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A Glucose binding lectin from *Leucaena leucocephala* seeds and its mitogenic activity against human lymphocytes

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ABSTRACT

Lectins are a specialized group of proteins with immense biological properties and applications. This study describes the purification and characterization of a lectin from *Leucaena leucocephala* seeds, a plant belonging to the Fabaceae family. *Leucaena leucocephala* lectin (LLL) was purified by a two-step purification method involving DEAE-cellulose anion exchange chromatography and Sephadex G-75 size exclusion chromatography. The isolated lectin displayed a high haemagglutination titre upon treatment with rabbit erythrocytes. SDS-PAGE and Reverse-Phase High performance liquid chromatography (RP-HPLC) analysis experimentally revealed the presence of three bands corresponding to 37, 27 and 20 kDa indicating the presence of isolectins. Periodic Acid Schiff's (PAS) staining of LLL confirmed the presence of glycoprotein. Various biochemical parameters were analysed to study its effect on the haemagglutination activity. Sugar inhibition studies experimentally revealed that Glucose was the most potent inhibitor. Fluorescence spectrometric analysis of LLL and Glucose indicated a strong interaction with an association constant of $0.159 \times 10^3\ M^{-1}$. Circular Dichroism spectroscopy indicated a higher alpha helical content (25.27%). LLL was observed to possess mitogenic activity against Peripheral blood mononuclear cells (PBMC). The present investigation reports the isolation of a novel lectin from this plant which could contribute towards the diagnostic studies of certain diseases and for its therapeutic potential.

1. Introduction

Lectins are a group of proteins or glycoproteins that can bind sugars or sugar derivatives reversibly and possess at least one catalytic domain. Lectins have been found ubiquitously in plants, animals, bacteria, fungi, algae, insects, fishes, and higher-order animals. Lectins are heterogeneous in nature owing to their differences concerning molecular structure, carbohydrate specificity and biological applications [1]. Lectins have been reported from a wide variety of plant families such as Aracaceae, Euphorbiaceae, Fabaceae, Passifloraceae, Graminae, Moraceae and Sapotaceae. Legume lectins are a class of highly conserved proteins and have exhibited several physiological roles in plants such as recognition of nitrogen-fixing bacteria at root surface, destruction of growth pathogenic organisms, insecticidal activity, mitogenic and anti-tumour activities [2,3].

Leucaena leucocephala is a fast-growing tree belonging to Fabaceae family native to the Central America and the Yucatan Peninsula of Mexico where it was used mainly for fodder [4]. It was subsequently introduced into Australia and in the seventies, it was used as an agroforestry crop to meet the urgent requirement for fodder, fuel, and timber. Due to its multiple utilities, it earned the reputation as a 'miracle tree' [5]. Despite its high protein content, the use of Subabul as a green fodder should be controlled due to the presence of a toxic anti-metabolite Mimosine [6]. Even though several lectins have been successfully characterized from legumes, there is a paucity of information regarding the reports of lectins from wild legumes. Inglum et al., proved that Leucocephalin, a new lectin from the seed extracts of L. *leucocephala* possessed antimutagenic activity against *E. coli* KU26 [7]. Hence, the present investigation conducted is a first detailed report on the characterization of the lectin from *Leucaena leucocephala* seeds. Here, in this study we isolated the lectin by standard procedures like Ammonium sulphate precipitation, ion exchange chromatography and Gel filtration chromatography. After two rounds of purification, the isolated lectin exhibited a high fold of purification of 5.6×10^4 with a corresponding protein concentration of 0.84 mg/mL. The purified *Leucaena leucocephala* lectin displayed the presence of 3 bands with molecular mass corresponding to 37, 27 and 20 kDa respectively under both reducing and non-reducing conditions. Reverse-phase HPLC analysis further corroborated the results indicating the presence of 3 peaks confirming the presence of co-precipitating lectins. The isolated lectin exhibited a high haemagglutination titre with rabbit erythrocytes. The carbohydrate binding specificity studies of LLL with various sugars indicated its affinity towards Glucose and mannose sugars. Fluorescence spectroscopic studies showed a strong affinity between the lectin and D-glucose. Hence, it can be concluded that LLL belongs to the group of glucose/mannose specific lectins. As a further extension of the current

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study, an effort was undertaken to evaluate the mitogenic activity of *Leucaena leucocephala* lectin and it was observed that the lectin stimulated the growth of human peripheral blood lymphocytes and thus it could be employed as a candidate for future studies in Glycobiology and biomedical applications.

2. Materials and methods

2.1. Materials

Seeds of *Leucaena leucocephala* were collected from Kalkeri village in Kumta Taluk, Uttar Kannad District of Karnataka State during early March and stored at 4 °C for further use. Sephadex G-75, Periodic acid, Bovine Serum Albumin (BSA) and Phytohemagglutinin-M(PHA-M) were obtained from Sigma Aldrich, USA. All the fine sugars and ovalbumin utilized for the Haemagglutination inhibition studies were procured from Sisco Research Laboratories (SRL), India. Standard protein molecular weight marker was acquired from Merck Life Sciences, Mumbai, India. DEAE-Cellulose was obtained from Himedia labs, India. All the other chemicals and reagents used in the experimental procedures were of high purity and analytical grade.

2.2. Isolation and purification of Leucaena leucocephala lectin (LLL)

The lectin was extracted and purified by successive steps involving crude extract preparation, ammonium sulphate precipitation, DEAE-cellulose ion exchange chromatography and Sephadex G-75 chromatography. All purification steps were carried out at 4 °C. The steps involved in purification were as follows:

(i) Crude extract preparation: *Leucaena leucocephala* seeds (150 g) were cleaned well with distilled water, air dried and homogenised in 600 ml of 50 mM Tris-HCl containing 0.15 M NaCl, pH 7.4 at 4 °C overnight. The extract was passed through a muslin cloth and centrifuged at 6941 ×g for 30 min at 4 °C. The haemagglutination titre, specific activity and protein concentration was determined.

(ii) Ammonium sulphate precipitation: The crude extract was treated with ammonium sulphate to 20, 40, 60, 80 and 100% saturation and the protein pellets were collected by centrifugation as described above. The pellets were further re-dissolved in minimum amount of 50 mM Tris-HCl buffer and dialysed extensively to remove the ammonium sulphate.

(iii) Ion exchange chromatography: The dialysate obtained was then subjected to anion exchange chromatography using DEAE-cellulose column (25 × 1.5 cm) pre-equilibrated with 50 mM Tris-HCl, pH 7.4. Unbound proteins were washed off with the same ion-exchange buffer until the baseline was reached indicated by zero absorbance at 280 nm. The bound fraction was eluted by a step gradient of 0.5 M NaCl and the bound protein fractions were collected. Fractions exhibiting haemagglutinating activity were pooled and concentrated using Amicon UF-10 kDa membrane (Merck Millipore, USA).

(iv) Sephadex G-75 chromatography: The ion exchange concentrated fraction was then further purified by a Sephadex G-75 column (BioRad Econo column 50 × 1.5 cm) using a flow rate of 0.88 mL/min. The column was pre-equilibrated with 50 mM Tris buffer containing 0.15 M NaCl pH 8.4. The peak fractions were analysed for lectin activity and the active fractions were pooled and concentrated to obtain the final purified lectin preparation.

2.3. Preparation of rabbit erythrocyte suspension

Rabbit blood (1 ml) was withdrawn from the marginal ear and collected in an EDTA vacutainer tube. The procedure was performed causing minimal pain to the animal. The blood was mixed with 2 volumes of PBS buffer (pH 7.4) and centrifuged at 825 ×g for 10 min at 4 °C. The supernatant was discarded, and the Red blood cell (RBC) pellet was resuspended in PBS buffer and subsequent centrifugation was repeated 2 more times to obtain washed RBC. The washed RBC (0.2 mL) was diluted with normal saline (9.8 mL) to constitute a 2% v/v erythrocyte suspension.

2.4. Determination of haemagglutination activity and carbohydrate specificity

Haemagglutination titre was determined for the various sequential stages of purification by performing the assay in a 96 well microtitre assay plate using the 2% rabbit erythrocyte suspension by serial two-fold dilution as described previously [8]. The highest dilution of the extract displaying complete, visible agglutination was considered as the haemagglutination titre and the minimum concentration of lectin required for complete agglutination under the assay conditions was considered as one haemagglutination unit (HAU). Specific activity is expressed as one activity unit mg^{-1} of protein.

Carbohydrate binding specificity of the purified lectin was determined by the hapten inhibition assay using a standard serial dilution procedure [9]. Each sugar solution (25 μL) was serially diluted two-fold in 50 mM Tris-buffered saline (pH 7.4) in a micro titre plate and an equal volume of lectin with 4 haemagglutination units was added. After incubation for 1 h, 50 μL of 2% rabbit erythrocyte suspension was added and the haemagglutination inhibition was visualized by the presence of a red button instead of the agglutinated mat of erythrocytes. The inhibitory activity of the specific sugar is defined as the minimum concentration required for the complete inhibition of two doses of the lectin [10].

Protein assay was performed according to the method of Bradford using BSA as the standard at varying concentrations [11].

2.5. Characterization of the purified lectin

2.5.1. Determination of molecular weight

The purified lectin was subjected to electrophoretic analysis by Native, non-reducing and reducing SDS-PAGE (with β-mercaptoethanol) in a vertical slab electrophoresis unit (Mini Dual Vertical Electrophoresis Unit, Tarsons). Electrophoresis was carried out in 12.5% polyacrylamide gel as per the procedure described by Laemmli [12]. Coomassie blue staining was carried out for both reducing and non-reducing SDS-PAGE. However, for Native PAGE, silver staining was performed according to the modified method by Blum [13].

2.5.2. Periodic Acid Schiff (PAS) staining

The glycoprotein nature of the purified *Leucaena leucocephala* lectin was confirmed by PAS staining. IgM (anti-D blood grouping serum) and Ovalbumin were used as positive control. BSA served as negative control. The electrophoresed gel was immersed in 12.5% TCA solution for 30 min and lightly rinsed with distilled water for 15 s. The gel was then incubated in 1% periodic acid for 30 min. The gel was extensively washed with distilled water and the bands were developed in dark by immersing the gel in Schiff's reagent at 4 °C for 50 min. Finally, the gel was washed in freshly prepared 0.5% sodium metabisulphite and the gel was stored in 3% acetic acid.

2.6. Reverse-phase HPLC analysis

The purified fraction obtained after Size Exclusion Chromatography was subjected to HPLC analysis (Schimadzu LC 20AD, Japan) using C18 column (250 × 5 mm-length × internal diameter, pore size 4.6 μM) at a flow rate of 1 mL/min for 10 min using Acetonitrile: Water (50:50% v/v). The proteins were detected by monitoring the absorbance at 280 nm using the Photodiode array (PDA) detector.

2.7. Effect of pH and temperature on the stability of LLL

The pH stability of the lectin was determined by incubation of the LLL (0.1 mg/mL) in various buffers: Na_2HPO_4-Citric acid buffer (pH 4–5), Sodium phosphate buffer (pH 6–7), Tris-HCl buffer (pH 8–9) and Gly-NaOH (pH 10) for 24 h at 4 °C and the haemagglutination activity was analysed. The thermal stability of the lectin from *Leucaena leucocephala* was evaluated by incubating the lectin (0.1 mg/mL) at different temperatures ranging from 4 to 100 °C for 1 h and then brought back to room temperature before testing the haemagglutination activity.

2.8. Effect of denaturing and reducing agents on the stability of lectin

LLL was treated with Urea and Guanidine hydrochloride to investigate the effect of these denaturing agents on the haemagglutination activity. Urea at a final concentration of 10–200 mM was incubated with the lectin for 3 h at 4 °C. Similarly, Guanidine hydrochloride at a concentration ranging from 0.2–1 M was treated and the change in the haemagglutination titre was studied. The effect of reducing agents 2-Mercaptoethanol (2-ME) and Dithiothreitol (DTT) was used and its effect on the haemagglutination activity was analysed by incubating the lectin with different concentration of 2-ME (10–50 μM) and DTT (50–250 μM) for 3 h at 4 °C after which the haemagglutination activity was analysed.

2.9. Effect of EDTA and metal ions on the stability of LLL

To investigate the effect of metal ions on the haemagglutination, the purified lectin was demetallized by treatment with EDTA at a final concentration of 100 mM and incubated overnight at 4 °C. Control samples were incubated with an equal volume of Tris buffer (50 mM) pH 8.4. Eventually, the lectin was treated with 100 mM of various cations and its effect prior and post ion treatment was studied by the haemagglutination assay.

2.10. Fluorescence spectroscopic analysis of Leucaena lectin

Fluorescence emission of *Leucaena leucocephala* lectin was scanned in the excitation wavelength range 200–700 nm. Steady state fluorescence emission spectra of *Leucaena leucocephala* lectin (0.19 mg/ml) was analysed using a Fluorescence Spectrophotometer (Agilent Technologies Cary Eclipse MY16260002, path length 10 mm) in the presence of different concentrations of D-glucose at pH 8.4 at 298 K using the excitation wavelength of 280 nm as described by Bose et al. [14]. The fluorescence spectra were evaluated, and the data was plotted according to Scatchard [15] using the OriginLab software (OriginPro 9.0.0, OriginLab Co., USA). Two independent titrations were performed to observe the consistency of the experimental results.Eqn 1

$$\mathrm{Log}\left[\frac{(F_0-F)}{F}\right] = \log K_a + n \log [Q] \quad [15] \tag{1}$$

where F_0 and F is the steady state fluorescence emission intensity in the absence and presence of the quencher respectively, K_a is the binding constant, n is the number of binding stoichiometry as indicated by the slope of the Scatchard plot and Q is the molar concentration of the quencher.

The Stern Volmer plot was generated for the determination of quenching constant and binding stoichiometry according to Eqn 2 [16].

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_q \tau_0 [Q] \tag{2}$$

In Eq. (2), K_{SV} denotes the quenching constant as determined from the slope of Stern-Volmer plot obtained at a lower concentration of the quencher, K_q is the bimolecular rate constant of the quenching constant and τ_0 is the average integral fluorescence lifetime of tryptophan which is approximately 4.31×10^{-9} s [17].

2.11. Circular dichroism (CD) spectroscopic analysis of Leucaena leucocephala lectin

Far-UV circular dichroism (CD) measurements were performed using a JASCO J-815 spectropolarimeter (JASCO, Easton, MD, U.S.A.) using a 1 mm cell at 25 °C in a thermostatic cell holder at a concentration of 0.25 mg/mL lectin solution in 15 mM Tris Buffer pH 8.0 using a quartz cuvette recorded in the far-UV region (260–190 nm). All spectra were recorded in triplicates and the average is plotted. The mean residue ellipticity was computed as reported previously [18]. K2D3 software was used to predict the percentages of secondary structure based on the ellipticity values obtained for the corresponding wavelength range.

2.12. Mitogenic activity of Leucaena leucocephala lectin

The mitogenic activity of LLL was investigated using isolated Human Peripheral Blood Mononuclear cells (PBMCs) by the prescribed method [19]. Human Peripheral blood Mononuclear cells were isolated from healthy human blood and cultured in RPMI media and 100 μL of purified lectin was added in aseptic conditions. Appropriate controls were maintained wherein Phytohemagglutinin (PHA) was used as a positive control. All the samples were incubated at 37 °C in a water bath for 69 h and the tubes were mixed intermittently to disrupt the cell clumps. At the end of 69th hour, the mitotic arrest was induced by Colchicine treatment and further incubated at 37 °C for another 2 h. The cells were harvested after centrifugation at 108 ×g for 5 min. The cell pellet obtained was resuspended in 1 ml of freshly prepared fixative solution and mixed gently and this step was

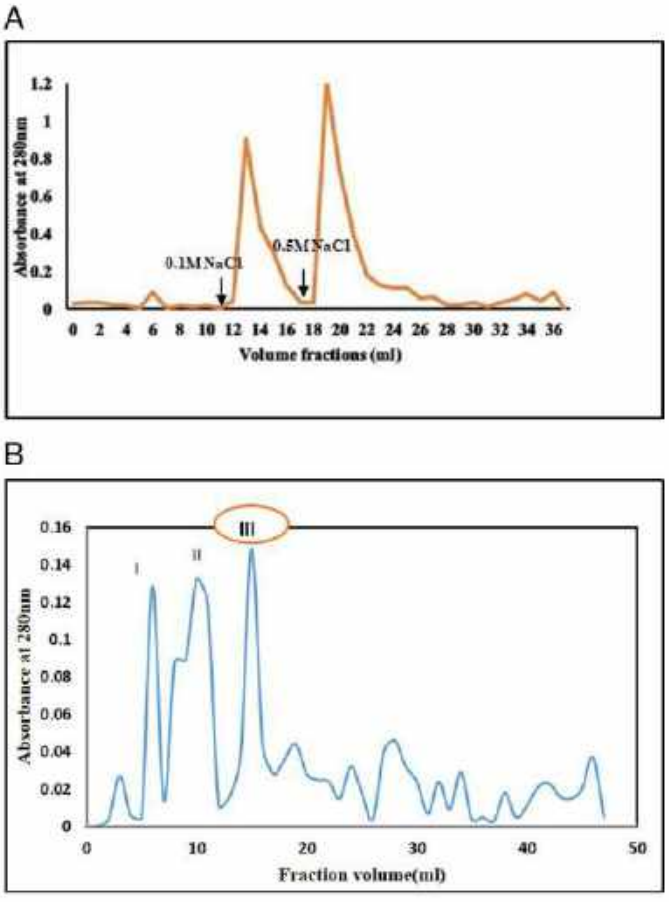


Fig. 1.

Table 1
Purification profile of *Leucaena leucocephala* lectin.

Sample	Volume (ml)	Concentration of protein (mg/ml)	Titre	Total activity (HU)[a]	Specific activity (HU/mg)[b]	Yield of protein (%)	Yield of activity (%)	Purification fold
Crude	300	221	1×10^3	8×10^8	1×10^1	100[c]	100[c]	1[c]
60–80% AS fraction	40	131	8×10^5	7×10^9	1×10^4	7.9	853	1×10^3
Ion exchange purified	14	0.7	2×10^7	5×10^8	5×10^8	0.014	598	4×10^4
Gel filtration chromatography	3	0.84	2×10^7	8×10^8	7×10^8	0.002	107	6×10^4

[a] Total activity: hemagglutinating activity in total protein.
[b] Specific activity: hemagglutinating activity mg^{-1} of protein.
[c] Values taken arbitrarily.

repeated a couple of times until the supernatant became clear for fixation. The cells were then suspended in 500 μL fixative solution for chromosome preparation. Chromosome spread was prepared by the splash technique as reported by Ulsh et al. [20]. The heat fixed spread was stained with 10% Giemsa staining solution and incubated for 30 min, washed with clean distilled water and air dried. The chromosome spreads were examined microscopically using a System Microscope (Leica DM6B, Germany) under a bright field.

3. Results

3.1. Isolation and purification of Leucaena lectin

Leucaena leucocephala lectin was purified by subjecting the crude extract to ammonium sulphate precipitation. The 60–80% fraction was purified by a two-step process involving ion exchange chromatography and Sephadex G-75 chromatography. After DEAE-cellulose chromatography, two peaks which possesses lectin activity was obtained (Fig. 1a). The concentrated ion-exchange eluted fraction was subsequently subjected to Sephadex G-75 chromatography and the lectin was purified with a high fold of purification of 5.6×10^4 (Fig. 1b). The specific activity, % yield and purification fold of the purified lectin are summarized in Table 1. Native PAGE followed by silver staining showed the presence of a diffused band (Fig. 2a). Electrophoretic analysis of the purified fraction displayed the presence of 3 bands corresponding to 37, 27 and 20 kDa under both reducing and non-reducing conditions which indicates the possibility of isolectins (Fig. 2b & c). The PAS staining of L. *leucocephala* lectin indicated the presence of 3 pinkish bands confirming the presence of glycoprotein (Fig. 3). The presence of 3 isolectins was further indicated by the presence of three peaks at the different retention time of 2.8, 3.3 and 3.8 min when the purified lectin was subjected to Reverse- HPLC analysis (Fig. 4).

3.2. pH dependence and thermal inactivation studies

When the purified LLL was subjected to a various temperature ranging from 4 to 100 °C for 1 h, it was observed that the lectin was relatively stable up to 37 °C. A gradual decrease of specific activity was seen when the temperature was raised and above 80 °C the lectin lost all its activity completely (Fig. 5a). LLL was observed to be relatively stable in the pH range between 4 and 8, with the most optimum pH range being 7 and 8 (Fig. 5b).

3.3. Effect of denaturing and reducing agents on Leucaena lectin stability

The treatment of reducing agents like DTT and 2-ME resulted in the initial decrease in the lectin activity and a subsequent increase in the activity at a higher concentration of the reducing agent. In the case of both the denaturing agents the haemagglutination activity was observed to decrease up to a concentration of 0.4 M (Fig. 6a, b, c, d).

3.4. Studies on the effect of divalent cations on the lectin activity

The effect of EDTA and divalent ions on the stability of *Leucaena* lectin was evaluated. It was observed that EDTA did not possess any effect

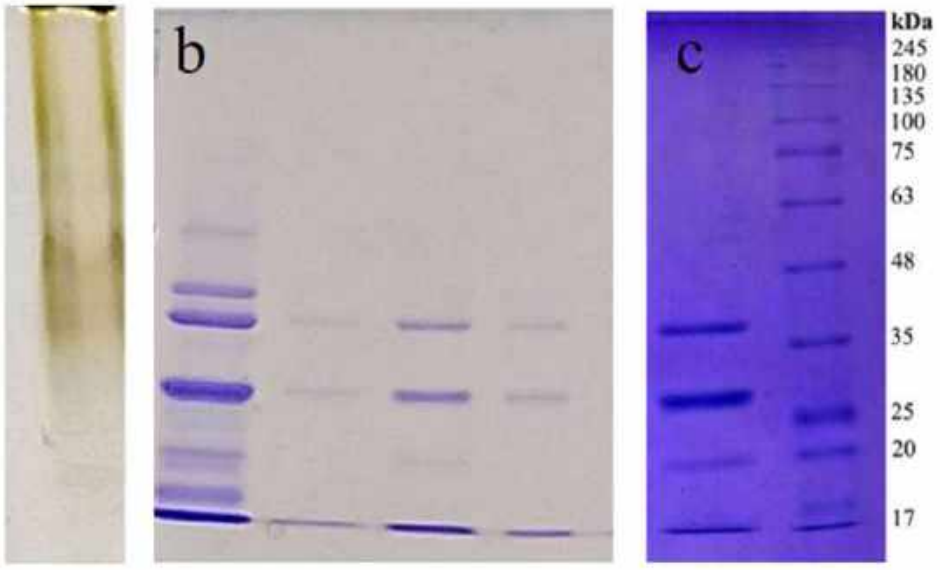


Fig. 2. Electrophoretic analysis of LLL purified by Gel filtration chromatography. Leucaena leucocephala lectin was analysed by Native, reducing and non-reducing SDS PAGE. (a) **Native PAGE** shows a diffused band (b) **Non-reducing PAGE.** Lane 1: DEAE-cellulose ion exchange purified on DEAE-cellulose ion exchange column Lane 2-4: Purified fractions from Sephadex G-75 gel filtration chromatography (c) **Reducing SDS-PAGE (with β-mercaptoethanol)** Lane 1: Three bands corresponding to 37, 27 and 20 kDa obtained after Sephadex G-75 gel filtration chromatography. Lane 2: Molecular weight marker.

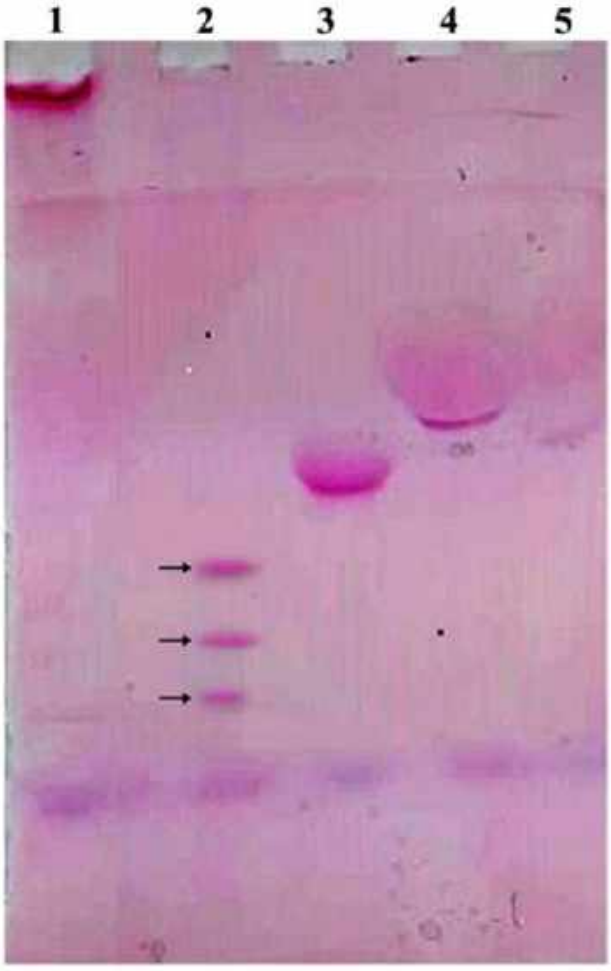


Fig. 3. Periodic acid-Schiff (PAS) staining of LLL by gel filtration chromatography. Lane 1) 60-80% AS fraction 2) Purified LLL by Gel filtration chromatography 3) Ovalbumin (7.7mg/mL) 4) IgM 5) BSA (4mg/mL). Lane 1 corresponds to 60-80% AS fraction showing the presence of a faint pink smear. Lane 2 shows the presence of three bands corresponding to 37, 27 and 20kDa obtained after Sephadex G-75 gel filtration chromatography. Lane 3 and 4 displays the presence of Ovalbumin and IgM used as positive controls. Lane 5 consists of BSA used as the negative control.

on the lectin activity. However, it was observed that $MgCl_2$, $CaCl_2$, $BaCl_2$, $MnCl_2$ and $CoCl_2.6H_2O$ enhanced the activity of LLL.

3.5. Haemagglutination and carbohydrate specificity assay

Haemagglutination assay of *Leucaena leucocephala* lectin demonstrated a special preference for rabbit erythrocytes as indicated by a high titre value of 131,072 as compared to human and mice erythrocytes (Table 2). Carbohydrate specificity assays showed that the haemagglutinating ability of LLL was inhibited by D-Glucose and Mannose at a concentration of 0.024 mM. Several Mono- and disaccharides such as Mannitol, D-galactose, Fructose, Raffinose, Melibiose, Raffinose, Arabitol D-trehalose, D-xylose, D-fucose, *N*-acetyl glucosamine and D-rhamnose were used for studying the sugar inhibition assay. However, none of the sugars exhibited inhibition up to 200 mM and the results are summarized in Table 3.

3.6. Fluorescence spectroscopic analysis of Leucaena lectin

Fluorescence spectroscopy on *Leucaena leucocephala* lectin displayed the highest emission intensity at an excitation wavelength of 280 nm as evident from the fluorescence spectra and the contour map (Fig. 7a & b). Steady state fluorescence emission spectra of *Leucaena leucocephala* lectin in 150 mM NaCl was recorded in the presence of different concentrations of glucose at pH 8.4 at 298 K using the excitation wavelength of 280 nm. The fluorescence emission peak of *Leucaena leucocephala* lectin was found to be quenched regularly with an increase in the concentration of glucose up to a concentration of 4.8 mM as illustrated in (Fig. 7c). The data obtained were plotted according to Scatchard [15] as in (Fig. 7d). From the Scatchard analysis the association constant (K_a) of Glucose for the *Leucaena leucocephala* lectin was calculated to be $0.159 \times 10^3\ M^{-1}$. Stern Volmer plot was generated for the determination of quenching constant and binding stoichiometry which were found to be $3.55 \times 10^{11}\ M^{-1}\ S^{-1}$ and 2.3, respectively. The Stern Volmer constant was found to be $1.53 \times 10^3\ M^{-1}$ (Fig. 7e).

3.7. Secondary structure analysis of LLL by CD spectroscopic analysis

Far UV-CD analysis was performed to derive an idea about the secondary protein structure of the purified lectin. The purified lectin was concentrated in 15 Mm Tris buffer pH 8.0 to a concentration of 0.25 mg/mL. Five scans were performed with the protein sample and the ellipticity values were obtained. The ellipticity values were then

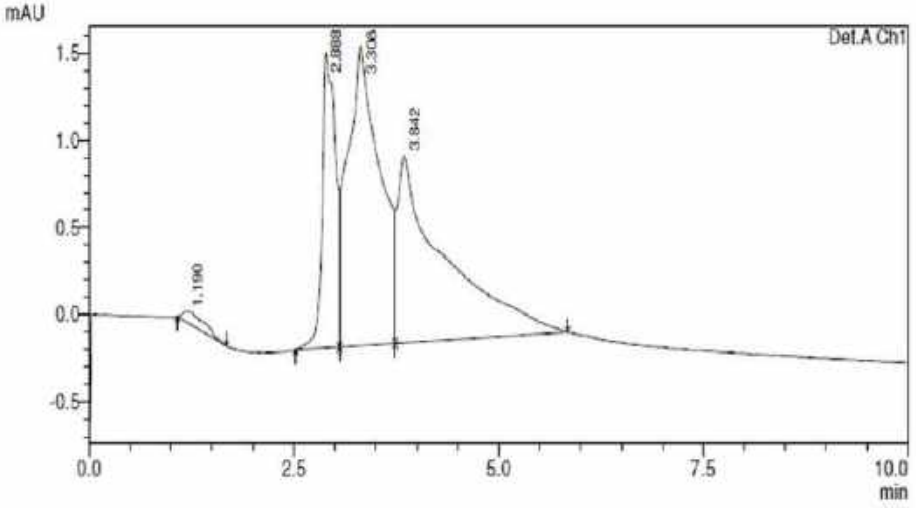
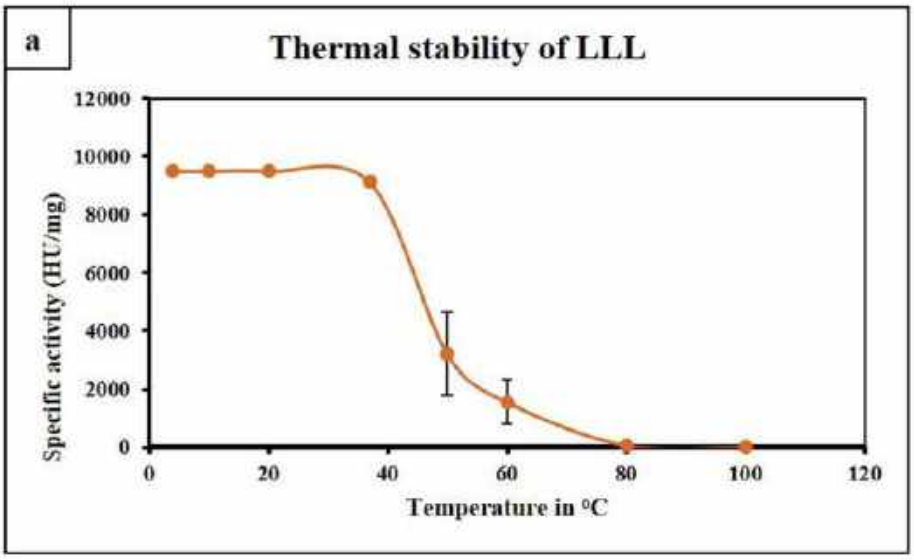


Fig. 4. HPLC analysis of purified LLL. LLL (20μL) with a concentration of 0.1mg/mL was further purified using a reversed phase C18 column with a linear gradient (50:50%) solvent (Acetonitrile: Water) over 10 minutes. Flow rate of 1ml/min and absorbance and retention time was recorded at 280nm.Three peaks with a retention time corresponding to 2.88, 3.3 and 3.84 min emerged in the chromatogram corresponding to the 3 bands obtained after gel filtration chromatography of LLL.



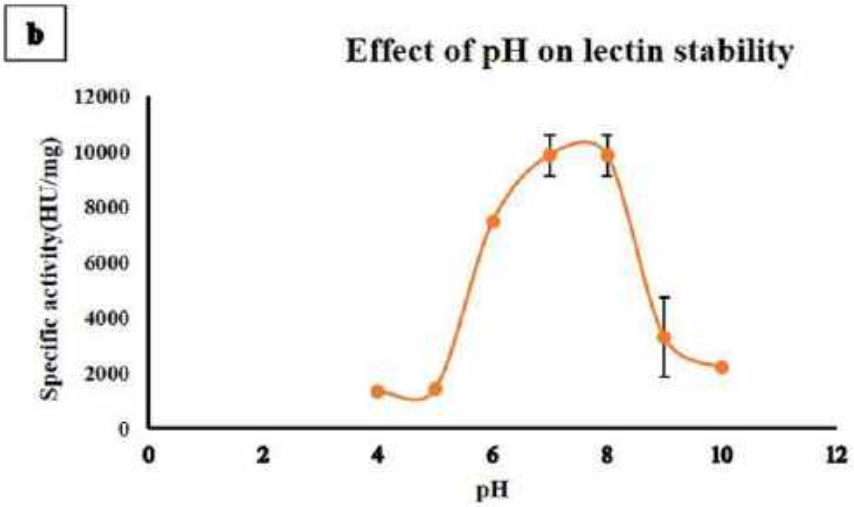


Fig. 5. Temperature and pH stability studies of purified LLL. a) Thermal stability curve for specific activity of LLL. The lectin was incubated at various temperatures for 60 min followed by monitoring its effect of heat treatment on lectin stability. b) pH stability curve for specific activity of LLL. Changes in specific activity upon incubation of LLL with various buffers: Na_2HPO_4-Citric acid buffer (pH 4-5), Sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9) and Gly-NaOH (pH 10) at 4°C after 24-h treatment was studied. Data represent the mean of three individual experiments and error bars represent standard deviation.

analysed by K2D3 software. The analysis revealed that the protein was composed of a higher percentage of alpha-helix (25.27%) and only 5.32% of beta-strand (Fig. 8).

3.8. Evaluation of the mitogenic activity of the purified LLL on human

To evaluate the role of *Leucaena leucocephala* lectin in promoting mitogenic activity in human lymphocytes, PHA-M was used as a positive control, and cells without lectin or PHA-M treatment were utilized as the negative control. It was observed that *Leucaena leucocephala* lectin at a concentration of 2.4 μg/mL displayed an increase in cell proliferation indicated by a mitotic index of 52.5% as compared to PHA-M with a mitotic index of 76.2% as represented in the Table 4 and the chromosome spread post lectin treatment is provided in Fig. 9.

4. Discussion

Plant lectins are important biological agents and are multifariously possessing numerous applications not only restricted to plant defense and symbiotic associations but provide useful insights on cell proliferation, cell arrest, antimicrobial processes, cell characterization and separation [21]. The present study describes the isolation and characterization of a lectin from the seeds of the fodder plant *Leucaena leucocephala*. Even though several lectins belonging to the Fabaceae family have been well characterized, the current study is a first detailed report on the presence of a lectin from this seed. Conventional methods of protein purification involving ammonium sulphate (AS) precipitation, ion exchange chromatography followed by size exclusion chromatography was utilized in obtaining a highly active lectin with haemagglutination specificity towards rabbit erythrocytes. A two-step purification strategy was required to purify the lectin from the crude seed sample. Likewise, several lectins have been purified by multi-step chromatographic techniques [22–24]. In the present study, a high percentage yield was obtained with 5.6×10^4 fold of purification. The use of a multi-step purification method ensures a high purification fold and recovery yield. Lectins exhibiting preference towards rabbit erythrocytes have been previously reported from the seeds of Caesalpinoideae plant, *Bauhinia variegata* [25]. In another study, a seed lectin from *Quercus ilex* L was found to be 16 times more

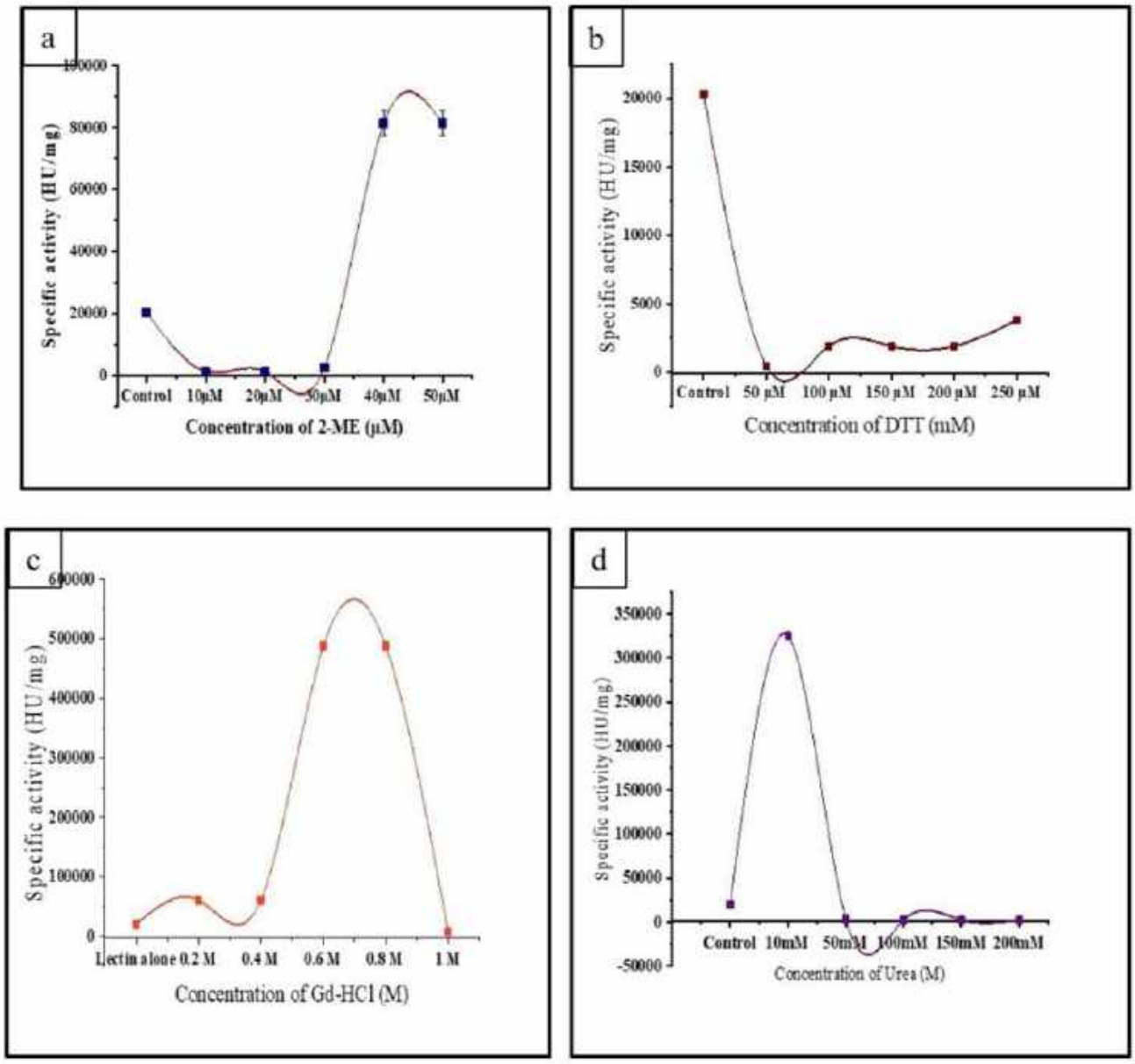


Fig. 6. Effect of reducing and denaturing agents on the activity of LLL. a,b) LLL was incubated with reducing agents- 2-Mercaptoethanol and Dithiothreitol at a concentration ranging from 50-250 μM for 3 hours at 4°C and its effect on the specific activity was monitored. c,d) LLL was treated with denaturing agents – Guanidine hydrochloride and Urea at various concentration ranging from 10mM- 200Mm for 3 h at 4 °C and the change in the specific activity was monitored.

Table 2
Agglutination of different blood types by LLL.

Type of blood group	Titre
Human O erythrocytes	4
Human B erythrocytes	4
Human AB erythrocytes	4
Rat erythrocytes	8
Chicken erythrocytes	12
Mice erythrocytes	48
Rabbit erythrocytes	1.3×10^{5a}

[a] As indicated in this table rabbit erythrocytes were found to be the most sensitive towards LLL with a corresponding high titre value of 1.3×10^5 HAU.

Table 3
Inhibition of haemagglutination activity of LLL by mono- and disaccharides.

Sugar	Minimum inhibitory concentration of carbohydrates (mM*)
Glucose	0.024
Mannose	0.024
Galactose	No inhibition
Fructose	
Raffinose	
Arabinose	
Trehalose	
Xylose	
Fucose	
N-acetyl Glucosamine	
Rhamnose	
Mannitol	
Melibiuse	
Arabitol	

NI: no inhibition.
* The concentration of the various sugar solutions were prepared in mM.

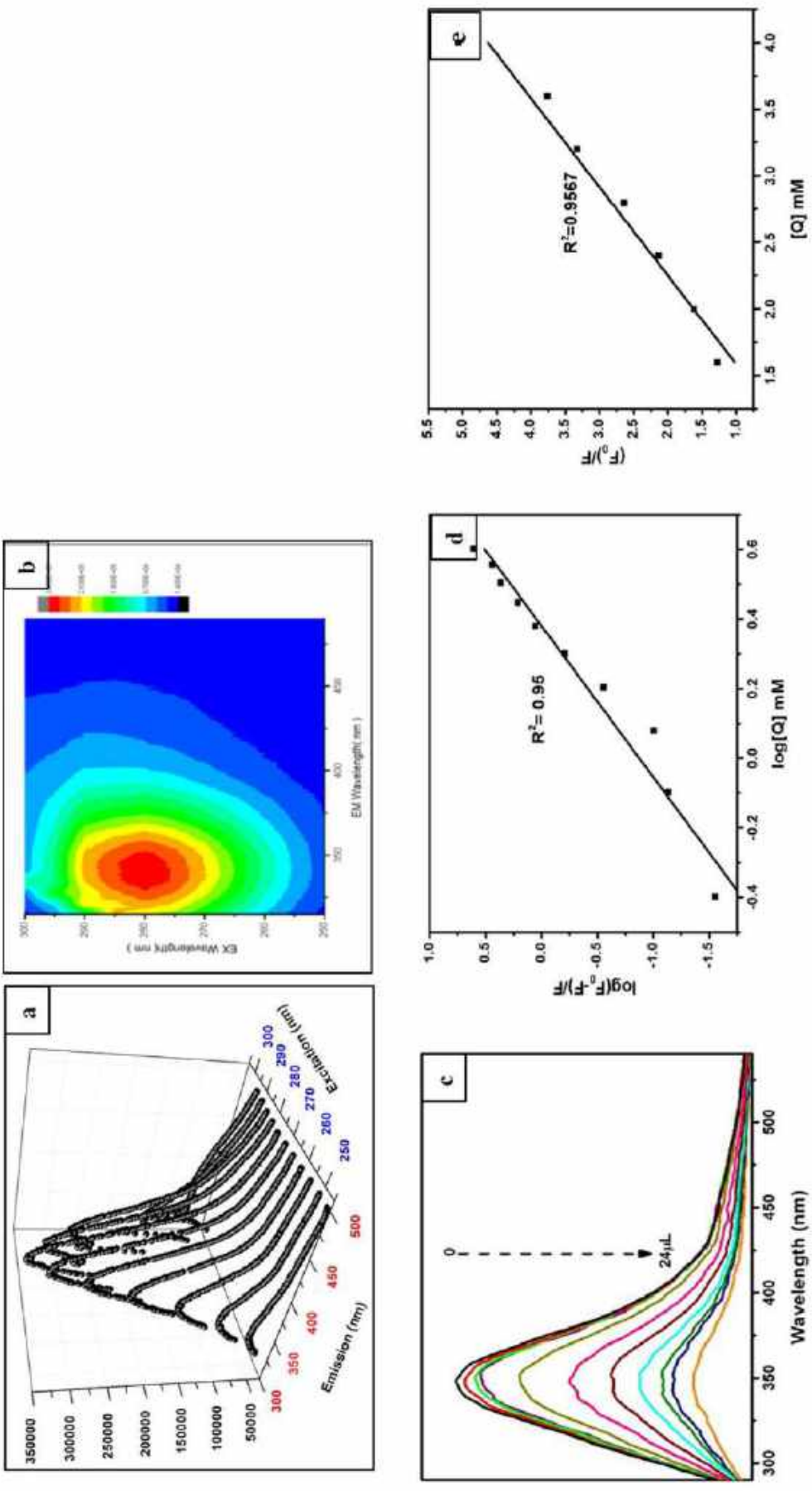


Fig. 7. (a) 3-D fluorescence spectra of *Leucaena leucocephala* lectin (0.2mg/ml). (b) 3-D Contour map of LLL (c) Fluorescence emission spectra of *Leucaena leucocephala* lectin (0.2mg/mL) in the presence of Glucose at different concentrations: (1) 0.4 mM (2) 0.8mM (3) 1.2 mM (4) 1.6 mM (5) 2 mM (6) 2.4 mM (7) 2.8 mM (8) 3.2 mM (9) 3.6 mM (10) 4 mM (11) 4.4 mM (12)4.8 mM at 298K in 150 mM NaCl pH 8.4. (d) Scatchard plot for binding of D-glucose to *Leucaena leucocephala* lectin and the binding was monitored at pH 8.4 at 298K with excitation wavelength set at 280 nm. From the slope, Ka value was calculated. (e) Stern-Volmer plot from steady-state fluorescence emission intensity measurements of *Leucaena leucocephala* lectin in the presence of glucose at 298K, pH 8.4.

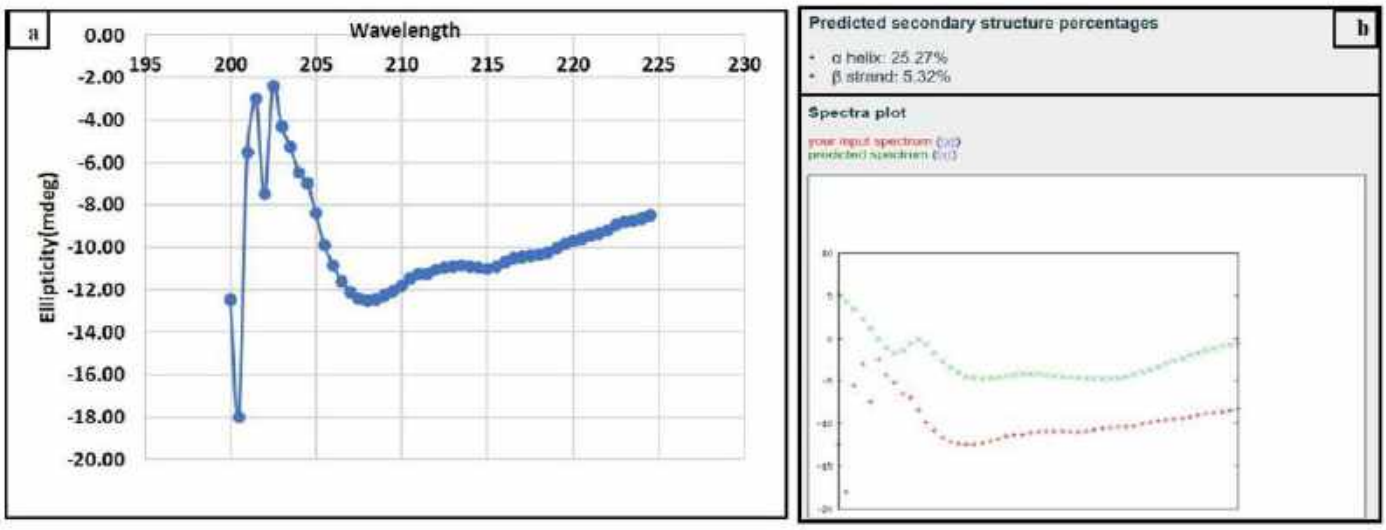


Fig. 8. (a) Far UV-CD spectrum of *Leucaena leucocephala* lectin. Analysis was performed in 15 mM Tris buffer, pH 8.0, at 25 °C. Measurements are the average of five scans using a solution containing 0.25 mg of protein/ml. **(b) Prediction of secondary structure of *Leucaena leucocephala* lectin by K2D3 software.** The average ellipticity values obtained from 5 scans was given as input in the K2D3 software with the corresponding wavelength range and the secondary structure was predicted. The red indicates the input spectrum and the green indicates the predicted spectrum.

Table 4
Effect of *Leucaena leucocephala* lectin and PHA-M on the Mitotic Index (MI) of PBMC (Peripheral Blood Mononuclear cells). The number of interphase cells and mitotic cells were studied. Data is represented as mean ± SD (n = 2).

Lectin used	Concentration (μg/ml)	Duration of incubation - 72 h at 37 °C			
		Mitotic cells	Interphase cells	Total cells	Mitotic index (%)
PHA-M	10	115 ± 2.8	36 ± 4.2	151 ± 1.4	76.2 ± 2.6
L. leucocephala lectin	2.4	80 ± 9.9	73 ± 10.6	153 ± 0.7	52.5 ± 6.6
Negative control	0	13 ± 3.5	140 ± 4.9	152 ± 1.4	8.2 ± 2.4

specific towards rabbit erythrocytes [26]. Lectins from *Fusarium* sp. agglutinates only rabbit erythrocytes as reported previously [27]. This high specificity of certain lectins towards rabbit erythrocytes is attributed towards the presence of 9-O-acetyl neuraminic acid on the glycoconjugate surface of the erythrocytes [28]. Electrophoretic analysis of the purified lectin exhibited the presence of three bands corresponding to 37, 27 and 20 kDa under both reducing and non-reducing conditions. This similar banding patterns under both reducing and non-reducing conditions indicates the possibility of isolectins in the purified sample. Similarly, to our observations, the SDS PAGE profile of the purified lectin from *Arisaema utile* Schott showed the presence of a single band under both reducing and non-reducing conditions [29]. In another similar study, purified *Clitorea ternatea* lectin (CTL) when subjected to SDS-PAGE analysis gave a single protein band in the presence and absence of β-mercaptoethanol [30]. Hence, the presence of three bands under both the conditions can be presumably due to the absence of inter-chain disulphide bridges despite subjecting it to heat treatment accompanied with extreme reducing conditions such as 2ME. The glycoprotein nature of *Leucaena* lectin was verified by performing PAS staining and the purified lectin showed the presence of 3 pink bands indicating the glycoprotein nature of the lectin. HPLC analysis of the purified lectin further validates the existence of the three lectins in close conjunction as specified by the presence of three peaks at different retention times. The study conducted by Graças et al. (2001) on *Talisia esculenta* revealed that the four isolectins, TEL-, TEL-II, TEL-III and TEL-IV indicated the presence of four major peaks confirming them to be isolectins [31]. Hence, in the present study three closely related lectins possessing nearly the same retention time was obtained during HPLC analysis with a specificity towards Glucose and Mannose. In another similar study, *Cajanus cajan* root lectin was also found to be inhibited by mannose and glucose [32]. Hence LLL could be considered to belong to glucose/mannose- specific group of lectins. Therefore, the lectins from LLL could be treated as isolectins since they were difficult to separate yet possessed similar sugar specificity.

The presently purified lectin was found to be highly thermolabile at a temperature above 37 °C as indicated by a decrease in the specific activity. This agrees with previous studies conducted on the lectin from Mulberry seeds-MSL1, MSL-2 and MSL-3 depicting a gradual decrease of activity above 40 °C with a maximum haemagglutinating activity noted at a temperature of 20–35 °C [33]. In general, legume lectins are heat labile and cannot survive processing or drastic changes caused by high temperatures resulting in the protein unfolding which may be due to the breakdown of hydrogen bonds, required to maintain the protein structure [34]. LLL was found to possess a narrow range of pH optima (pH 3–7). The decrease in specific activity upon treatment with extreme pH changes may be attributed to the disruption of the various non-covalent interactions that stabilize a protein structure resulting in the loss of structural and biological activity [35]. The effect of reducing agents such as 2-ME and DTT revealed an initial decrease in lectin activity at a lower concentration of the reducing agent and a subsequent increase at high concentration which might be due to the reformation of disulphide bonds via air oxidation or certain other factors like a shift in the equilibrium facilitating the reformation of disulphide bonds and free 2-ME. Upon treatment with denaturing agents like Urea and Guanidine hydrochloride, a differential behaviour was observed for the LLL activity. Guanidine hydrochloride treated lectin showed an initial decrease in haemagglutination activity but proceeded with an increase in specific activity above 0.4 M whereas in the case of Urea, a denaturing effect was prominently seen at high concentration. This differential behaviour of lectins towards denaturing agents might be associated with

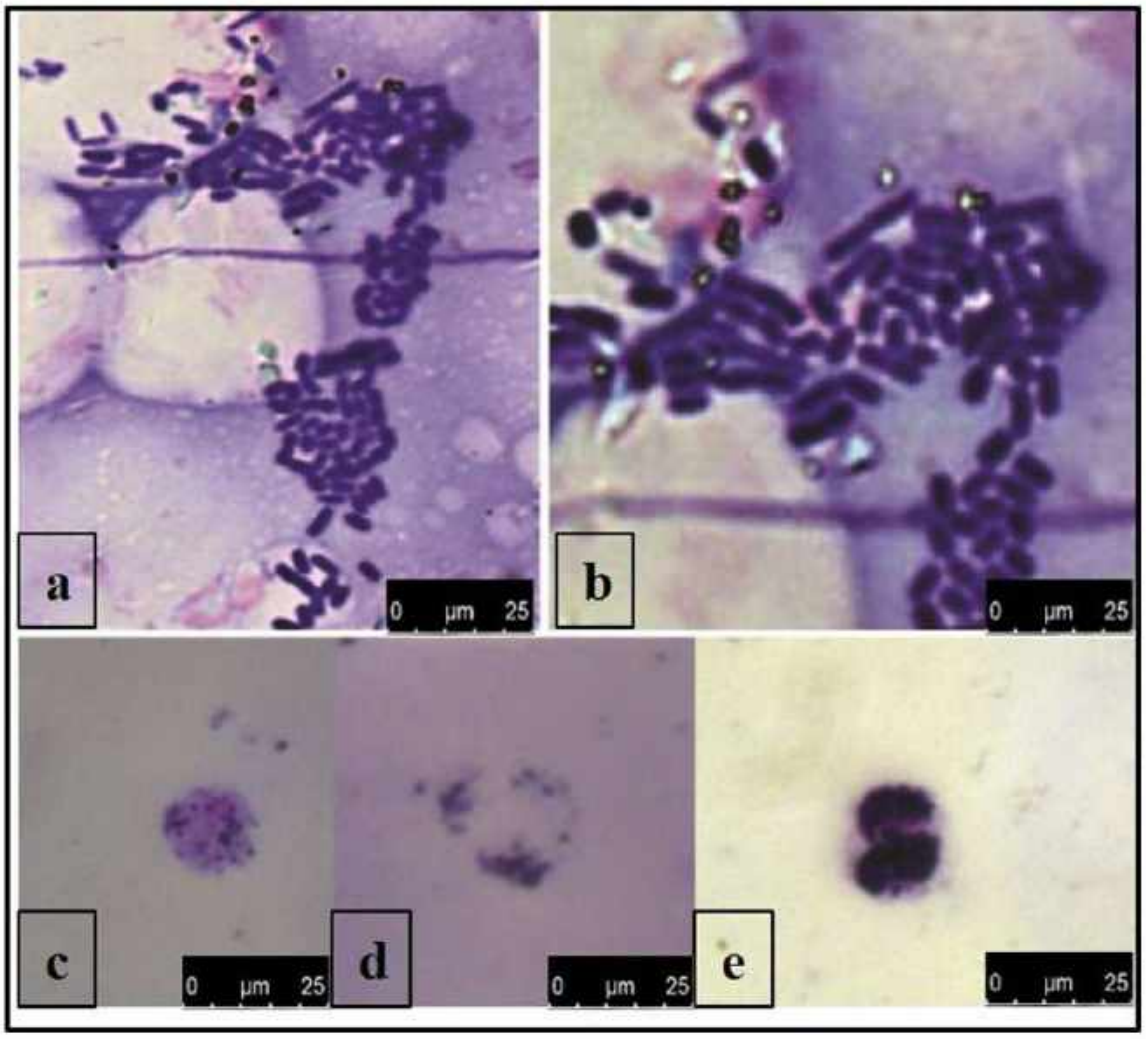


Fig. 9. Chromosome spread of LLL treated lymphocytes after culturing at 37°C for 72 h. Stages of cell division a, b) Metaphase c) Prophase d) Anaphase e) Telophase.

the difference in hydrophobicity and exposure of new hydrophobic binding pockets resulting in complete denaturation.

LLL was found to be preferential towards rabbit erythrocytes as compared to the various other blood groups verified in the study (Table 2). Similar results have been indicated in the case of two lectins from the Sea Cucumber, *Stichopus japonicus* which agglutinated rabbit erythrocytes more prominently when compared to human erythrocytes [36]. Carbohydrate inhibition assay revealed that among the various mono- and di-saccharides tested Glucose and mannose were found to be good inhibitors among which glucose had a more pronounced effect. The structural analogues of glucose used for the study are the epimers, mannose and galactose. Mannose and galactose are the C2 and C4 epimers of glucose, respectively. Hydrogen bonding and hydrophobic interactions are the main underlying forces involved in lectin-sugar interactions [37]. Together with the amines and carboxyl groups of substituted carbohydrates, the occurrence of several hydroxyl groups in sugar molecule plays a crucial role to hydrogen bond with the lectin receptor [38]. Hence, it could be concluded that the positioning of –OH group around C2 position does not have a role in hydrogen bonding with lectin receptor, whereas the –OH group on C4 position may have a role in hydrogen bonding to lectin. As its positioning is changed in case of galactose, the sugar probably could not interact with the lectin.

The results were further substantiated by the fluorescence emission spectra of LLL with varying concentrations of Glucose suggesting a strong interaction between the sugar and LLL. Excitation maximum of the lectin was observed at around 350 nm indicative of the presence of Tryptophan residues at the sugar binding site. The emission spectra exhibited quenching regularly with a concomitant increase in glucose concentration. A similar quenching mechanism has been observed in the case of Litchi seed lectin and mannose residue as the concentration of sugar increased gradually [14]. Similar results were obtained in the case of a legume lectin from *Trigonella foenumgraecum* seeds which were also found to glucose/mannose specific [39].

Far UV-CD spectroscopic analysis aided us to attain an idea regarding the nature of the secondary structure of the purified lectin which revealed that it was composed of a higher percentage of alpha-helix (25.27%) as compared to β-strand (5.32%). Similar results have been observed in the case of *Dolichos lablab* seeds (DL-II) and *Moringa oleifera* lectin reporting a higher alpha-helical percentage [40,41]. Given the fact that lectins have been reported to possess the unique property of inducing proliferation of human lymphocytes, it was interesting to delve into questioning if LLL possessed mitogenic activity. As expected, it was observed that *Leucaena leucocephala* lectin was effective as a mitogen towards peripheral blood lymphocytes at a concentration of 2.4 µg/mL with the mitotic index of 52.5 ± 6.6.

5. Conclusion

In conclusion, a new glucose specific lectin has been isolated from *Leucaena leucocephala* seeds which exhibits mitogenic activity thereby triggering cell stimulation and division as evidenced by a high mitotic index. Since it binds to glucose with high specificity, it could be targeted

towards the investigation of cell surface glycosylation changes associated with certain diseases. Hence, the immense potential of *Leucaena leucocephala* lectin can be directed towards the area of therapeutics and cancer cell glycosylation studies.

Author contributions

Deepti Madayi: Conceptualization, Investigation, Resources, Writing- Original draft.

Surya P.H: Formal analysis, Methodology, Data curation.

Elyas K K: Validation, Writing- Review and editing, Supervision.

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Declaration of competing interest

The authors declare no conflict of interest.

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