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Purification and Characterization of a Small Chito-specific Lectin from *Datura innoxia* Seeds Possessing Anti-microbial Properties

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Authors' contributions

This work was carried out in collaboration between both authors. Authors RS and CGS have envisaged the study, designed the protocols and performed the data analysis. Author RS carried out the experiments and wrote the first draft of the manuscript. Author CGS checked the analyses and edited the manuscript. Both authors read and approved the final form of manuscript.

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Original Research Article

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ABSTRACT

Aims: To purify and characterize the smallest lectin from *Datura innoxia* (*Di*L9) and to evaluate its antimicrobial application potential.
Study Design: Protein purification and characterization using biochemical and biophysical techniques. MALDI-MS/MS for peptide mass finger printing. *In vitro* assay of antimicrobial activity.
Place and Duration of Study: Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune, India, From Jan 2014 to July 2015.
Methodology: Dried *Datura* seeds were taken as source of lectin and purified using ion-exchange and gel filtration chromatographic techniques. The rabbit erythrocytes were used for the study of hemagglutinating activity. Structural properties were studied using Fluorescence and Circular dichroism spectroscopic methods. The studied lectin was also examined for its antimicrobial activity using agar disc diffusion method and minimum inhibitory concentration (MIC) was calculated using microdilution method.

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Results: The lectin was purified from dried seeds of *Datura innoxia*. It was characterized as a chito-specific glycoprotein with 4% neutral carbohydrate content and molecular weight of 9 kDa. Circular dichroism and fluorescence studies showed it to be structurally different from other known lectins isolated from *Datura*. Protein was found to be highly thermostable with sugar binding capacity spread over a broad pH range (1-12), and showed resistance to effects of organic solvents, chemical denaturation and proteolytic digestion. *Di*L9 also demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria; however, more effective against *Enterococcus faecalis* and *Bacillus cereus* with MIC values at 0.325 and 0.15 mg ml^{-1}, respectively.
Conclusion: The present study revealed that the lectin purified from *D. innoxia* is a glycoprotein and agglutinates pronase treated rabbit erythrocytes. The lectin is highly thermostable with respect to its structure and activity at all denaturing conditions, also showed significant antibacterial activity. This data also supports the fact of its traditional use in folk or alternative medicines for treatment of bacterial infections.

Keywords: Lectin; Datura innoxia; chito-specific; thermostable; circular dichroism; fluorescence; antibacterial activity.

ABBREVIATIONS

DiL9: *Datura innoxia lectin 9 kDa;* ***GDn-HCl:*** *Guanidine hydrochloride;* ***GDn-SCN:*** *Guanidine-thiocyanate;* ***GlcNAc:*** *N-acetyl glucosamine;* ***DMSO:*** *Dimethyl sulfoxide;* ***ACN:*** *Acetonitrile*.

1. INTRODUCTION

Plants have been used as a remedy for human diseases owing to the presence of various therapeutic components in them capable of acting as antimicrobial agents. *Datura innoxia* (Family: *Solanaceae*) or toloache, is one such plant which has been used for various purposes such as pain killer, fever reducing agent, cure for diarrhea and skin diseases [1]. All parts of the plant possess hallucinogenic, hypnotic and narcotic effects and are considered traditional medicines around the world. An Indian traditional medical practice, despite toxicity, recognized the medicinal effects of *Datura* as depended on the dosage and method of medicine preparation. The plant is originally a native of Central and South America; it is later introduced in Asia. It is cultivated in southeastern Europe mainly for the production of scopolamine and other alkaloids [2].

Among the many therapeutic components present in plants, lectin is one of the most potent antimicrobial agents known. Lectin is a hemagglutinating protein which can bind sugars present on cell surfaces and can precipitate as glycoconjugates [3]. Lectins are mostly extracted from the plant storage organs like seeds, and may constitute upto 10% of its total protein content [4]. Other parts of the plant also contain lower amounts of lectins, sometimes with different carbohydrate specificities or having enzymatic function. In the field of biomedicine, lectins have been used as antimicrobial and diagnostic tools. Lectin through its interaction with bacterial cell wall components like teichoic acids, peptidoglycans and lipopolysaccharides, inhibits the growth of Gram-positive and Gram-negative bacteria by agglutinating and immobilizing the microorganisms [5,6]. Lectins are also known to inhibit the growth of fungi by attaching to hyphas, and thus interfering with its nutrient absorption and spore germination process [7], disturbing the synthesis or deposition of chitin monomers in the cell wall. Many pathogens have become resistant to multiple antibiotics and threaten to develop immunity against the currently used antimicrobial drugs and therefore infection becomes untreatable. Thus, the search for new antimicrobial agent is of utmost importance. In the present work we have purified and characterized *Datura innoxia* lectin and explored its antimicrobial activity.

There are only few reports available on the characterization of lectins from *Solanaceae* family and their medical applications. For instance, a lectin purified from *Solanum tuberosum* showed antimicrobial and antibiofilm activities [8] whereas one from *Datura stramonium* was shown mitogenic [9]. Previously, few lectins from *Datura innoxia* have been reported which are of high molecular weight, around 150 and 300 kDa [10]. Other high molecular weight *Datura innoxia* lectins (82, 54, 49 and 41 kDa) are also reported having N-acetyl

glucosamine oligomers specificity [11]. This is the first report of a small chito-specific agglutinin purified from the seeds of *Datura innoxia* (*Di*L9) and characterized using biochemical and biophysical techniques, which is part of an effort to expand the knowledge of lectins for novel applications.

2. MATERIALS AND METHODS

2.1 Biological Materials

The seeds of *Datura innoxia* were collected locally from the premises of CSIR-National Chemical Laboratory (NCL), Pune, India. The rabbit blood was procured from National Toxicology Centre (NTC), Pune, India. The bacterial and fungal strains were obtained from National Collection of Industrial Microorganism (NCIM), CSIR-NCL, Pune, India.

2.2 Chemicals and Reagents

Q-sepharose, Sephacyrl S-200, bovine serum albumin (BSA), urea, guanidine hydrochloride, guanidine thiocyanate, chemical modifiers like diethylpyrocarbonate (DEPC), 2,4,6-trinitro-benzenesulphonic acid (TNBS), Woodward's reagent K (WRK); phenylglyoxal (PGO), N-acetylimidazole (NAI), phenylmethylsulfonyl fluoride (PMSF), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB); gel filtration markers, sugars and sugar derivatives were purchased from Sigma Chemicals Co. (USA). Markers used in SDS-PAGE were procured from Bio-Rad. All other reagents and buffers used in the study were of analytical grade.

2.3 Isolation and Purification of Lectin from *Datura innoxia* Seeds

Twenty grams of dried seeds of *Datura innoxia* were soaked and ground in 20 mM sodium phosphate buffer, pH 7.4. The homogenate was filtered through double layered muslin cloth and centrifuged at 10000x rpm for 20 min at 4ºC. The supernatant was harvested and precipitated with 60% ammonium sulphate at 4ºC, dialyzed against 20 mM sodium phosphate buffer, pH 7.4 and then subjected to Q-sepharose column chromatography, column equilibrated with the same buffer. The lectin was eluted in the unbound fractions. The eluted fractions showing hemagglutinating activity were pooled, dialyzed against TBS (50 mM Tris-Cl, pH 8.0 containing 150 mM NaCl) and loaded on gel filtration column (Sephacyrl S-200) equilibrated previously with TBS. On the basis of absorbance at 280 nm (A_{280}) and detectable hemagglutinating activity the fractions were pooled, dialyzed against deionized water and stored at -20ºC for further use.

2.4 Determination of Protein and Carbohydrate Content

The amount of protein was monitored by measuring the absorbance at 280 nm of the column eluate. Protein concentration was estimated using Lowry method with BSA as standard [12]. Phenol-Sulphuric method was used to determine the neutral carbohydrate content with slight modification using mannose as standard, as reported [13].

2.5 Molecular Weight Determination

The molecular weight was estimated by running 12% SDS-PAGE and native subunit structure was determined using Gel filtration chromatography (Sephacryl S-200) and MALDI-TOF/TOF.

2.6 Hemagglutination and Sugar Specificity Test

A 2-fold serial dilution of purified *Datura* lectin in 10 mM phosphate buffered saline (PBS, pH 7.4, 50 µl) were mixed with 50 µl of 3% rabbit erythrocyte suspension in 96-well U-shaped microtiter plates at room temperature and the agglutination was observed after 1 h of incubation. Reciprocal of the highest dilution exhibiting detectable agglutination gives hemagglutination titre (HU) and the specific activity was calculated as HU mg^{-1} protein. Sugar specificity of *Di*L9 was examined by estimating the ability of sugars to inhibit agglutination. Various glycoproteins, carbohydrates and their derivatives were incubated with *Di*L9 and the assay was carried out.

2.7 Chemical Modification Studies

Chemical modification is one of the most widely used techniques to study ligand/sugar binding sites and identification of the residues involved in substrate binding. To determine the role played by tryptophan residues in lectin activity, NBS modification method was attempted [14]. Modification of histidine residues with DEPC prepared in absolute ethanol was carried out

[15]. PMSF was used for serine modification in 50 mM Tris-HCl buffer [16]. Lysine residues were modified with TNBS [17]. Modification of the carboxylate groups with WRK was carried out in 50 mM citrate-phosphate buffer pH 6.0, with different concentration range (5-20 mM) of WRK for 30 min [18]. Arginine residues were modified with phenylglyoxal [19]. Free cysteine residues were modified using DTNB [20]. NAI was used as chemical modifier for tyrosine residues [21]. The native lectin without any reagent was used as a positive control for hemagglutination activity in each case of chemical modification. Each assay was performed in triplicates.

2.8 Effect of pH on Lectin Activity and Stability

Various buffers were used to determine the lectin stability in range of pH 1-12 and activity at room temperature for 12 h. Buffers used were: 20 mM glycine-HCl (pH 1-3), 20 mM citrate-phosphate (pH 4-6), 20 mM sodium- phosphate (pH 7), 20 mM tris-HCl (pH 8-9) and 20 mM glycine-NaOH (pH 10-12). The lectin sample at room temperature served as positive control and buffer was taken as negative control. Each assay was performed in triplicates.

2.9 Thermal Inactivation Assay

The effect of temperature on lectin activity was determined by incubating the sample at different temperatures (25-100ºC) for 30 min. The kinetics of inactivation to deduce thermostability of the lectin was carried out by first incubating the sample at higher temperature in PBS for fixed intervals of time (20-120 min) followed by cooling to room temperature and assaying the activity, as mentioned earlier. For lectin heat denaturation process, the free energy change (ΔG) of activation was also determined using Arrhenius equation [22], a simple way to understand temperature dependence of reaction rates.

$$\Delta G = RT \ln(kT/k_1h) \quad (1)$$

where R is the gas constant (1.987 cal mol^{-1} K^{-1}), T is the absolute temperature (K), k is the Boltzmann constant (1.37 x 10^{-6} erg K^{-1}), k_1 is the velocity constant and h is Planck's constant (6.25 x 10^{-27} erg s^{-1}).

The velocity constant (k_1) can be calculated from the slope of the curve obtained from the following equation,

$$k_1t = - \ln (A/A_o) \quad (2)$$

where t is the time of heat treatment (s), A is the residual hemagglutinating activity and A_o is the initial hemagglutinating activity before heat treatment.

2.10 Effect of EDTA, Metal Ions and Chemical Denaturants

*Di*L9 was incubated with EDTA, various divalent metal ions, chemical denaturants like urea, guanidine hydrochloride (GDn-HCl) and guanidine thiocyanate (GDn-SCN) and reducing agents like β- mercaptoethanol (βME) and dithiothreitol (DTT) for 24 h at different concentrations. After incubation, 50 µl aliquot was withdrawn and residual activity was scored after 1 h in triplicates as described above.

2.11 Stability of *Di*L9 in Organic Solvents

To study the stability of *Di*L9 in various organic solvents, agglutination assay was carried out. The lectin was incubated in ethanol, methanol, acetonitrile (ACN), dimethylsulphoxide (DMSO) and propanol (25% and 50% v/v) at pH 7.4 for 24 h. All these solvents were completely miscible in deionized water. Presence of organic solvents can modify/alter the sugar binding sites of the lectin. The incubation was performed at room temperature in tightly closed vials to prevent evaporation. 50 µl aliquots were withdrawn at regular intervals of time for the hemagglutination assay.

2.12 Resistance to Proteolysis

Proteases such as pepsin, trypsin, chymotrypsin and pronase were used for proteolytic digestion of *Di*L9 at 37°C in 20 mM sodium phosphate buffer at pH 7.4. Lectin and each proteolytic enzyme were incubated at 10:1 molar ratio for 24 h. 50 µl aliquots were removed at regular intervals and assayed for hemagglutination activity.

2.13 Spectroscopic Measurements

2.13.1 Fluorescence studies

The intrinsic fluorescence of *Di*L9 was recorded using a Perkin Elmer Life Sciences LS50 spectrofluorimeter. The protein solution (0.05 mg ml^{-1}) was excited at 295 nm and emission was measured in the range of wavelengths 310-400 nm with slit width of 7 nm for both monochromators. The spectrum was recorded at

28ºC and the contribution of the solvent was subtracted from the protein spectra for further analysis. The barycentric mean (BCM) was calculated according to the equation:

$$BCM_\lambda = \sum(F_\lambda)x\ \lambda / \sum F_\lambda \quad (3)$$

where F_λ is the fluorescence emission at wavelength λ, and the summation was carried out over the range of appreciable values of *F*.

2.13.2 Circular dichroism (CD) studies

CD measurements of *Di*L9 were performed on a JASCO J-815-150S (Jasco, Tokyo, Japan) spectropolarimeter. Far-UV CD spectrum of the lectin (0.2 mg ml^{-1}) was recorded in a rectangular quartz cell of 1 mm path-length in the wavelength range 190-250 nm. Each recorded spectrum was an average of 3 accumulations. The baselines (buffer spectra) were subtracted from the protein spectra for further analysis. Results were expressed in terms of mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as:

$$MRE = M\ \theta_\lambda /10\ d\ c\ r \quad (4)$$

where ***M*** is the molecular weight of the protein, $\boldsymbol{\theta_\lambda}$ is the observed ellipticity in millidegree, ***d*** is the cell pathlength in cm, ***c*** is the protein concentration in mg ml^{-1} and ***r*** is the average number of amino acid residues in the protein. CDPro software was used to determine the secondary structure elements of the lectin (http://lamar.colostate.edu/~sreeram/CDPro/main.html). Low NRMSD values were observed for analysis with CONTINLL. The scans were also taken for lectin incubated in the presence of chemical modifiers and organic solvents as described in section 2.7 & 2.11.

2.14 Mass Spectrometry

To identify the protein, the in gel digestion protocol was carried out using mass spectrometry [23]. The trypsin digested peptides were mixed with a solution of α-cyano-4-hydroxycinnamic acid (10 mg ml^{-1}) prepared in 50% acetonitrile and 0.3% trifluoroacetic acid. The samples were analyzed in a mass spectrometer MALDI-TOF/TOF in reflection mode. On an average 100 MS/MS spectra were measured for the digested protein and were analyzed using Protein pilot software. The plant lectin database was created using Uniprot online software.

2.15 Evaluation of Antimicrobial Activity

2.15.1 Antibacterial assay

Antimicrobial activity of the lectin was examined using disc diffusion method [24] on sterile nutrient agar (NA) plates. Total of 6 species (listed in Table 2) of pathogenic bacteria were selected and were seeded separately onto the surface of NA plates. The lectin sample was aseptically added on the sterile disc placed on the nutrient agar plates and the organisms were allowed to grow at 37°C for 12 h. The transparent ring around the disc shows zone of inhibition measured in millimeters (mm) indicating the antimicrobial activity of the lectin. Anti-mycobacterial activity of lectin was also evaluated for its *in vitro* effects against dormant and active phases of *M. tuberculosis* H37Ra using XTT Reduction Menadione Assay (XRMA) protocol [25]. All experiments were performed in triplicates.

2.15.2 Antifungal Assay

Antifungal bioassays were performed against *Candida albicans, Trichoderma viride, Gibberella saubinetii, Fusarium oxysporum, Cephalosporium* sp*, Saccharomyces cerevisiae, Fusarium moniliforme* and *Aspergillus falvus* using agar well diffusion method [24], in order to determine the susceptibility of these pathogenic fungi towards *Di*L9. Amphotericin B (as positive control) and lectin were dissolved in deionized water and added to the well (8 mm in diameter) of potato dextrose agar plates, incubated at 28°C. The diameter of the clear zone around the well was measured and considered as the inhibitory power of the lectin against the particular test pathogen.

3. RESULTS AND DISCUSSION

Since lectins are known for their potential in applications such as anti-microbials, anticancer; characterization of lectins and understanding the structure-function relationship are highly essential to determine their applications in clinical microbiology and pharmaceutical industry. A combination of various biochemical and biophysical techniques were used to characterize the smallest lectin purified from *Datura innoxia* to understand the protein structure-function relationship. Antimicrobial assays were also carried out to explore its antibacterial and antifungal activity.

3.1 Purification and Structural Characterization of *Di*L9

The *Di*L9 agglutinin was purified using Q-sepharose ion exchange and gel filtration chromatography. On gel filtration column, the protein was resolved into two peaks, P1 and P2 (Fig. 1) and lectin activity was observed for both the peaks. The lectin from P1 peak corresponded to a 88 kDa lectin which was already reported from *Datura* seeds [11], while lectin (*Di*L9) obtained from P2 peak was of the size 9 kDa. We understand that this is the smallest agglutinin, which is characterized in this study. Table 1 summarizes the purification step; specific activity and enrichment fold of *Di*L9 lectin. It is important to note that *Datura* seeds are rich in different types of lectins, and hence we observed a decrease in activity per unit mass of protein at each step of chromatographic techniques presumably by the removal of other lectins from the preparation.

3.1.1 Molecular weight determination and estimation of carbohydrate content

The purity of *D. innoxia* lectin was evaluated at each step of purification using SDS-PAGE under reducing conditions. The ammonium sulphate precipitated protein showed many bands ranging from 6-97 kDa. After Q-sepharose chromatography, the unbound fractions gave only two bands of 9 and 88 kDa possessing lectin activity. As already discussed, the 88 kDa lectin was previously reported, so we focused mainly on the small agglutinin which was finally purified using sephacryl S-200 (Fig. 2A). The native polyacrylamide gel electrophoresis showed that *Di*L9 moved as a single band. MALDI-TOF/TOF provided the exact molecular weight measured to be 9656.6 Da, a monomeric lectin (Fig. 2B). Hence, *Di*L9 is representation for *Datura innoxia* lectin of molecular weight 9 kDa. It is a glycoprotein containing 4% neutral carbohydrate estimated using phenol-sulphuric acid assay. This lectin has lower content of sugar as compared to the other lectins characterized from the same source. For instance, the sugar content reported for *Datura innoxia* seed agglutinin (DIA) of molecular weight 88 kDa was 25 % [11]. The other Solanaceae lectins like potato lectin [26] and Jimson weed lectin [9] had 50% and 40% carbohydrate content, respectively.

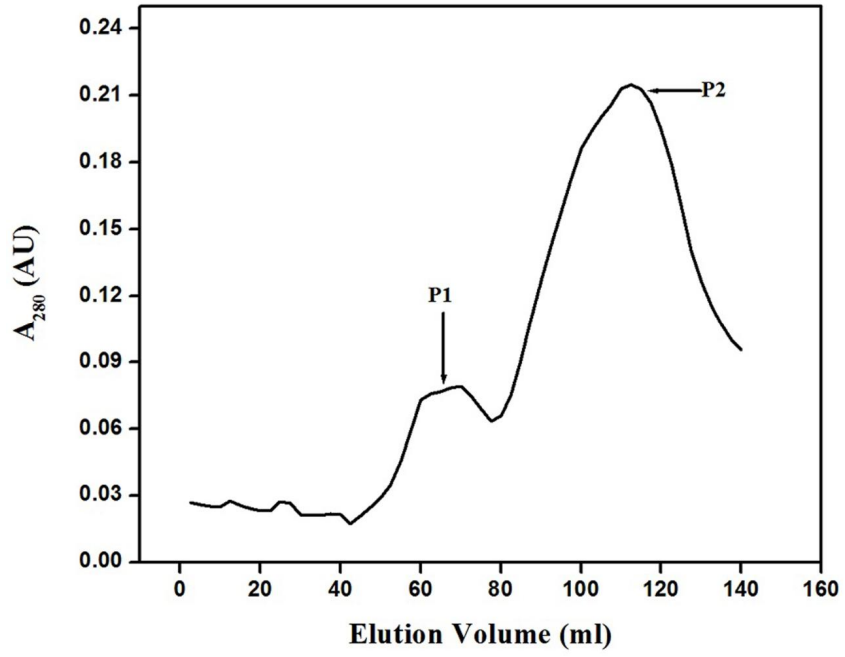

Fig. 1. Purification profile of *Di*L9 using Sephacryl S-200 gel filtration chromatography, equilibrated with TBS at flow rate of 1 ml/min. The column resolved into two different peaks (P1 & P2). P2 corresponds to *Di*L9. Data shown are representative of three such repeats

Table 1. Summary of *Di*L9 purification from 20 g seeds of *D. innoxia*

Fraction	Volume (ml)	Total protein(mg)[1]	Total activity	Specific activity[2]	Fold purification[3]	% yield
Crude extract	230	529	36800	69.56	1.0	100
Q- Sepharose	70	62	11200	180.6	8.5	30.4
Sephacryl S-200	22	26.4	880	34	20	2.4

[1] *Crude protein extract from 20 g seeds of D. innoxia*

[2] *Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg ml^{-1}) of the assay solution*

[3] *Purification fold was calculated as the ratio of total protein in the extract to the purified protein at each purification step*

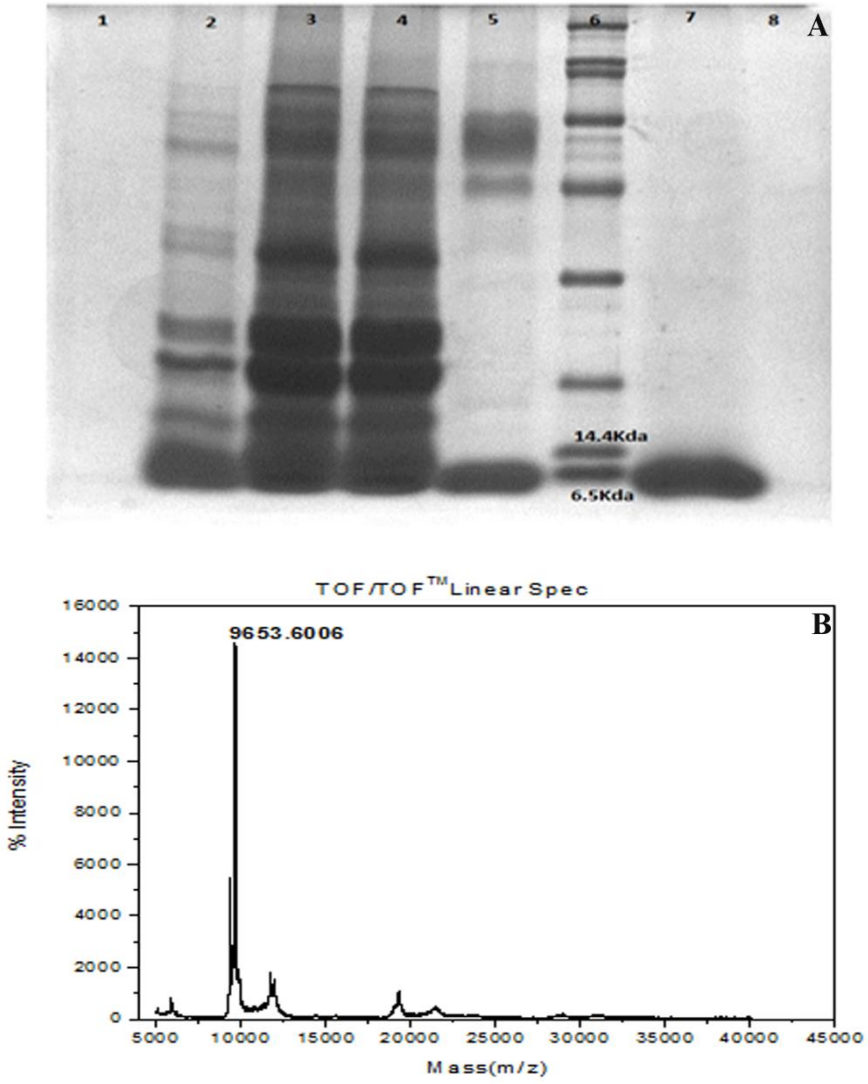

Fig. 2. Estimation of molecular weight of *Di*L9. (A) Purity check on 12% SDS-PAGE. Lane 2: crude extract; lane 3-4: 60% ammonium sulphate precipitate; lane 5: loaded on Sephacryl S-200 column; 6-Protein molecular weight marker; 7- Pure *Di*L9. (B) MALDI-TOF/TOF spectra

3.1.2 Mass spectrometry analysis

The MALDI TOF-MS/MS data analysis gave us the average masses of several peptides which were obtained after trypsin digestion. However, few sequence similarity was observed with Con A lectin and lectin fragment from *Caragana frutex* and *Penaeus japonicas,* which are distinctly related (Table 2). Due to lack of sufficient homology data for protein sequences in the databases, it was not possible to elucidate primary structure of the lectin by peptide mass fingerprinting. Hence, different techniques need to be applied to determine the primary sequence.

3.1.3 Characterization using fluorescence and CD

The intrinsic fluorescence of native *Di*L9 gave λ_{max} (emission wavelength) of 354 nm indicating that the tryptophan residues were exposed on the protein surface. A total of 4 Trp residues have been estimated through NBS titration, of which three are present on the surface and one is buried. In the presence of 6M GDn-HCl, a shift of 3 nm was observed in the barycentric mean (Fig. 3A). This shows that the lectin has been unfolded and the buried Trp residues have become exposed to the polar solvent resulting in the shifting of λ_{max} towards longer wavelengths.

The far-UV CD spectrum of *Di*L9 is shown in Fig. 3B. The calculation of secondary structure elements by CONTINLL program of CDPro analysis showed α-helix: 31.5%, β-sheet: 17.9%, turns: 20.7% and random coil: 29.8%. Previously, two high molecular weight lectins (150 kDa and 300 kDa) from *Datura* have been characterized using CD spectral studies and were shown to have conformations of polyproline II type [27], which was found to be absent in *Di*L9. Thus, these lectins are different with respect to their structural features.

3.2 Biochemical Characterization

3.2.1 Carbohydrate specificity of *Di*L9

*Di*L9 exhibited low agglutination activity when assayed with rabbit erythrocytes even at higher concentration of the protein whereas a minimal concentration of the protein was required for agglutination with pronase treated erythrocytes. Pronase is a nonspecific protease that cleaves membrane proteins present on erythrocytes and exposes other receptor glycoproteins. This difference in the agglutination activity could be due to unavailability of or weak binding to the receptor sites on erythrocytes by *Di*L9. No change in lectin agglutination activity was observed when incubated with EDTA and divalent metal ions, thus suggesting that it could be a metal ion independent protein for hemagglutination. The sugar specificity of *Di*L9 was tested against several sugars and glycoproteins by hemagglutination inhibition assay. Of all the carbohydrates tested, only N-acetyl glucosamine (GlcNAc) oligomers, i.e., *N,N',N''-triacetylchitotriose* (2.5 mM) showed inhibitory action. The monomer glucosamine, GlcNAc and N,N'-diacetylchitobiose did not show inhibition even at higher concentrations (Table 3). This confirms that the sugar binding site requires at least three β (1→ 4)-linked N-acetylglucosamine units. Similarly, other lectins from *Datura stramonium* [28], *Solanum tuberosum* [29] and *Urtica dioica* [30] *were* also known to show GlcNAc oligomer sugar specificity. The glycoproteins tested such as fetuin, ovalbumin, mucin, casein, etc., also showed hemagglutination inhibition at low concentrations.

3.2.2 Chemical modification studies

As a consequence of protein microenvironment within the folded structure, protein side chains frequently exhibit reactivity different from those expected from studies with model compounds. Here, purified lectin, *Di*L9, was subjected to various chemical modifications in order to determine the amino acid residues that are responsible for the hemagglutinating activity. It was observed that in the presence of 2 mM NBS, there was a complete loss of activity, implying direct involvement of tryptophan in sugar binding. This observation could be correlated with the intrinsic fluorescence study which showed presence of exposed tryptophan residues which might be directly involved in sugar binding. About 50% and 25% residual hemagglutination activity was observed on NAI and WRK treatment indicating possible role of tyrosine and aspartate/glutamate residues near the sugar binding region of the protein (Table 4). The far-UV CD spectra also showed loss of secondary structure in the presence of these chemical modifiers (2 mM, 4 h incubation), thus resulting in the loss of lectin activity. Incubating the lectin with NBS resulted in complete loss of structure, and hence no activity was observed as mentioned before (Fig. 4). Other chemical modifiers did not show any significant change in

the hemagglutination activity of *Di*L9. From these findings it can be concluded that Trp, Tyr and Asp/Glu are involved in carbohydrate binding site of *Di*L9 lectin.

3.2.3 Effect of pH on lectin activity

The hemagglutination activity of *Di*L9 was found to be stable in the pH range 1-12, retaining full activity even after incubating for 24 h. Similar behavior was observed in other lectins as well, like lectin from *Allium sativum* [31] *and Hericium erinaceum* [32]

3.2.4 Effect of temperature on lectin activity and stability

*Di*L9 was found to be thermostable till 90ºC. The lectin retained 100% of its hemagglutination activity at 60ºC for 2 h of incubation. With increase in temperature, the lectin showed sigmoidal decay curve with 50%, 25% and 12.5% activity at 70, 80 and 90ºC, respectively (Fig. 5A). The thermostability of *Di*L9 in terms of hemagglutinating activity varies with both temperatures as well with incubation time i.e., lectin retained 50% of its maximum activity at 70ºC even after 2 h of incubation, but rapidly inactivated at 100ºC. This implies that the lectin undergoes conformational changes under extreme temperatures resulting in the loss of activity (Fig. 5B). Examples of other lectins with similar thermostability with respect to hemagglutinating activity includes lectin from edible mushroom *Agaricus arvensis* [33], *Pinellia ternate* [34] and *Trichosanthes dioica* [35].

Table 2. Analysis of MALDI-MS/MS data using protein pilot software

Sl.no	Species	Sequence	Accessions	Homology (%)
1.	Lectin fragment from Caragana frutex	DWDPTGDR	tr|Q9ZRN7|Q9ZRN7_9FABA	9
2.	Calcineurin B-like protein 2 OS=Oryza sativa subsp. japonica	GLEDPQVLAR	sp|Q3HRP5|CNBL2_ORYSJ	4.4
3.	C-type lectin from Penaeus japonicus	GGVWFDTTMR	tr|I2C0B3|I2C0B3_PENJP	3
4.	REVERSED Concanavalin A-like lectins	EILDYTMGR	RRRRRtr|S3DKM5|S3DKM5_GLAL2	1.2
5.	Coatomer subunit beta OS=Setaria italica GN=Si000204m.g PE=4 SV=1	LLCNTGDDVR	tr|K3XE87|K3XE87_SETIT	1

Table 3. Hemagglutination inhibition activity of *Di*L9 by saccharides and glycoproteins

S.no	Sugar / Glycoprotein	Minimum concentrations required to inhibit *Di*L9 activity (µg ml^{-1})
1.	Glucosamine	NI*
2.	N-acetyl glucosamine	NI
3	N,N'-Diacetylchitobiose	NI
4.	N, N',N"- Triacetylchitotriose	750
5.	Fetuin	62.5
6.	Ovalbumin	78
7.	Bovine submaxillary mucin	156
8.	Thyroglobulin	9

**NI: No inhibition*

Table 4. Effect of chemical modification on *Di*L9 activity

Sl.no	Chemical modifier	Concentration (mM)	Residual hemagglutinating activity (%) #
1.	Native	0	100
2.	DTNB	10	100
3.	TNBS	10	100
4.	WRK	5	25
5.	NAI	10	50
6.	NBS	2	0
7.	PMSF	20	100
8.	PGO	10	100
9.	DEPC	10	75

#Full activity (100%) of DiL9 corresponds to titre of 2^6

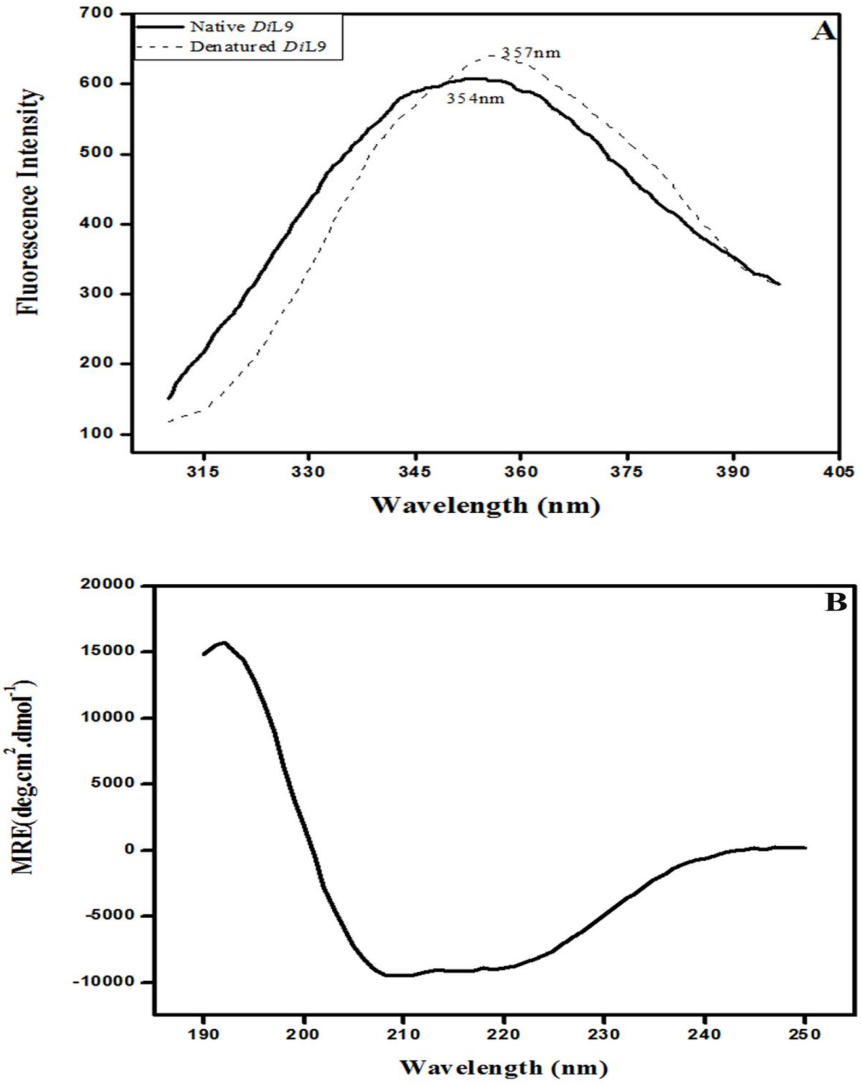

Fig. 3. Biophysical characterization of *Di*L9. (A) Intrinsic fluorescence spectra of native (λ_{max} at 354 nm) and denatured (λ_{max} at 357 nm) *Di*L9 (0.05 mg ml^{-1} at pH 7.4, 28°C). (B) Far-UV CD spectra of *Di*L9 (0.2 mg ml^{-1})

The ΔG value or the Gibb's free energy of thermostability at different incubation temperatures and time intervals were calculated. For example, the ΔG value at 60ºC was found to be zero, due to no change in hemagglutination activity implying no thermal enthalpy (ΔH) change. But, with increase in temperature the ΔG values were found to be positive indicating that energy is absorbed in a non-spontaneous process. Considering this phenomenon, the maximum residual hemagglutinating activity observed at 90ºC for 20 min corresponded to ΔG of 43 kcal mol^{-1}; the lectin is considered stable at this temperature where the entropies (S) of the native and denatured states are equal. Since many lectins are known to be toxic and show anti nutritional effects on mammals, including humans, the free energy of activation of lectin denaturation process remains an important physicochemical parameter when considering lectin's applications in various fields like medicine, agriculture and other related fields of human and animal nutrition [36].

3.2.5 Effect of chemical denaturants

Chaotropic agents such as urea, GDn-HCl and GDn-SCN at high concentrations disrupt the hydrophobic interactions in the interior of lectin that are essential to maintain the native conformation. Urea had no effect on *Di*L9 activity. However, complete loss of hemagglutination activity of *Di*L9 was observed in the presence of GDn-SCN (2M) but retained 12.5 % of residual activity in the presence of GDn-HCl (6M) for 24 h (Fig. 6). It could be concluded that GDn-SCN is the most potent denaturing agent for this lectin. In the presence of βME and DTT (2-5 mM), complete loss of lectin activity was observed implying presence of disulphide bridges that play important role in maintaining the possible tertiary structure of the protein.

3.2.6 Stability of *Di*L9 in organic solvents

Physical properties like miscibility, hydrophobicity and hydrogen bonding capacity of organic solvents can have drastic influence on the structural and functional aspects of proteins. Here, *Di*L9 showed good tolerance towards high concentrations of organic solvents. The hemagglutinating activity of the lectin was retained completely in the presence of methanol and ACN (all 50% v/v), and remained constant up to 24 h of incubation. The far-UV CD spectra of *Di*L9 in presence of organic solvents also showed increase in negative ellipticity implying enhancement in the compactness of the lectin structure.

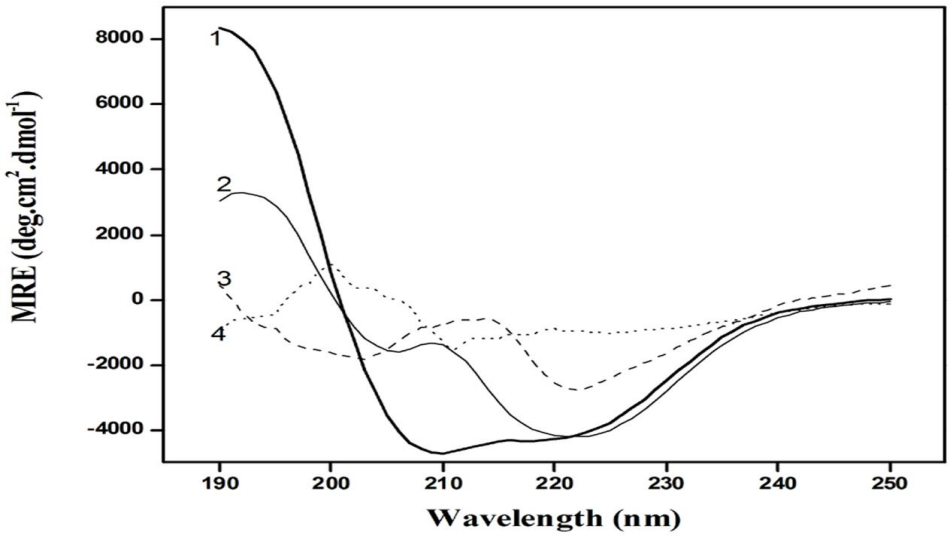

Fig. 4. Far-UV CD spectra of chemical modification effects on *Di*L9; 1- Native *Di*L9, 2-NAI, 3-WRK and 4- NBS treated *Di*L9. The lectin (0.2 mg ml^{-1}) was incubated with 2 mM of each chemical modifier for 4 h

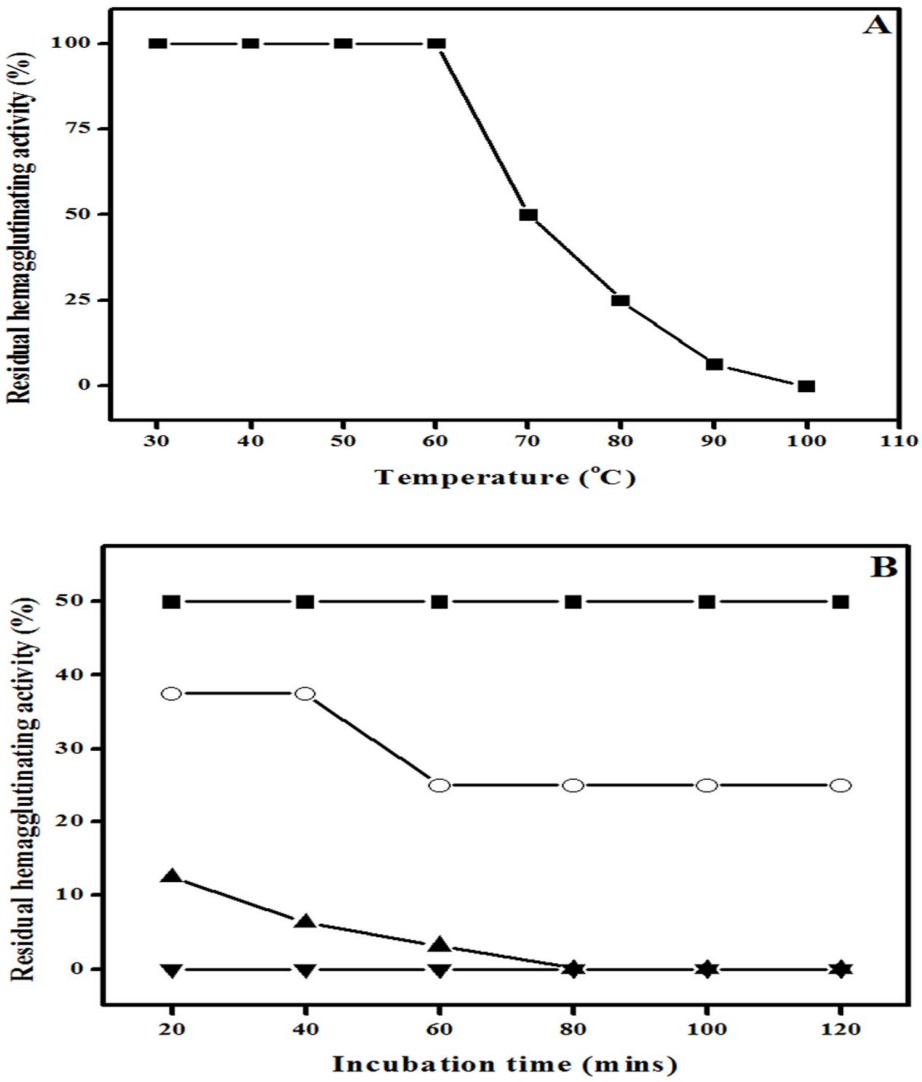

Fig. 5. Thermal denaturation of *Di*L9. (A) Effect of temperature on agglutinating activity of purified *Di*L9 towards a rabbit erythrocyte suspension in PBS. (B) Thermostability of *Di*L9 at: (-■-) 70ºC; (-○-) 80ºC; (-▲-) 90ºC; and (-▼-) 100ºC. Each point on the line is the mean (+1 SD) derived from three replicates. Full activity (100%) corresponds to a titre of 2^6

The hemagglutinating activity of *Di*L9 in organic solvents was observed to decrease with an increase in the carbon chain length of the solvent like ethanol as well as in DMSO. The possible reason could be that the solvents compete for hydrogen bonding interaction with protein atoms, thus disrupting the structure [37]. These observations were also reflected in the far-UV CD spectra of *Di*L9 incubated with 50% organic solvents for 4 h, where *Di*L9 in the presence of methanol and ACN retained its structure, on the other hand lost complete structure on incubating with ethanol and DMSO (Fig. 7).

3.2.7 Proteolytic effects on *Di*L9 activity

As part of interrelationship between the structure and activity of the lectins, it is important to understand the conditions under which the molecule resists the conformational changes in order to retain its native conformation and biological activity. Capacity of the *D. innoxia* lectin for being digested by various proteolytic enzymes with different specificity was also investigated. No change in the hemagglutination activity of the lectin was observed when incubated with trypsin (EC 3.4.21.4),

chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1) and papain (EC 3.4.21.62) at 37ºC for 24 h (data not shown). However, in the presence of protease (Nagarse; 3.4.21.62), 50% loss in lectin activity was observed. This feature was found to be in accordance with reported susceptibility of other *Datura* lectins to proteolysis [38]. The resistance of *Di*L9 and other lectins to proteolysis was attributed to the fact that it's a glycoprotein and is well stabilized by numerous disulphide bonds, because of which the native structure is retained under denaturing conditions.

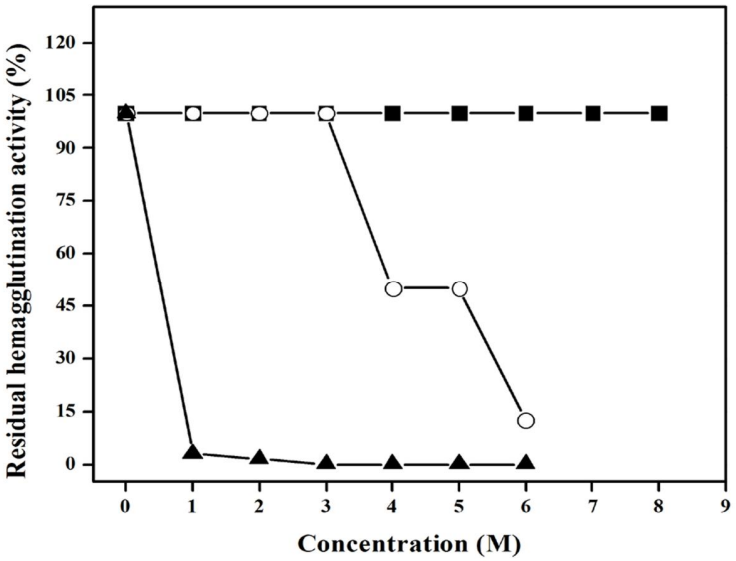

Fig. 6. Effect of chemical denaturants on *Di*L9. Lectin was incubated with required denaturant concentration, 20mM phosphate buffer, pH 7.4 for 16 h at room temperature. (-■-) Urea; (-O-) GDn-HCl and (-▲-) GDn-SCn

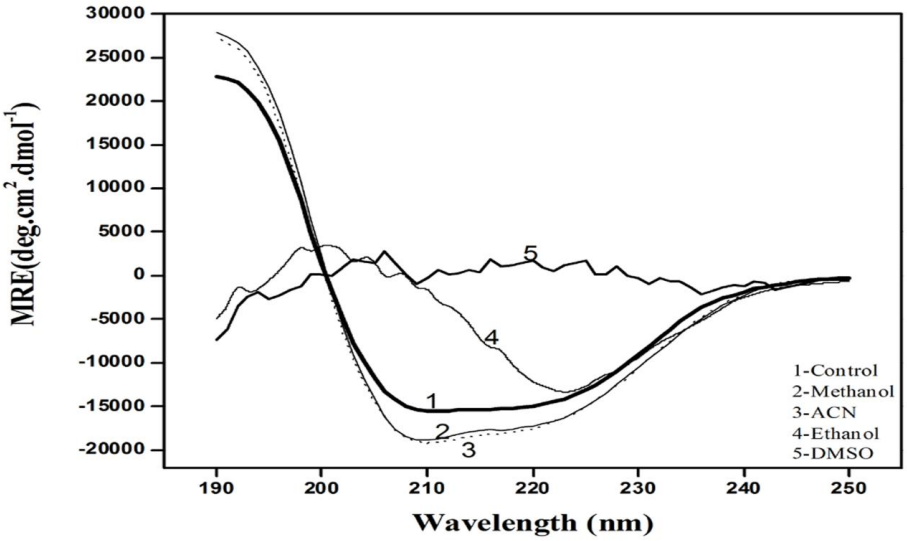

Fig. 7. Effect of organic solvents on far-UV CD spectra of *Di*L9. The lectin (0.2 mg ml^{-1}) was incubated with 50% of organic solvent for 4 h at 28°C

3.2.8 Antimicrobial activity

Lectins are known to play defensive role against bacteria, fungi and insects. Their ability to inhibit microbial growth varies from species to species. In the present study, *Di*L9 was subjected to screening for *in vitro* antibacterial inhibition growth against 6 pathogenic bacteria and the inhibitory action was compared to that of ampicillin, an antibacterial antibiotic. The lectin agglutinated both Gram-positive and Gram-negative bacteria (data not shown). *Di*L9 exhibited significant antibacterial activity against *E. faecalis* and *B. cereus* with 13 and 11 mm of diameter (Fig. 8) of inhibition zone, and MIC of 150 and 250 µg ml^{-1}, respectively (Table 5). No activity was found against mycobacterium, indicating *Di*L9 has specificity towards Gram positive/negative bacteria only. This antibacterial function of *Di*L9 could be related to its observed ability to bind to GlcNAc oligomers as already discussed, being responsible for bacterial recognition, where GlcNAc is a part of peptidoglycan and murien constituting the cell wall components. *In vitro* antifungal susceptibility by *Di*L9 was also determined against eight pathogenic fungi by considering amphotericin B, an anti-fungal agent used as positive control. But *Di*L9 showed some, though not significant, inhibition of growth only against *F. oxysporum.* From these findings, we would suggest that *Di*L9 has potential antibacterial activity though less of antifungal activity. The resistance to currently used antibiotics by microorganisms posed threat towards prevention of infectious diseases, and thus stimulating researchers to discover new antimicrobial agents. Owing to the bacteriostatic effect, *Di*L9 characterized here could be considered for use as an alternative antimicrobial agent. The results from this study imply the application potential of this lectin in clinical microbiology and its plausible therapeutic potential. The present study also gave useful insights into structure-function relationship of this small lectin and its high stability under denaturing conditions. Further studies might be required to explore more of its antibacterial action and design lectin preparation for potential application to control pathogens. With the advancement in protein engineering and nanotechnology, the protein bioavailability can be increased to optimize for target delivery strategies with more efficacies.

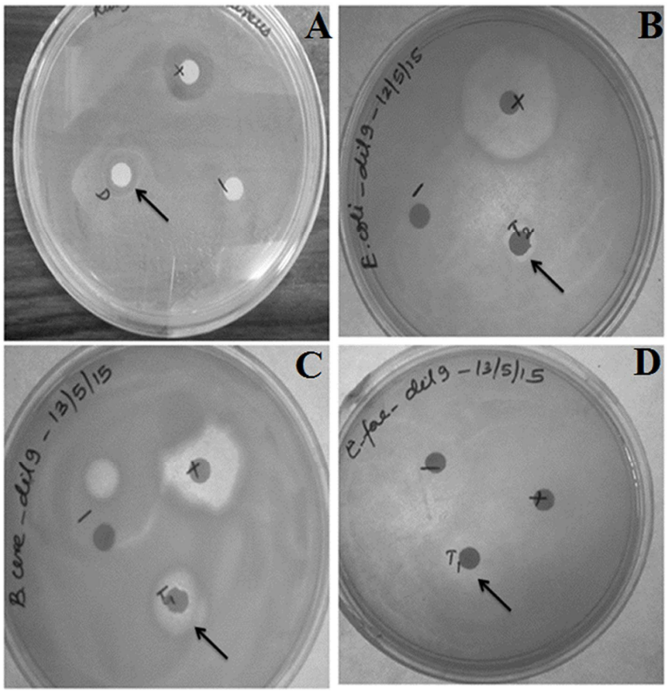

Fig. 8. Inhibitory effect of purified *D.innoxia* seed lectin on the *in vitro* growth on nutrient agar plates of (A) *S. aureus*, (B) *E. coli,* (C) *B. cereus* and (D) *E. faecalis*. For each plate 5 mm diameter discs were seeded with 10 µl of PBS (-) alone as the negative control, (+) 5 µg of ampicillin and (T_1) purified *Di*L9. Data shown are representative of three repeat assays

Table. 5 *In vitro* antibacterial activity of *Datura innoxia* Lectin

SL.no	Pathogen name	Gram staining feature	Zone of inhibition (mm)*	MIC (mg ml^{-1})
1	*Staphylococcus aureus*	Gram positive	10	0.325
2	*Bacillus cereus*	Gram positive	11	0.25
3	*Enterococcus faecalis*	Gram positive	13	0.15
4	*Escherichia coli*	Gram negative	7	0.5
5	*Salmonella typhimurium*	Gram negative	-	-
6	*Pseudomonas aeruginosa*	Gram negative	-	-

**Zone of inhibition (in mm diameter): includes 5 mm diameter of disc placed in agar disc diffusion assay*

4. CONCLUSION

In summary, a chito-specific agglutinin was purified from the seeds of *D. innoxia* using ion exchange and gel filtration chromatography. MALDI-TOF/TOF analysis gave 9653.6 Da monomeric protein with 4% neutral carbohydrate content. The far-UV CD spectra showed structural features different from other *Datura* lectins, thus, showing presence of diverse lectins in the same plant species. Only chitotriose sugar and glycoproteins showed hemagglutination inhibition implying presence of extended sugar binding site in the lectin. *Di*L9 was found to be highly stable in a wide range of pH, at high temperature, in the presence of chemical denaturants, organic solvents and resistant to proteolytic digestion, implying its structural and functional rigidity. *Di*L9 also showed significant antibacterial activity against certain pathogenic bacteria.

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COMPETING INTERESTS

The authors certify that they have no conflict of interests with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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