were carried out at 500 and 125 MHz Bruker Avance DPX-500 spectrometer, respectively, using a 5 mm broad-band probe. For NMR studies, PS-1 was dried in vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dueñas Chaso et al., 1997) by lyophilizing with D_2O for three times. The deuterium-exchanged polysaccharide (4 mg) was dissolved in 0.7 mL D_2O (99.96% atom ^{2}H, Aldrich). The ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) NMR spectra were recorded at 27 °C. Acetone was used as an internal standard (δ 31.05) for ^{13}C spectrum. The ^{1}H NMR spectrum was recorded fixing HOD signal at δ 4.73 at 27 °C using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). 2D (DQF-COSY) NMR experiment was performed using standard Bruker software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay were 300 ms. The delay time in the HMBC experiment was 80 ms.

2.13. Test for macrophage activity by Nitric oxide assay

Peritoneal macrophages (5×10^5 cells/mL) after harvesting were cultured in complete RPMI (Rose well Park Memorial Institute) media in 96-well plate (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). 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% CO_2 incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction (Green et al., 1982). Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine 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.

2.14. 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 μL) were plated in 96-well flat-bottom plates and incubated with 20 μL of various concentrations (10–200 μg/mL) of the polysaccharide with lipopolysaccharide (LPS, which is positive control) of 4 μg/mL. Cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method (Ohno et al., 1993). Data were reported as the mean standard deviation of six different observations and compared against PBS control (Maiti et al., 2008; Sarangi et al., 2006).

3. Results and discussion

3.1. Isolation, purification and chemical analysis of the polysaccharide

The polysaccharide was isolated from the leaves of *C. 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-I only. The total carbohydrate of this fraction was estimated to be 98.5% using phenol–sulphuric acid method (York et al., 1985). The pure polysaccharide had $[\alpha]_D^{25.6}$ +98.74 (*c* 0.094, water). The molecular weight (M.W.) (Hara et al., 1982) of the polysaccharide was found to be $\sim 2.0 \times 10^2$ kDa. Paper chromatographic analysis (Hoffman et al., 1972) of the hydrolyzed polysaccharide showed the presence of arabinose, galacturonic acid, rhamnose, and a slow moving spot nearer to glucose. The absolute configuration of the sugar units was determined by the method of Gerwig et al. (1978) and confirmed by the NMR spectroscopy. GC analysis of the alditol acetates of the sugar showed the presence of rhamnose, 6-*O*-methyl-glucose, and arabinose in a molar ratio of nearly 1:1:2. The carboxy methyl reduced (Maness et al., 1990) polysaccharide on hydrolysis, followed by GC analysis of corresponding alditol acetates showed the presence of rhamnose, 6-*O*-methyl-glucose, arabinose, and galactose in a molar ratio of nearly 1:1:2:2. This result indicated that galacturonic acid residues were present in the polysaccharide. The polysaccharide was methylated using the method of Ciucanu and Kerek (1984). The alditol acetates of methylated product were analyzed by GC and GC–MS which showed the presence of 2,5-Me_2-Ara; 2,3-Me_2-Ara; 2,3,4,6-Me_4-Glc, and 3-Me-Rha in a molar ratio of nearly 1:1:1:1. The above result indicated the presence of one unit of 3-acetyl arabinose and methoxy-glucose as terminal residues. It also indicated the presence of (1→5)-linked arabinofuranosyl or (1→4)-linked arabinopyranosyl and (1→2,4)-linked rhamnopyranosyl. The GC–MS analysis of the alditol acetates of methylated carboxyl-reduced (Abdel-Akher & Smith, 1950) polysaccharide showed two new peaks of 3-Me-Gal and 2,3-Me_2-Gal. This result indicated that galacturonic acids were present as (1→2,4)-linked and (1→4)-linked moieties. For further linking information, a periodate oxidation experiment (Goldstein et al., 1965; Hay et al., 1965) was carried out with the carboxyl-reduced and intact polysaccharide. The periodate-oxidized, $NaBH_4$ reduced material upon hydrolysis with trifluoroacetic acid followed by GC analysis showed the presence of rhamnose only. The GC–MS analysis of carboxyl-reduced periodate-oxidized, methylated (Abdel-Akher & Smith, 1950) polysaccharide showed that 3-Me-Rha and 3,6-Me_2-Gal were present in a molar ratio of nearly 1:1. Thus the periodate oxidation experiment confirmed that rhamnose and one galacturonic acid residue were present as (1→2,4)-linked moieties.

3.2. NMR and structural analysis of polysaccharide

The ^{1}H NMR spectrum (500 MHz, Fig. 1) of this polysaccharide at 27 °C showed signals at δ 5.37, 5.22, 5.06, and 4.93 for six anomeric protons where δ 5.06 and 4.93 accommodated two protons for each signal and the rest for another two protons. The spectrum had an unusually downfield chemical shift of one of the ring proton in the anomeric region, indicated by the signal at 5.12 ppm. The singlet at

Table 1
^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of polysaccharide, isolated from *Catharanthus rosea*[a,b] recorded in D_2O at 27 °C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5a, H-5b/C-5	H-6a, H-6b/C-6	6-O-Me	$COOCH_3$	3-*O*-$COCH_3$
α-D-Glc*p*-6-OMe (1→	5.37	3.57	3.66	3.53	3.83	3.63, 3.93	3.75		
A	100.0	72	73.7	70.9	71.1	68.1	53.3		
→5)-α-L-Ara*f* (1→	5.22	4.2	4.07	4.09	3.86, 3.91				
B	109.6	82	76.9	82.8	68.1				
→2,4)-α-L-Rha*p* (1→	5.06	3.93	3.79	3.48	3.87	1.23			
C	100.8	76.0	68.3	79.4	68.0	17.5			
3-OAc-α-L-Ara*f* (1→	5.06	4.1	5.12	4.07	3.77, 3.82				2.03[c]
D	107.8	81.3	79.4	84.3	61.6				171.0[d], 20.2[e]
→2,4)-α-D-Gal*p*A6Me (1→	4.93	3.7	3.81	3.99	4.43			3.78[f]	
E	100.8	75.5	70.9	77.1	70.9	171.0[d]		53.3[g]	
→4)-α-D-Gal*p*A6Me (1→	4.93	3.55	3.85	3.98	4.43			3.78[f]	
F	100.8	68.9	69.0	77.1	70.9	171.0[d]		53.3[g]	

[a] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.73 at 27 °C.
[c] Value of the acetyl methyl proton.
[d] Value of the carbonyl carbon of ester and acetyl group.
[e] Value of the acetyl methyl carbon.
[f] Proton value ester group.
[g] Value of the ester methyl carbon.

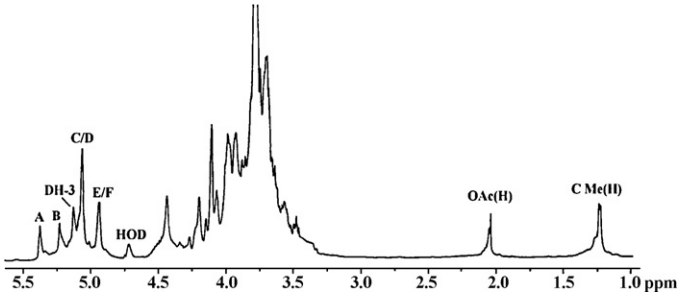


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide, isolated from *Catharanthus rosea*.

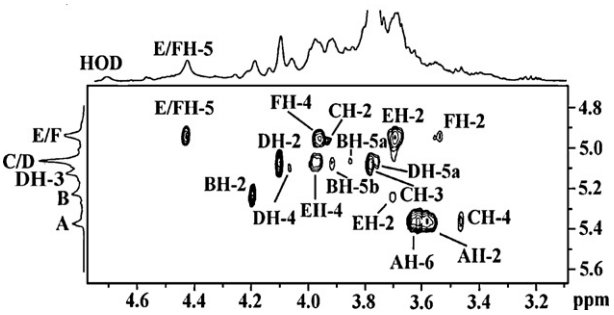


Fig. 3. The ROESY spectrum of polysaccharide, isolated from *Catharanthus rosea*. The ROESY mixing time was 300 ms.

2.03 was indicative of CH_3 proton of an acetic ester. The doublet at 1.23 ppm may be for the CH_3 proton of deoxy sugar, rhamnose. The carbomethoxy proton was observed at 3.78 ppm. The sugar residues were designated as **A**–**F** according to their decreasing anomeric proton chemical shifts (Table 1). In the ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C signals appeared at δ 109.6, 107.8, 100.8, and 100.0 for six anomeric carbons where signal at δ 100.8 consisted of three carbon and the rest for another three carbon signals. The signals at δ 17.5 and 20.2 were assigned as methyl carbon of rhamnose and methyl carbon of acetyl group, respectively. Furthermore the signal at δ 53.3 was assigned for methyl carbon of ester group and for O-methyl carbon. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, NOESY, ROESY (Fig. 3, Table 2), and HMQC NMR experiments.

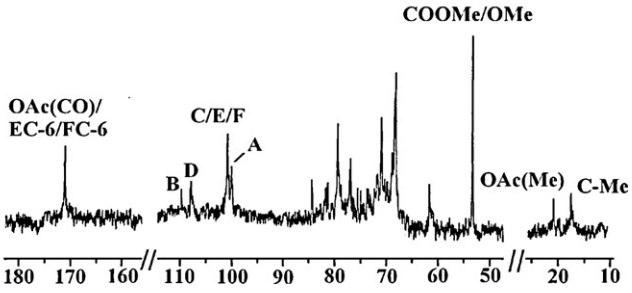


Fig. 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of polysaccharide, isolated from *Catharanthus rosea*.

Table 2
ROESY data for the polysaccharide isolated from *Catharanthus rosea*.

Glycosyl residue	Anomeric proton δ_H (ppm)	NOE contact protons δ_H (ppm)	Residue, atom
α-D-Glc*p*-6-OMe (1→	5.37	3.48	**C** H4
A		3.57	**A** H2
		3.63	**A** H6a
→5)-α-L-Ara*f* (1→	5.22	3.7	**E** H2
B		4.2	**B** H2
→2,4)-α-L-Rha*p* (1→	5.06	3.99	**E** H4
C		3.79	**C** H3
3-OAc-α-L-Ara*f* (1→	5.06	3.86	**B** H5a
D		3.91	**B** H5b
		4.1	**D** H2
		4.07	**D** H4
		3.77	**D** H5a
→2,4)-α-D-Gal*p*A6Me (1→	4.93	3.98	**F** H4
E		4.43	**E** H5
		3.7	**E** H2
→4)-α-D-Gal*p*A6Me (1→	4.93	3.93	**C** H2
F		3.98	**F** H4
		3.55	**F** H2
		4.43	**F** H5

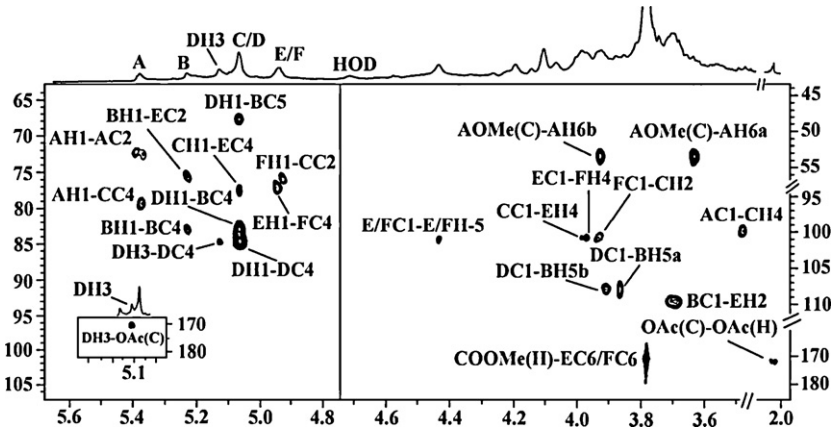


Fig. 4. Part of the HMBC spectrum of polysaccharide, isolated from *Catharanthus rosea*. The delay time in the HMBC experiment was 80 ms.

Residue **A** had an anomeric proton signal at 5.37 ppm and $J_{H\text{-}1,H\text{-}2} \sim 3.8$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz indicating that it was an α-linked residue. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed for **A**, supporting that it was D-glucosyl moiety. The anomeric carbon signal of residue **A** appeared at δ 100.0 and the other carbon signals from C-2 to C-6 were assigned from HMQC experiment. The presence of methoxy group at C-6 position of residue **A** was confirmed by appearance of cross-couplings between methoxy carbon at δ 53.3 and its H-6a, H-6b atoms [**A** OMe(C), **A** H-6a and **A** OMe(C), **A** H-6b] in the HMBC experiment (Fig. 4, Table 3). Thus considering the result of methylation analysis and NMR experiment, it may be concluded that residue **A** was a α-linked, terminal 6-*O*-Me D-glucopyranosyl moiety.

The residue **B** was assigned as α-L-arabinofuranosyl moiety. The furanose conformation was confirmed by high chemical shift of C-1 (δ 109.6), C-2 (δ 82.0), C-3 (δ 76.9), and C-4 (δ 82.8). The coupling constants $J_{H\text{-}1,H\text{-}2}$ (~3.4 Hz) and $J_{H\text{-}1,C\text{-}1}$ (~163 Hz) indicated that arabinofuranose was α-glycosidically linked residue supported by the unresolved anomeric proton at δ 5.22. The downfield shift of C-5 (δ 68.1) and other carbon values with respect to the standard value of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **B** was (1→5)-linked glycosides. Thus considering the result of methylation analysis and NMR data, it was confirmed that residue **B** was (1→5)-α-L-Ara*f*.

Residue **C** had an anomeric proton chemical shift at δ 5.06 ($J_{H\text{-}1,H\text{-}2} \sim 1.8$ Hz and $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz) indicating that it was an α-

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *Catharanthus rosea*.

Residue	Sugar linkage	H1/C1		Observed connectivities	
		δ_H/δ_C	δ_C/δ_H	Residue	Atom
A	α-D-Glc*p*-6-OMe (1→	5.37	79.4	**C**	C-4
			72	**A**	C-2
		100	3.48	**C**	H-4
B	→5)-α-L-Ara*f* (1→	5.22	75.5	**E**	C-2
			82.8	**B**	C-4
		109.6	3.7	**E**	H-4
C	→2,4)-α-L-Rha*p* (1→	5.06	77.1	**E**	C-4
		100.8	3.99	**E**	H-4
D	3-OAc-α-L-Ara*f* (1→	5.06	68.1	**B**	C-5
			82.8	**B**	C-4
			84.3	**D**	C-4
		107.8	3.86	**B**	H-5a
			3.91	**B**	H-5b
E	→2,4)-α-D-Gal*p*A6Me (1→	4.93	77.1	**F**	C-4
		100.8	3.98	**F**	H-4
			4.43	**E**	H-5
F	→4)-α-D-Gal*p*A6Me (1→	4.93	76.0	**C**	C-2
		100.8	3.93	**C**	H-2
			4.43	**F**	H-5
		6-OMe (δ_C)	δ_H		
A	α-D-Glc*p*-6-OMe (1→	53.3	3.63	**A**	H-6a
			3.93	**A**	H-6b
		COOMe (δ_H)	δ_C		
E	→2,4)-α-D-Gal*p*A6Me (1→	3.78	171.0	**E**	C-6
F	→4)-α-D-Gal*p*A6Me (1→	3.78	171.0	**F**	C-6
		3-OAc (δ_C)	δ_H		
D	3-OAc-α-L-Ara*f* (1→	171.0	5.12	**D**	H-3

linked residue. Residue **C** was assigned as Rha*p* due to the signals for an exocyclic methyl group and weak couplings of H-2 with H-1 and H-3. The ^{13}C signal for the anomeric carbon of residue **C** was observed at δ 100.8. The downfield shift of C-2 (δ 76.0) and C-4 (δ 79.4) indicated that residue **C** was present as (1→2,4)-α-L-Rha*p*.

Residue **D** was assigned as α-L-Ara*f* since it showed coupling constant $J_{H\text{-}1,H\text{-}2} \sim 3.4$ Hz and high anomeric carbon chemical shift (δ 107.8). The anomeric proton chemical shift of residue **D** appeared at δ 5.06. Cross-peaks were found between H-3 (5.12 ppm) of residue **D** and C-4 (84.3 ppm) [**D** H-3, **D** C-4] as well as H-3 (5.12 ppm) of residue **D** and acetyl carbonyl carbon (171.0 ppm) [**D** H-3, OAc (C))] in HMBC experiment (Fig. 4, Table 3) strongly suggesting that C-3 of residue **D** is the site of *O*-acetylation. All the carbon chemical shifts of residue **D** were more or less same to the standard value of methyl glycosides and also confirmed by the HMQC experiment. Thus, considering the experimental data of both methylation and NMR experiment it was confirmed that residue **D** was α-linked, terminal 3-OAc-L-Ara*f*.

Residue **E** was assigned as D-Gal*p*A6Me as its spin system consisted of five protons with the high chemical shift of H-5 signal (4.43 ppm). The galacturonic acid configuration was confirmed by the small coupling constants $J_{H\text{-}3,H\text{-}4}$ (~3.0 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~3.3 Hz). The anomeric proton signal of moiety **E** at δ 4.93 and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicated that D-galacturonosyl residue was α-linked anomer. ^{13}C signal for the anomeric carbon of the residue **E** was observed at δ 100.8. The downfield shift of C-2 (δ 75.5) and C-4 (δ 77.1) with respect to the standard value of methyl glycosides indicated that residue **E** was 2,4-linked-α-D-Gal*p*A. The presence of carboxymethyl group in residue **E** was confirmed by the appearance of intra-residual coupling between ester carbonyl carbon (δ 171.0) and the carboxymethyl proton (δ 3.78) [$COOCH_3$ (H), **E** C-6] in the HMBC experiment (Fig. 4, Table 3). Thus, the result indicated that residue **E** was methyl ester of 2,4-linked-α-D-Gal*p*A.

Residue **F** was assigned as (1→4)-linked galacturonic acid moiety as this spin system consisted of only five protons with a high chemical shift of H-5 (δ 4.43) and week coupling between H-3, H-4 and H-5 as well as a high chemical shift of C-4 signal at δ 77.1. The anomeric proton chemical shift for residue **F** at δ 4.93 ppm and coupling constants $J_{H\text{-}1,H\text{-}2} \sim 3.2$ Hz and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicated that residue **F** was α-linked. The carbon signals of residue **F** were observed at δ 68.9, 70.9, 70.9, and 171.0 for C-2, C-3, C-5, and C-6 (ester carbonyl), respectively. The appearance of intra-residual coupling between carbonyl carbon (δ 171.0) and carboxymethyl proton (δ 3.78) in HMBC experiment (Fig. 4, Table 3) clearly indicated that carbonyl group of galacturonic acid was present as methyl ester.

The sequence of glycosyl residues of the polysaccharide was determined from ROESY (Fig. 3, Table 2) as well as NOESY (not shown) experiments followed by confirmation with HMBC experiment (Fig. 4, Table 3). Residue **B** had interresidue ROESY contracts from H-1 to H-2 of residue **E**. Residue **D** had interresidual ROESY contacts from H-1 to H-5a and H-5b of residue **B**. Hence, the sequence in between the residues **B**, **D**, and **E** was established as

α-D- Gal*p*A6Me
2 **E**
↑
1
3-OAc-α-L-Ara*f*(1 →5)-α-L-Ara*f*
D **B**

Again, from the interresidue ROESY contacts from **E** H-1 to **F** H-4, **F** H-1 to **C** H-2, **C** H-1 to **E** H-4, and A H-1 to **C** H-4 the following connectivities were established

→4)-α-D-Gal*p*A6Me (1→4)-α-D-Gal*p*A6Me (1→2)-α-L-Rha*p* (1→
2 **E** **F** 4 **C**
↑ ↑
1
α-D-Glc*p*-6-OMe
A

So, from the ROESY and NOESY connectivities the repeating unit of the polysaccharide was established as

→4)-α-D-Gal*p*A6Me (1→4)-α-D-Gal*p*A6Me (1→2)-α-L-Rha*p* (1→
2 **E** **F** 4 **C**
↑ ↑
1 1
3-OAc-α-L-Ara*f* (1→5)-α-L-Ara*f* α-D-Glc*p*-6-OMe
D **B** **A**

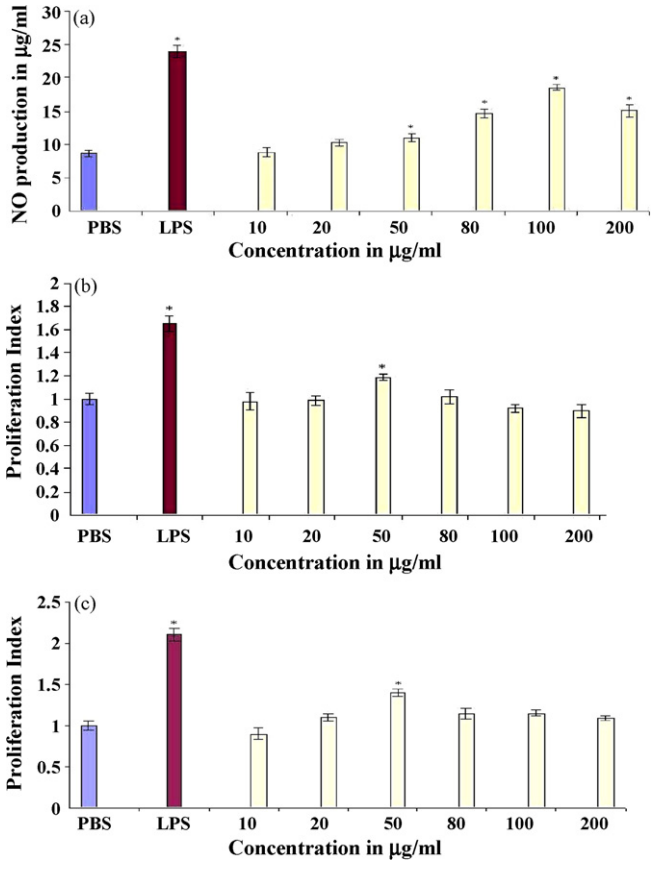


Fig. 5. (a) In vitro activation of peritoneal macrophage stimulated with different concentration of the polysaccharide in terms of NO production. Effect of different concentration of the polysaccharide on (b) splenocyte, and (c) thymocyte proliferation (asterisks indicate the statistically significant compared to PBS control).

A long range HMBC experiment (Fig. 4, Table 3) was carried out to confirm the NOESY and ROESY connectivities. The cross-peaks of both anomeric protons and carbon of each sugar residues of the polysaccharides were examined, and both inter- and intra-residual connectivities were observed from HMBC experiments (Fig. 4, Table 3). Cross-peaks were found between H-1 (5.06 ppm) of residue **D** and C-5 (68.1 ppm) of residue **B** (**D** H-1, **B** C-5); C-1 (107.8 ppm) of residue **D**, and H-5a and H-5b of residue **B** (**D** C-1, **B** H-5a; **D** C-1, **B** H-5b), with other inter- and intra-residual coupling between H-1 of residue **D** and C-4 of residue **B** (**D** H-1, **B** C-4) and H-1 of residue **D** with its own C-4 atom (**D** H-1, **D** C-4). Similarly cross-peaks between H-1 (5.22 ppm) of residue **B** and C-2 (75.5 ppm) of residue **E** (**B** H-1, **E** C-2); C-1 (109.6 ppm) of residue **B** and H-2 (3.7 ppm) of residue **E** (**B** C-1, **E** H-2), with other intra-residual coupling between H-1 of residue **B** with its own C-4 (**B** H-1, **B** C-4), were observed. Cross-peaks were also observed between H-1 (4.93 ppm) of residue **E** and C-4 (77.1 ppm) of residue **F** (**E** H-1, **F** C-4); C-1 (100.8 ppm) of residue **E** and H-4 (3.98 ppm) of residue **F** (**E** C-1, **F** H-4). Cross-peaks between H-1 (4.93 ppm) of residue **F** and C-2 (76.0 ppm) of residue **C** (**F** H-1, **C** C-2); C-1 (100.8 ppm) of residue **F** and H-2 (3.93 ppm) of residue **C** (**F** C-1, **C** H-2) were observed. The cross-peaks between H-1 (5.06 ppm) of residue **C** and C-4 (77.1 ppm) of residue **E** (**C** H-1, **E** C-4), C-1 (100.8 ppm) of residue **C** and H-4 (3.99 ppm) of residue **E** (**C** C-1, **E** H-4) were observed. Again the cross-peaks between H-1 (5.37 ppm) of residue **A** and C-4 (79.4 ppm) of residue **C** (**A** H-1, **C** C-4); C-1 (100 ppm) of residue **A** and H-4 (3.48 ppm) of residue **C** (**A** C-1, **C** H-4) were observed. Intra-residual coupling between methoxyl carbon (53.3 ppm) and H-6a and H-6b proton of residue **A** [OMe (C), **A** H-6a] and [OMe (C), **A** H-6b] were observed. The cross-coupling of carbonyl cabon (171.0 ppm) of *O*-acetyl group with methyl proton (2.03 ppm) [OAc (C), OAc (H)] and H-3 (5.12 ppm) of residue **D** [OAc (C), **D** H-3] were found. Again cross-peak was also observed between carbomethoxyl proton (3.78 ppm) and ester carbonyl carbon (171.0 ppm) of residues **E** and **F**.

3.3. Assay for macrophage activity by NO

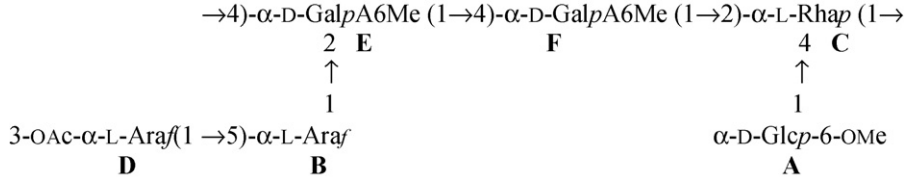
Some biological studies were carried out with this polysaccharide. Macrophage activation of the polysaccharide was observed in vitro. On treatment with different concentrations of the polysaccharide an enhanced production of NO was observed in a dose dependent manner with optimum production of 20.5 μM NO per 5×10^5 macrophages at 100 μg/mL of the polysaccharide (Fig. 5a). The various types of polysaccharides like lentinan inhibits tumor growth by stimulating the immune system (Chihara, 1978) through activation of macrophages, T-helper, NK, and other cells.

3.4. 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 (Ohno et al., 1993). The polysaccharide was tested to proliferate splenocyte and thymocyte as shown in Fig. 5(b) and (c), respectively, and the asterisks on the columns indicated the statistically significant differences compared to PBS (phosphate buffer solution) control. At 50 μg/mL of the polysaccharide, splenocyte proliferation index was observed maximum as compared to other concentrations. Hence, 50 μg/mL of the polysaccharide can be considered as efficient splenocyte proliferators. Again 50 μg/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 (Mondal, Chakraborty, & Rout, 2006; Rout, Mondal, Chakraborty, Pramanik, & Islam, 2004, 2005; Roy et al., 2009) and plant (Das et al., 2009; Ojha et al., 2009) polysaccharides had also shown similar type of splenocyte activation as well as phagocytic response of macrophages as reported earlier by our group.

4. Conclusion

A water soluble heteropolysaccharide was isolated from aqueous extract of the leaves of *C. rosea* and purified by gel-filtration chromatography. The reported material is not a pectic polysaccharide but the fragment [→4)-α-D-Gal*p*A6Me (1→4)-α-D-Gal*p*A6Me (1→] of pectin is present in the repeating unit of this polysaccharide. This molecule showed splenocyte and thymocyte activation and phagocytic response of macrophages in a dose dependent manner. On the basis of chemical analysis and NMR studies the structure of the repeating unit of the polysaccharide was established as



Acknowledgements

The authors are grateful to Professor S. Roy, Director, IICB, Dr. A.K. Sen (Junior), IICB, Kolkata, for providing instrumental facilities. Mr. Barun Majumder of Bose Institute, Kolkata, is acknowledged for preparing NMR spectra. DST, Government of India is acknowledged for sanctioning a project (Ref. No.: SR/S1/OC-52/2006 dated 19/02/2007). The author (S.P.) acknowledges CSIR for offering junior research fellowship.

References

Abdel-Akher, M., & Smith, F. (1950). Use of lithium aluminium hydride in the study of carbohydrates. *Nature*, *166*, 1037–1038.
Agrawal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, *31*, 3307–3330.
Antia, B. S., & Okokon, J. E. (2005). Effect of leaf juice of *Catharanthus roseus* Linn on cholesterol, triglyceride and lipoproteins levels in normal rats. *Indian Journal of Pharmacology*, *37*, 401–402.
Careew, D. P., & Patterson, B. D. (1970). The effect of antibiotics on the growth of *Catharanthus roseus* tissue cultures. *Lloydia*, *33*, 275–277.
Chattopadhyay, R. R., Sarkar, S. K., Ganguli, S., Banerjee, R. N., & Basu, T. K. (1991). Hypoglycaemic and antihypoglycaemic effect of leaf of *Vinca rosea* Linn. *Indian Journal of Physiology and Pharmacology*, *35*, 145–151.
Chattopadhyay, R. R., Sarkar, S. K., Ganguli, S., Banerjee, R. N., & Basu, T. K. (1992). Antiinflammatory and acute toxicity studies with leaves of *Vinca rosea* Linn in experimental animals. *Indian Journal of Physiology and Pharmacology*, *36*, 291–292.
Chihara, G. (1978). Antitumor and immunological properties of polysaccharides from fungal origin. *Mushroom Science*, *10*, 797–814.
Ciucanu, I., & Kerek, F. (1984). Simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.
Das, D., Mondal, S., Roy, S. K., Maiti, D., Bhunia, B., Maiti, T. K., et al. (2009). Isolation and characterization of a heteropolysaccharide from the corm of *Amorphophallus campanulatus*. *Carbohydrate Research*, *344*, 2581–2585.

Dueñas Chaso, M. T., Rodriguez-Carvajal, M. A., Mateo, P. T., Franko-Rodriguez, G., Espartero, J. L., Iribas, A. I., et al. (1997). Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus*. *Carbohydrate Research*, *303*, 453–458.

El-Sayed, A., & Cordell, G. L. (1981). Catharanthus alkaloids. XXXIV. Catharanthamine, a new antitumor bisindole alkaloid from *Catharanthus roseus*. *Journal of Natural Product*, *44*, 289–293.

El-Sayed, A., Handy, G. A., & Cordell, G. A. (1983). Catharanthus alkaloids, XXXVIII. Confirming structural evidence and antineoplastic activity of the bisindole alkaloids leurosine-N'b-oxide (pleurosine), roseadine and vindolicine from *Catharanthus roseus*. *Journal of Natural Product*, *46*, 517–527.

Fornsworth, N. R, Svoboda, G. H., & Blomster, R. N. (1968). Antiviral activity of selected Catharanthus alkaloids. *Journal of Pharmacological Sciences*, *57*, 2174–2175.

Gerwig, G. J., Kamarling, J. P., & Vliegenthart, J. F. G. (1978). Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. *Carbohydrate Research*, *62*, 349–357.

Ghosh, R. K., & Gupta, I. (1980). Effect of *Vinca rosea* and *Ficus racemososus* on hyperglycemia in rats. *Indian Journal of Animal Health*, *19*, 145–148.

Goldstein, I. J., Hay, G. W., Lewis, B. A., & Smith, F. (1965). Controlled degradation of polysaccharides by periodate oxidation, reduction and hydrolysis. *Methods in Carbohydrate Chemistry*, *5*, 361–370.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Analytical Biochemistry*, *126*, 131–138.

Hara, C., Kiho, T., Tanaka, Y., & Ukai, S. (1982). Anti-inflammatory activity and conformational behavior of a branched (1→3)-β-D-glucan from an alkaline extract of *Dictyophora indusiata* Fisch. *Carbohydrate Research*, *110*, 77–87.

Hård, K., Zadelhoff, G. V., Moonen, P., Kamerling, J. P., & Vliegenthart, J. F. G. (1992). The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male Novel sulfated and novel *N*-acetylgalactosamine-containing *N*-linked carbohydrate Chains. *European Journal of Biochemistry*, *209*, 895–915.

Hay, G. W., Lewis, B. A., & Smith, F. (1965). Periodate oxidation of polysaccharides: General procedures. *Methods in Carbohydrate Chemistry*, *5*, 357–361.

Hoffman, J., Lindberg, B., & Svensson, S. (1972). Determination of the anomeric configuration of sugar residues in acetylated oligo- and polysaccharides by oxidation with chromium trioxide in acetic acid. *Acta Chemica Scandinavica*, *26*, 661–666.

Jaleel, C. A., Manivannan, P., Sarkar, B., Kishorekumar, A., Sankari, S., & Rajaram, P. (2007). Induction of drought stress tolerance by keto-conazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. *Colloids and Surfaces B: Biointerfaces*, *60*, 201–206.

Maiti, S., Bhutia, S. K., Mallick, S. K., Kumar, A., Khadgi, N., & Maiti, T. K. (2008). Antiproliferative and immunostimulatory protein fraction from edible mushrooms. *Environmental Toxicology and Pharmacology*, *26*, 187–191.

Maness, N. O., Ryan, J. D., & Mort, A. J. (1990). Determination of the degree of methyl esterification of pectins in small samples by selective reduction of esterified galacturonic acid to galactose. *Analytical Biochemistry*, *185*, 346–352.

Mondal, S., Chakraborty, I., & Rout, D. (2006). Isolation and structural elucidation of a water-soluble polysaccharide (PS-I) of a wild edible mushroom, *Termitomyces striatus*. *Carbohydrate Research*, *341*, 878–886.

Ohno, N., Hasimato, T., Adachi, Y., & Yadomae, T. (1996). Conformation dependency of nitric oxide synthesis of murine peritoneal macrophages by β-glucans in vitro. *Immunology Letters*, *52*, 1–7.

Ohno, N., Saito, K., Nemoto, J., Kaneko, S., Adachi, Y., Nishijima, M., et al. (1993). Immunopharmacological characterization of a highly branched fungal (1→3)-β-D-glucan, OL-2, isolated from *Omphalia lapidescens*. *Biological & Pharmaceutical Bulletin*, *16*, 414–419.

Ojha, A. K., Chandra, K., Ghosh, K., Bhunia, B., Maiti, T. K., & Islam, S. S. (2009). Structural analysis of an immunoenhancing heteropolysaccharide isolated from the green (unripe) fruits of *Solenum melongena* (Brinjal). *Carbohydrate Research*, *344*, 2357–2363.

Raja, M. L., Nasir, M., Abbas, T., & Naqvi, B. S. (2009). Antibacterial activity of different extracts from the *Catharanthus roseus*. *Clinical and Experimental Medical Journal*, *3*, 81–85.

Rinaudo, M., & Vincendon, M. (1982). ^{13}C NMR structural investigation of scleroglucan. *Carbohydrate Polymers*, *2*, 135–144.

Rout, D., Mondal, S., Chakraborty, I., Pramanik, M., & Islam, S. S. (2004). Structural characterisation of an immunomodulating polysaccharide isolated from aqueous extract of *Pleurotus florida* fruit-bodies. *Medicinal Chemistry Research*, *13*, 509–517.

Rout, D., Mondal, S., Chakraborty, I., Pramanik, M., & Islam, S. S. (2005). Chemical analysis of a new (1→3)-, (1→6)-branched glucan from an edible mushroom, *Pleurotus florida*. *Carbohydrate Research*, *340*, 2533–2539.

Roy, S. K., Das, D., Mondal, S., Maiti, D., Bhunia, B., Maiti, T. K., et al. (2009). Structural studies of an immunoenhancing water-soluble glucan isolated from hot water extract of an edible mushroom, *Pleurotus florida*, cultivar Assam Florida. *Carbohydrate Research*, *344*, 2596–2601.

Sarangi, I., Ghosh, D., Bhutia, S. K., Mallick, S. K., & Maiti, T. K. (2006). Anti-tumor and immunomodulating effects of *Pleurotus ostreatus* mycelia derived proteoglycans. *International Immunopharmacology*, *6*, 1287–1297.

Singh, S. N., Vats, P., Suri, S., Shyam, R., Kumria, M. M., Ranganathan, S., et al. (2001). Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology*, *76*, 269–277.

Ueda, J. Y., Tesuka, Y., Banskota, A. H., Haimaya, Y., Saiki, I., & Kadota, S. (2002). Antiproliferative activity of Vietnamese medicinal plants. *Biological & Pharmaceutical Bulletin*, *25*, 753–760.

York, W. S., Darvill, A. K., McNeil, M., Stevenson, T. T., & Albersheim, P. (1985). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology*, *118*, 33–40.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, *49*, 5165–5170.