CHAPTER 3

Isolation of a galactose specific lectin from *Moringa oleifera* seeds (MoSL): Affinity purification and biochemical characterization

3.1.0. Introduction

Lectins constitute a class of proteins which possess at least one non catalytic domain capable of specific recognition and reversible binding to carbohydrate [Peumans and Van Damme, 1995]. They are ubiquitously distributed in nature and most abundant in the Plant kingdom, where they are found predominantly in the seeds, leaves, barks, bulbs, rhizomes, roots and tubers depending on the plant species [Hankins *et al.*, 1988; Diaz *et al.*, 1990; Zhu *et al.*, 1996; Wright *et al.*, 1999; Van Damme *et al.*, 2000]. Lectins, by virtue of their exquisite sugar specificities are useful tools in widespread applications for monitoring the expression of cell-surface carbohydrates as well as for the purification and characterization of glycoconjugates [Goldstein and Poretz, 1986; Sharon and Lis, 1989; Cummings, 1997]. As a result of these studies, many plant lectins have become a very popular class of proteins because of their obvious potential in aiding researchers in various areas of biological sciences. Regarding their endogenous cellular functions, plant lectins appear to exert multiple physiological roles (described in Chapter 1). However, the majority of the studies on lectins have been carried out on legume species [Kocourek, 1986; Lakhtin, 1994] particularly from their seeds where they comprise up to 15% of the total protein. Extensive study of sequence homology and 3-D structure of various plant lectins suggests that they are conserved throughout evolution and thus may play, yet unknown, important physiological roles [Barondes, 1981; Etzler, 1992].

Research in the laboratory where this work was done is focused on developing new affinity methods for the large scale purification and characterization of biologically important proteins such as the lectins, glycosidases, receptors, with a long term objective to understand their structure and function. In view of their high potential as tools in biochemical and biomedical research, both for preparative and for analytical purposes, we are looking for new lectins in the tropical and subtropical flora that might have special properties in terms of sugar-binding and specificity and /or stability. *Moringa oleifera* (MO) (family: *Moringaceae*) is a tropical plant having many medicinal values which is largely grown in India [Fuglie, 1999]. The seeds contain high amounts of reserve storage proteins (MOCP), which has been used to purify water and has also been characterized in our laboratory [Tejavath and Nadimpalli, 2010]. Previously, there are reports on the identification and isolation of water soluble *M.oleifera* Lectin (WSMoL) acidic protein which is mainly active at pH

(4.5) [Santos *et al.,* 2005]. Moreover, other hemagglutinin proteins; coagulant MoL (cMoL) and MoL were reported from the same plant, which have distinct sugar specificity and molecular properties [Katre *et al.,* 2008; Santos *et al.,* 2009]. A recent study provided evidence that lectins from *Moringa oleifera* were associated with the insecticidal activity [Coelho *et al.,* 2009; Oliveira *et al.,* 2010]. In view of the diversified results on the lectins in literature [Ahuja *et al.,* 2007], it would be also interesting to study the fine sugar specificity of this lectin and naturally occurring and synthetic sugars have been used by us for this study.

The present investigation has been devoted to purify and characterize a D-galactose-binding ***M****oringa* ***o****leifera* **S**eed **L**ectin (MoSL) from seed kernels using galactose affinity gel. The strategy adopted was to prepare an affinity matrix that contained covalently linked galactose (Serlaose-divinyl sulfone-galactose) and use this for the purification of the *Moringa oleifera* seed lectin. This matrix was earlier successfully used by us for the purification of galactose-specific lectins from *Dolichos lablab* seeds, as well as from leaves and roots of the plant [Latha *et al.,* 2006; Rao and Nadimpalli, 2007]. The purified *Moringa oleifera* lectin was further characterized with respect to its glycoprotein nature, biological activity, and sugar specificity. These properties were compared to other known lectins in literature. Furthermore, a lectin-affigel was prepared to study the interaction of the glycosidases from the same plant with the lectin.

So, the present report also deals with the studies of interaction of synthetic glycoconjugates possessing hydrophobic moieties. The studies include the agglutination properties of MoSL by a variety of synthetic glycoconjugates modified with aromatic moiety as described by Kumar *et al.,* [2010].

3.2.0. Materials and methods

3.2.1. Materials

Dry mature seeds of *Moringa oleifera* (MO) PKM $_1$ variety were purchased locally. Synthetic sugars were kindly provided by Prof. C. P. Rao, IIT Mumbai. Seralose 6B, Sephadex G-150, divinyl sulfone (DVS), other chemical modification reagents used in the present study were of high purity and obtained from reputed firms.

3.2.2. Erythrocyte preparation

This was carried out according to Kumar and Rao [1986]. By ear vein puncture, rabbit blood was collected into Alsevier's solution. It was centrifuged at 3000 rpm for 10 min at 4°C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline and the pellet was made to 4% suspension with saline. Processed erythrocytes were treated with 0.05 % (w/v) pronase and incubated at 37°C for one hour. After pronase treatment the erythrocytes are washed to remove the pronase and the erythrocytes were made to 4% using saline.

3.2.3. Hemagglutinating Activity

This was performed in a plexiplate. Lectin samples (100 µL) were serially diluted in 100 µL of physiological saline (0.15 M NaCl). To each well 100 µL of 4% pronase treated rabbit erythrocytes. This reaction was incubated at 37°C for one hour and the hemagglutination was visually observed. Hemagglutination unit (HU) was expressed as the reciprocal of the highest dilution showing detectable agglutination of erythrocytes. The specific activity was calculated as the HU per mg protein (HU/mg). This pronase treated rabbit erythrocytes were used for the hemagglutination tests throughout this work unless otherwise stated.

3.2.4. Preparation of the chromatographic matrix (Seralose-DVS-Galactose-affinity gel)

Seralose-Galactose affinity column was prepared by activating Seralose-6B with divinyl sulfone as described by Latha *et al.*, [2006]. 25 mL of Seralose 6B was washed thoroughly with double distilled water on a sintered glass funnel and the wet cake was suspended in 0.5 M Na_2CO_3 buffer pH 11.0 in a falcon tube. 2.5 mL of DVS was added and the gel suspension rotated for 70 min at room temperature followed by washing with double distilled water. It was later washed with 0.5 M Na_2CO_3 buffer pH 10. Then, 5 g (20% w/v) of galactose in 20 mL of pH 10.0 buffer was added and rotated for 72 h at 4°C. The gel was washed with water and suspended in 0.5 M $NaHCO_3$ pH 8.5. To this, 0.5 mL of β-mercaptoethanol was added to block the unreactive sites and incubated for three hours at room temperature. Finally, the gel was washed with double distilled water and suspended in TBS until further use.

3.2.5. Extraction and purification of the lectin

All operations were done at 4°C unless otherwise mentioned. Freshly dehulled *Moringa oleifera* seeds were ground into a fine powder. The flour was defatted using acetone and air dried at room temperature. The powder obtained was extracted with 0.15 M NaCl overnight. The crude slurry was centrifuged at 12000 rpm for 25 min. Solid $(NH4)_2SO_4$ was added to the supernatant to attain 75% saturation, stirred for 2 h. The precipitate was collected by centrifugation (12000 rpm, 25 min), dissolved in a small amount of saline, and dialyzed extensively against saline. The dialyzed sample was centrifuged as above and the clear supernatant was subjected to affinity chromatography.

3.2.6. Affinity chromatography

Seralose-galactose gel was packed into a glass column and equilibrated with 0.15 M NaCl. The protein sample obtained above was then applied on the gel. The gel was washed extensively to remove unbound proteins and the bound protein eluted specifically using 0.3 M galactose in 0.15 M NaCl. Protein was monitored at 280 nm. Protein containing fractions were dialyzed and tested for hemagglutinating activity using pronase treated rabbit erythrocytes. The active fractions were pooled, concentrated and subjected to gel filtration.

3.2.7. Molecular mass determination

The fractions containing the lectin obtained above were pooled, concentrated and applied on to a Sephadex G-150 gel (75 cm x 1.3 cm) equilibrated with 0.15 mM NaCl at a flow rate of 20 mL/h. The column was calibrated with proteins of known molecular weight viz., BSA (66 kDa), ovalbumin (45 kDa) and lysozyme (14.7 kDa) respectively. MoSL eluted as a single peak and its native molecular mass was determined using the standard graph.

3.2.8. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 5% stacking gel and 10% resolving gel according to Laemmli [1970] under non-reducing and reducing conditions. After the electrophoretic run, the gels were stained with coomassie brilliant blue R-250. At low protein concentration, the gels were silver stained. The standard protein marker used is medium range Fermentas marker consisting of β- galactosidase (116.0 kDa), BSA

(66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25.0 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

3.2.9. Protein concentration

The protein content of the lectin samples obtained during the purification process was determined by the dye-binding method of Bradford with bovine serum albumin (1 mg/mL) as the standard [Bradford, 1976]. Protein in column fractions was monitored by measuring the absorbance at 280 nm.

3.2.10. Carbohydrate content

The neutral sugar content of MoSL was estimated by the phenol–sulfuric acid method using glucose as the standard [Dubois *et al.*, 1956].

3.2.11. Sugar inhibition studies

The assay with the purified lectin was performed as follows: 50 µL of different sugar solutions (0.4 M) (10 mM for synthetic sugars) were placed in the plexi plate and serially diluted. Then, 50 µL of the purified lectin (75 µg) was added to each well. The reaction mixture was incubated for 30 min at 37°C. To this pronase treated rabbit erythrocytes were added as described above and the agglutination visualized and titers noted.

3.2.12. Effect of pH, temperature and EDTA

To see the effect of pH on the lectin activity, 150 µg of MoSL was incubated for 6 h at various pH buffers ranging from pH 2.0 to 8.0, residual lectin activity was tested using standard hemagglutination assay. To investigate the thermostability, 1 mg/ mL concentration of the lectin was incubated at different temperatures of 4, 30, 40, 50, 60, 70, 80, and 90°C for a period of 30 min. The samples were brought back to room temperature and their ability to agglutinate the pronase treated rabbit erythrocytes were tested according to the method described above. To test the requirement of divalent cations for lectin activity, the sample was dialyzed against 100 mM EDTA in 100 mM sodium acetate buffer, pH 6.0 for 24 h. The solution was then dialyzed against distilled water for 24 h to remove EDTA. The lectin solution was then tested for hemagglutinating activity in the absence or presence of 10 mM $CaCl_2$, $MnCl_2$, or $MgCl_2$ in 0.15 M NaCl.

3.2.13. Lectin Affi-gel preparation

Coupling of the purified MoSL to Affigel-10 was carried out following manufacturer's instructions. Purified MoSL was dialyzed against 0.1 M HEPES buffer pH 7.4. 2.0 mL of Affigel-10 (Bio-rad labs) was thoroughly washed with chilled isoproponal followed by cold-water and 0.1 M HEPES buffer pH 7.4. To this 10 mg/mL MoSL in HEPES buffer, was added and the coupling reaction allowed to proceed at 4°C for 24 h by end over end rotation. At the end of this incubation period, the unbound fraction collected to determine the extent of binding and the unreacted sites in the gel were blocked with 0.1 M-ethanolamine-HCl pH 8.0 (200 µL/mL Affigel-10) for 1 h at 4°C. The gel was finally washed with PBS and equilibrated with sodium acetate buffer pH 5.0 for further experimentation.

3.2.14. Immobilized lectin-affinity chromatography

Lectin affigel prepared above was used in this study. The unbound fraction from the Seralose-galactose gel (described in methods section 3.2.6), devoid of lectin activity was dialyzed against 50 mM sodium acetate buffer pH 5.0 and analyzed for various glycosidase activities. Significantly α-D-galactosidase, α-D-mannosidase, β-D-glucosidase and α-D-glucosidase were detectable (Assay described in chapter 4A). This sample was passed through the lectin-affigel which was previously equilibrated with pH 5.0 buffer and the gel extensively washed with the same buffer until the A_{280} of the unbound fractions was <0.05. The bound proteins were eluted using 25 mM Tris-HCl buffer pH 8.0. Aliquots of the eluted fractions were analyzed by SDS-PAGE to know the nature of protein bands.

3.2.15. Proteomic analyses: in-gel digestion and mass spectrometry (MS)

After SDS-PAGE of the purified lectin, the protein was stained with CBB for detection of the lectin band. The excised gel pieces were destained with 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. In-gel digestion and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) according to the method described by Shevchenko *et al.* [1996] with slight modifications. The gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56°C for 1 h. This is followed by treatment with 55 mM

Iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2°C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac and rehydrated in 20 µL of 25 mM NH_4HCO_3 solution containing 12.5 ng μL^{-1} trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice for 10 min and kept overnight for digestion at 37°C. After digestion, a short spin for 10 min was given and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and were reconstituted in 5 µL of 1:1 ACN and 1% TFA. 2 µL of the above sample was mixed with 2 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate.

Protein identification: peptide mass fingerprinting and MS/MS analysis.

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics).The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT probability analysis ($P<0.05$), only significant hits were accepted for protein identification.

3.2.16. Serological cross reactivity

Ouchterlony's double immunodiffusion was performed to study serological cross-reactions with other galactose specific lectins. Immunodiffusion was carried out on 1% agar (in PBS) plates for 24-48 hours at 37°C in a humidified chamber and visualised for precipitin arcs [Ouchterlony, 1948]. Antiserum was placed in the central well and the lectin samples were placed in the other wells.

3.3.0. Results

3.3.1. Extraction and purification of MoSL

Seeds from *M.oleifera* contain a galactose binding lectin (MoSL) that was isolated by affinity chromatography, followed by gel permeation chromatography. The purification of the lectin from the seed extract is summarized [Table: 3.1]. The crude extract of the *M.oleifera* seeds after ammonium sulphate precipitation did not show any agglutination activity with untreated and trypsin treated rabbit erythrocytes. However, agglutination with titer value of 2^7 was observed with the pronase treated rabbit erythrocytes [Table: 3.2]. With the initial results of the sugar inhibition with the crude extract, galactose showed pronounced inhibition (MIC: 4mM) followed by lactose (MIC: 15 mM). Using this information we prepared a Seralose-galactose gel for the affinity purification of this lectin. MoSL was strongly bound to the gel and specifically eluted as a single peak from the Seralose-galactose gel using 0.3 M galactose in the column buffer [Figure: 3.1]. To further analyze the homogeneity of the purified lectin gel filtration was used. The same gel filtration column after calibrating with known standard protein is used to determine the native molecular mass of the MoSL. The protein was eluted as a single major protein peak which also exhibited the lectin activity [Figure: 3.2 A & B]. The purified lectin migrated as a single band corresponds to 27.0 kDa in SDS-PAGE under both reducing and non-reducing conditions [Figure: 3.3]. The results of SDS-PAGE and gel filtration indicated that this lectin is a monomer with an apparent molecular mass of 27 kDa. The calculated pI value of the MoSL lectin was 8.6, as suggested by MALDI report. Phenol sulphuric acid method did not show detectable range of carbohydrate indicating that this is not a glycoprotein.

3.3.2. Sugar inhibition

The influence of carbohydrates on hemagglutination activity of MoSL was studied by incubating the lectin with various simple and synthetic sugars [structures shown in Figure: 3.4] as described under methods. Agglutination mediated through MoSL has been studied in absence and in the presence of the synthetic glycoconjugates. Simple carbohydrates, viz., Mannose and Glucose don't show inhibition of agglutination activity up to a concentration of 200 mM and 400 mM respectively. Even the conjugates, viz., Glucosyl Naphthyl Imine (Glu2NI), Glucosyl Salicilyl Imine

(Glu2SI) and Mannosyl Naphthyl Imine (MNI), showed no inhibition until a concentration of 10 mM respectively. On the other hand, galactose and its conjugates, viz., Galactose (Gal), Galactosyl C1-Amine (GC1A), Galactosyl Salicilyl Imine (GSI) and Galactosyl Naphthyl Imine (GNI), were found to inhibit the agglutination even at a minimum concentration of 3.12, 1.56, 0.39, 0.04 mM respectively. Thus the inhibition of agglutination among the galactosyl based ones follow a trend, viz., GNI > GSI > GC1A > Gal [Table: 3.3].

3.3.3. Effect of temperature, pH and EDTA on hemagglutination activity

The lectin activity is stable from pH 4.0 to 6.0 [Figure: 3.5-A]. The effect of temperature on the hemagglutinating activity of MoSL is shown in Figure: 3.5-B. It is evident that MoSL was relatively stable below 70°C. However, the hemagglutinating activity of MoSL declined markedly at 80°C, and the lectin was completely inactive at 90°C. MoSL showed no change in its hemagglutinating activity after treatment with EDTA, suggesting that MoSL doesn't require metal ion for its activity.

3.3.4. Lectin affigel

Many plant lectins have been studied for their interactions with glycosidases. Immobilized lectin-affinity chromatography using MoSL affigel showed that there is some interaction between MoSL and glycosidases which are present in the same seeds [Figure: 3.6 A & B]

3.3.5. Denovo peptide sequencing

Peptide mass fingerprinting of the internal peptide sequence of the purified MoSL resulted after digestion with the trypsin was searched against the plant kingdom using Mascot search. MS/MS data obtained from this does not share similarities with any know lectin studied so far [Figure: 3.7]

3.3.6. Cross-reactivity of the MoSL with the anti-DLL antibodies

The degree of immunological homology between MoSL and a galactose specific lectin from *Dolichos lablab* (DLL-II) which was purified and characterized in our laboratory was assessed using double-immunodiffusion experiments, the lectin reacted with the antiserum to the galactose specific lectin DLL-II giving a single precipitin line, indicating that the antiserum specifically recognises the MoSL [Figure:

3.8-A]. Pre-immune serum did not give any precipitin line. This is further confirmed in western blot developed with DLL-II antibody [Figure: 3.8-B].

FIGURES

Figure: 3.1.

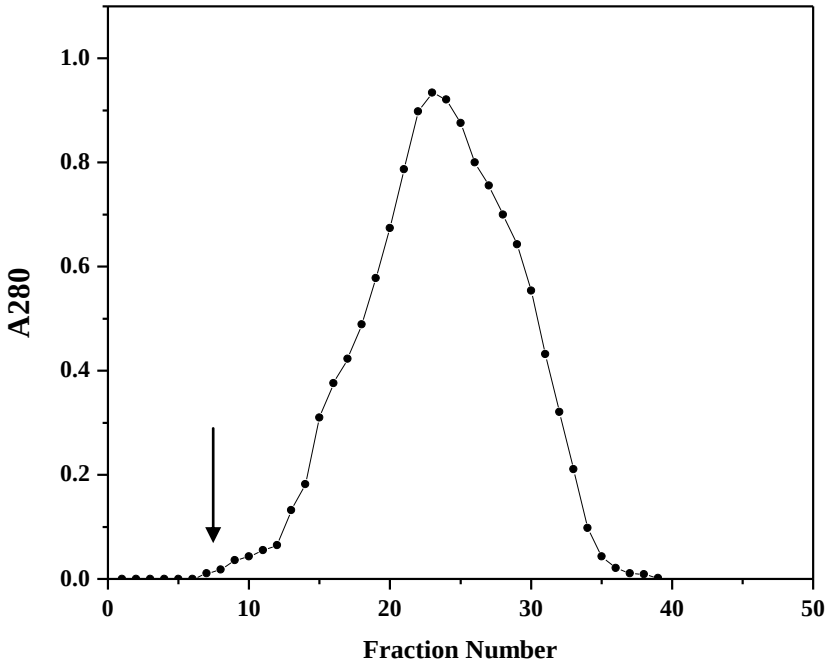
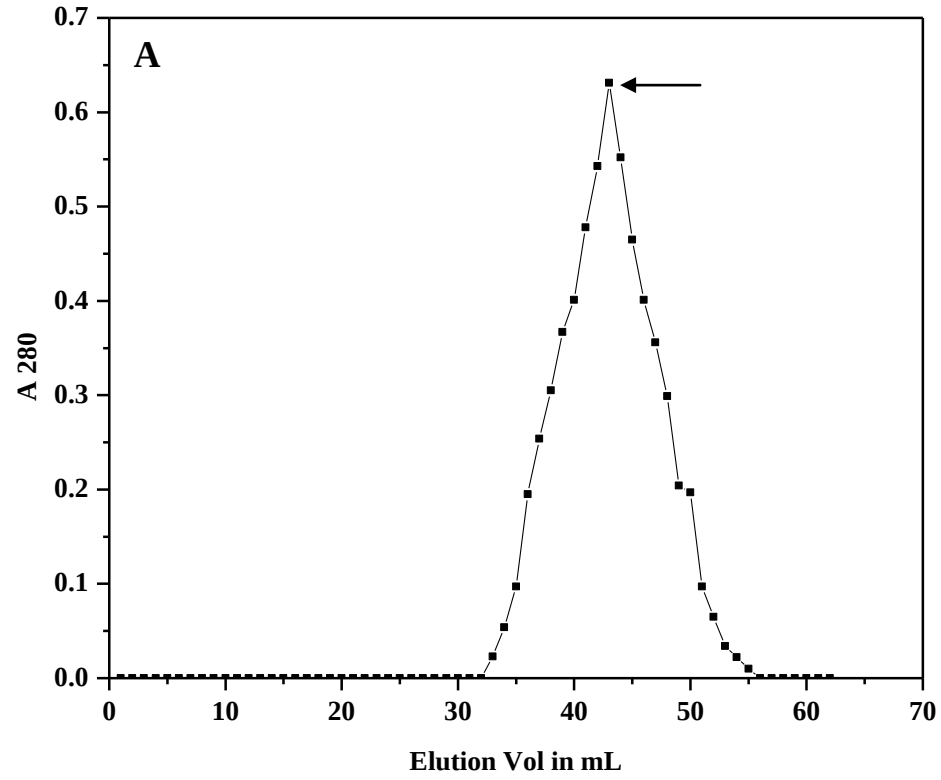


Figure: 3.1. Elution profile of MoSL from the affinity gel (3 x 15 cm): The ammonium sulphate fraction (70%) of *Moringa oleifera* saline extract was loaded on to the Seralose-galactose gel which was previously equilibrated with saline and washed to remove unbound proteins. The column was eluted with 0.3 M galactose in 0.15 M NaCl and fractions of 1 mL were collected manually. Protein was monitored at 280 nm and haemagglutinating activity checked after dialyzing the sample. Arrow indicates point where 0.3 M galactose was applied.

Figure: 3.2 A & B.


A
A 280
Elution Vol in mL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
10
20
30
40
50
60
70

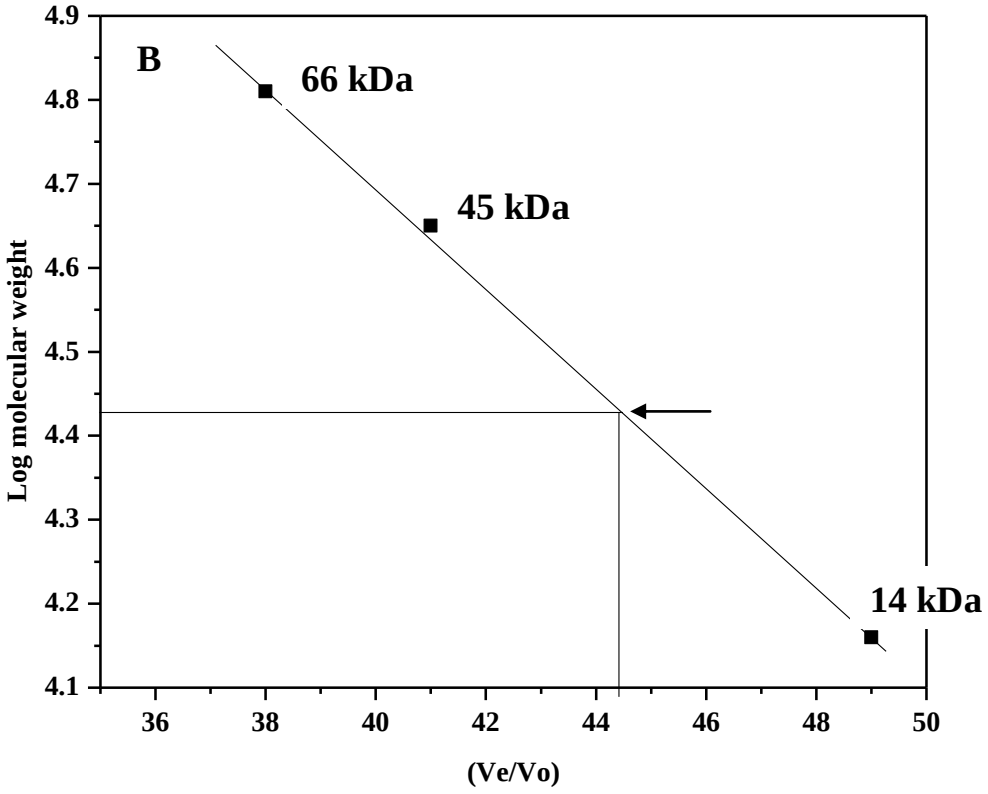

B
66 kDa
45 kDa
14 kDa
Log molecular weight
(Ve/Vo)
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
36
38
40
42
44
46
48
50

Figure: 3.2 A & B. Gel filtration and molecular weight determination of the purified lectin using G-150 column (75 x 1.3 cm): (A) The galactose gel eluted lectin sample was concentrated and passed through Sephadex G-150 column. Both equilibration and elution was carried with 0.15 M NaCl. Arrow indicates peak point where the lectin was eluted. **(B)** The same column was calibrated with known standard proteins i.e. BSA (66.0 kDa), Ovalbumin (45.0 kDa), Lysozyme (14.7 kDa) respectively. Arrow indicates fraction at which purified sample (Mr 27.0 kDa) was eluted.

Figure: 3.3

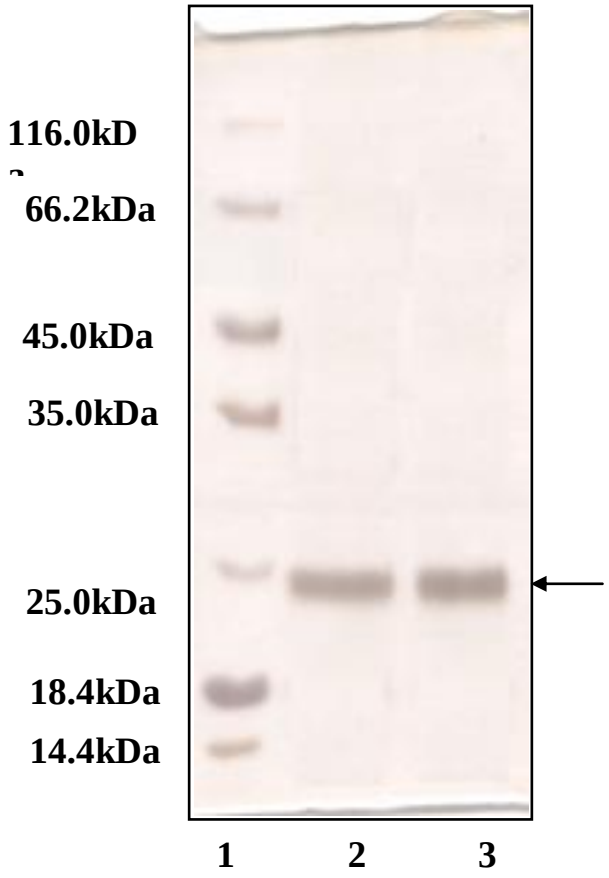
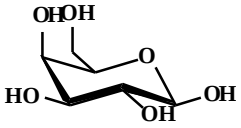


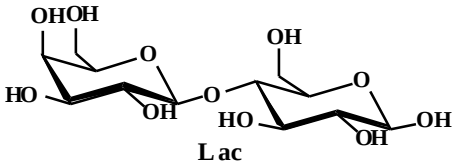
Figure: 3.3. 12.5 % Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of *Moringa oleifera* Seed Lectin (MoSL). Lane 1: - Medium range molecular weight marker. Lane 2 & 3: - *Moringa oleifera* Seed Lectin after gel filtration under reducing and non-reducing conditions. The arrow corresponds to purified lectin (Silver staining). The standard protein markers used are medium range Fermentas markers consisting of β- galactosidase (116.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

Figure: 3.4.



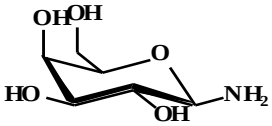
Gal

Galactose



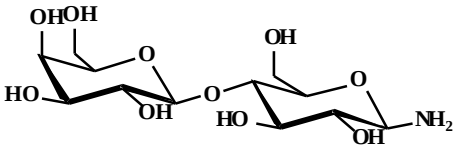
Lac

Lactose



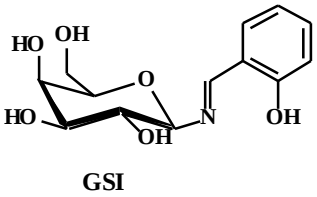
GC1A

Galactosyl C1 – Amine



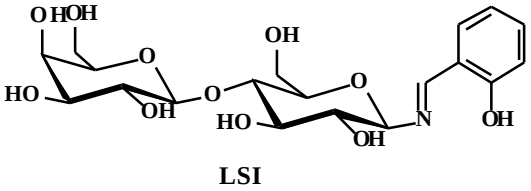
LC1A

Lactosyl C1 – Amine



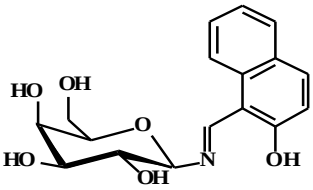
GSI

Galactosyl Salicilyl Imine



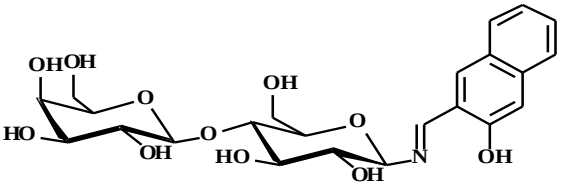
LSI

Lactose Salicilyl Imine



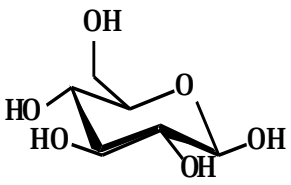
GNI

Galactosyl Naphthyl Imine



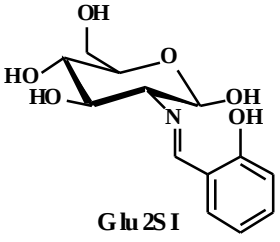
LNI

Lactose Naphthyl Imine



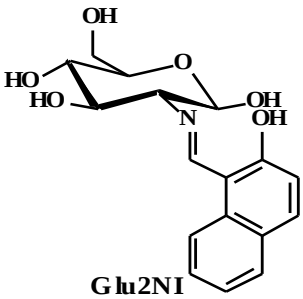
Glu

Glucose



Glu2SI

Glucosyl Salicilyl Imine

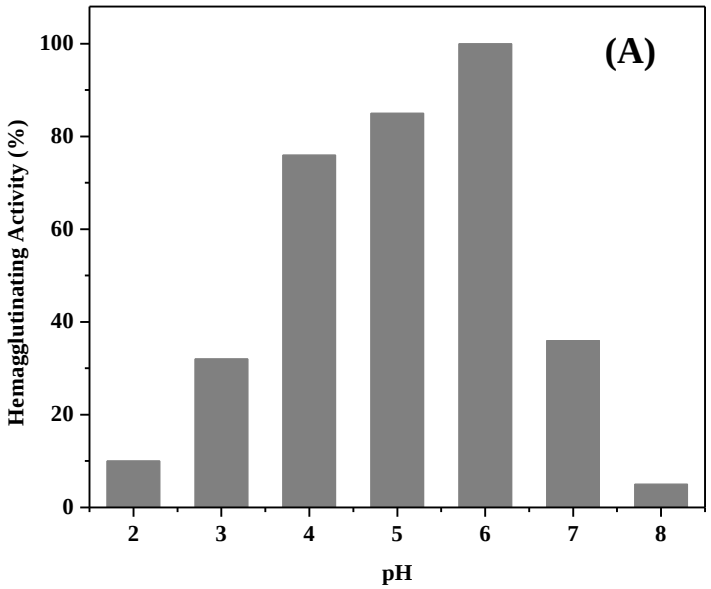


Glu2NI

Glucosyl Naphthyl Imine

Figure: 3.4. Synthetic glyco-conjugates used in the present study: Synthesis and characterization of glyco-conjugates are detailed in Kumar *et al.* (2009). These were kindly provided by Prof. C. P. Rao, IIT Mumbai.

Figure: 3.5 A & B


(A)
Hemagglutinating Activity (%)
0
20
40
60
80
100
2
3
4
5
6
7
8
pH

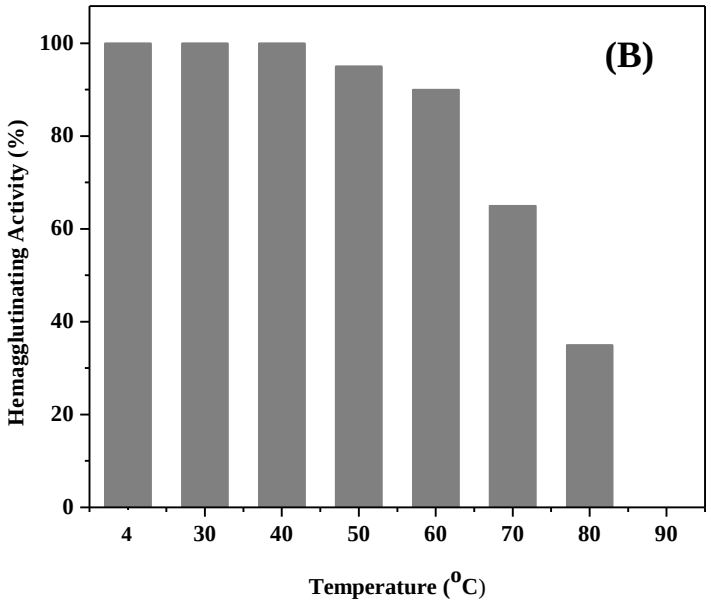

(B)
Hemagglutinating Activity (%)
0
20
40
60
80
100
4
30
40
50
60
70
80
90
Temperature (°C)

Figure: 3.5 A & B. (A) Effect of pH and (B) Temperature on the MoSL: The effect of pH and Temperature on MoSL activity was carried out at various pH and temperatures as described in the materials and methods. Same amount of protein was used at different pH values and at pH 6.0 the activity is taken as 100%.

Figure: 3.6 A & B

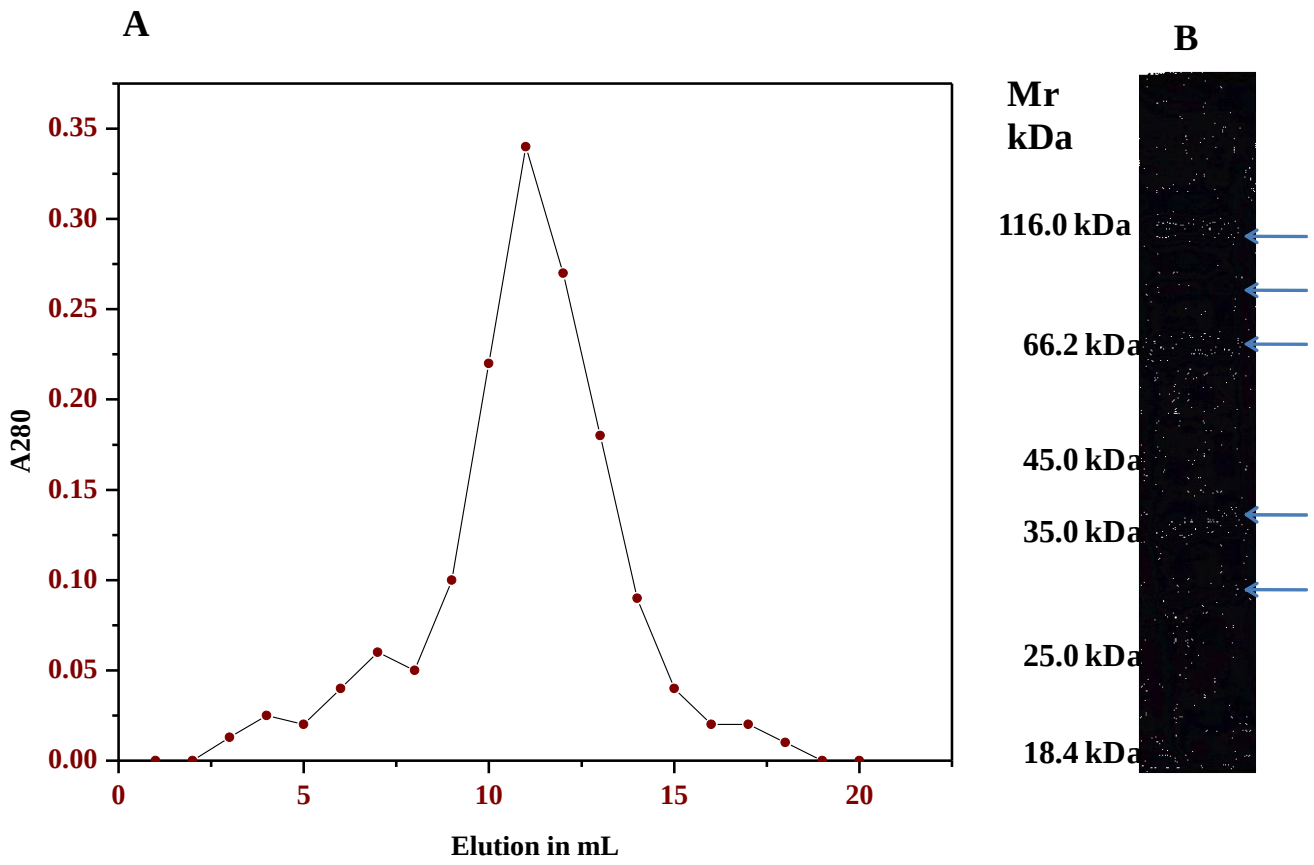

A
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A280
0
5
10
15
20
Elution in mL
B
Mr
kDa
116.0 kDa
66.2 kDa
45.0 kDa
35.0 kDa
25.0 kDa
18.4 kDa

Figure: 3.6 A & B. Immobilized lectin affinity chromatography: (A) Glycosidase rich crude seed extract in 20 mM sodium acetate buffer pH 5.0 containing 150 mM NaCl was loaded. The column was washed with equilibrating buffer until A_{280} was zero. Bound proteins were eluted with 20 mM Tris-Hcl buffer pH 8.0 containing 150 mM NaCl. As shown in the Figure A. **(B) SDS–PAGE of the eluted proteins**. Electrophoresis was carried out in a 10% gel as described in the Materials and methods. The arrows on the right indicates the proteins eluted from the gel, that also showed glycosidase activities.

Figure: 3.7

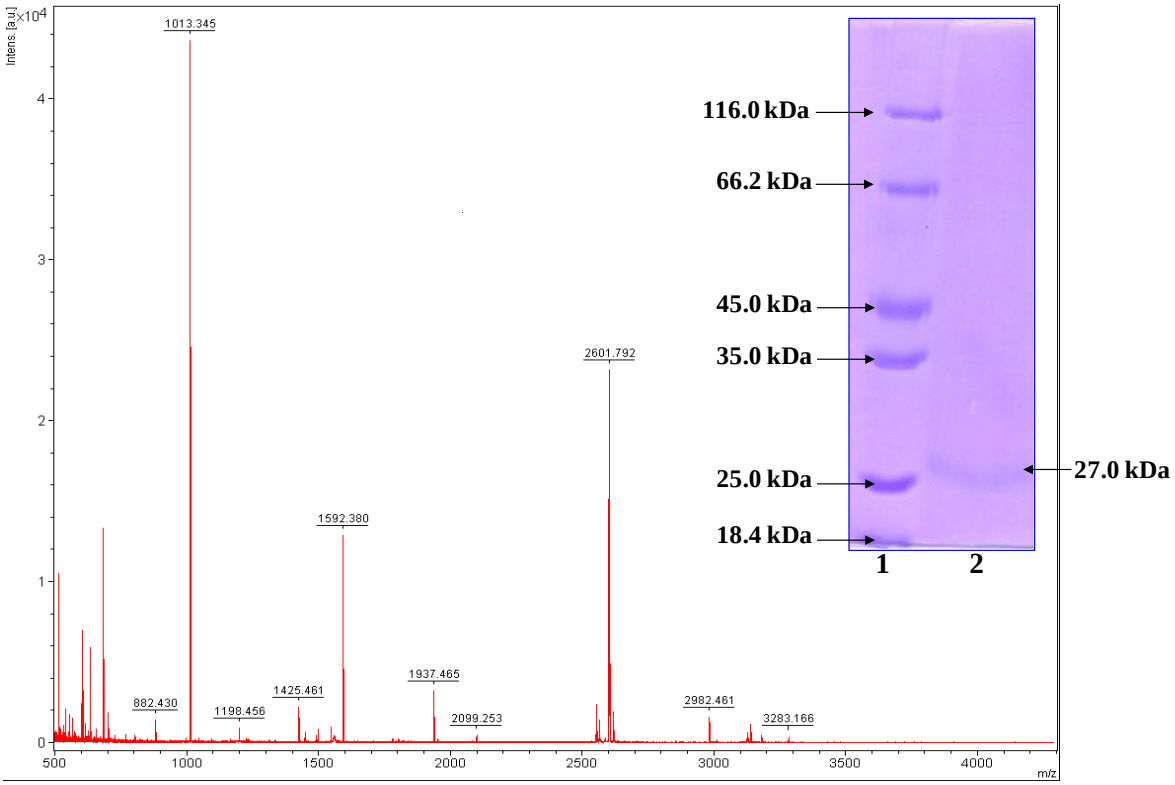
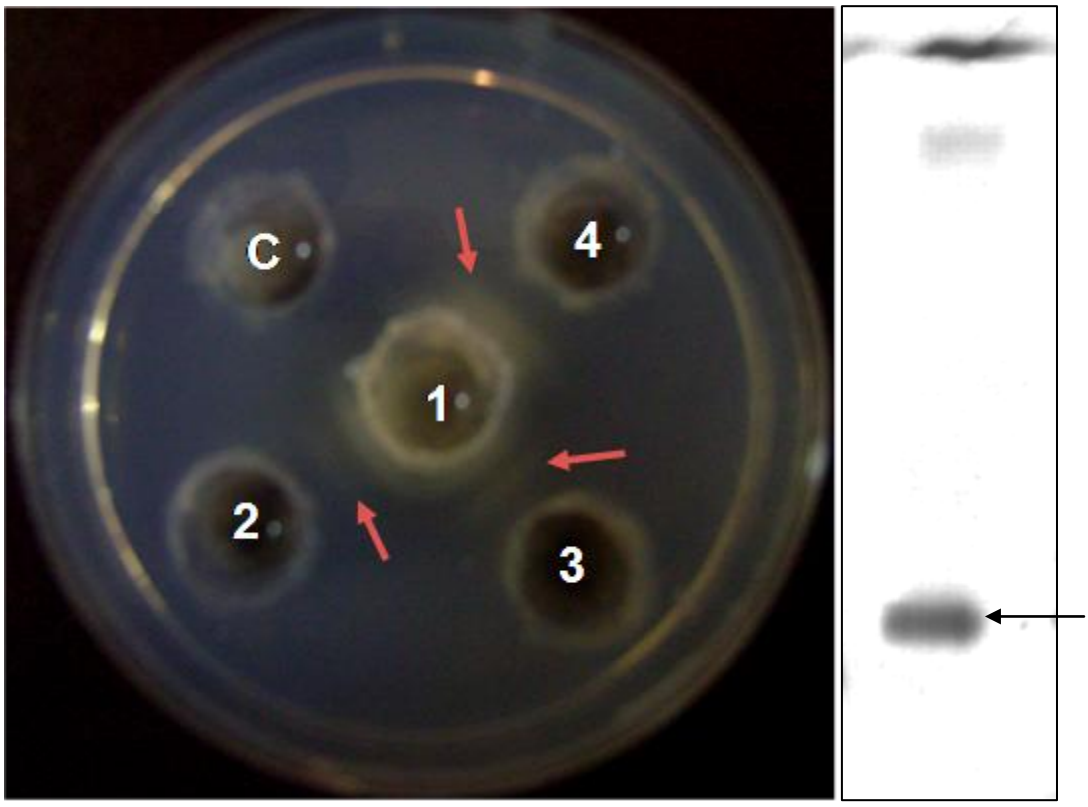

Intens. [a.u.]
×10^4
1013.345
2601.792
1592.380
1937.465
1425.461
882.430
1198.456
2099.253
2982.461
3283.166
m/z
116.0 kDa
66.2 kDa
45.0 kDa
35.0 kDa
25.0 kDa
18.4 kDa
27.0 kDa
1
2

Figure: 3.7. Internal peptide analysis of MoSL by MALDI-TOF. MALDI-TOF analysis was performed with proteolytic digests of the MoSL using trypsin. Displayed are the peptide mass fingerprints peaks. Inset 12.5% SDS-PAGE of the purified MoSL stained with Coomassie R250. Arrow represents the lectin band which is excised and given for the MALDI analysis.

Figure: 3.8 A & B



A **B**

Figure: 3.8 A & B. Serological cross-reactivity: (A) Immunodiffusion of MoSL with that of *Dolichos lablab* seed lectin antibody: Well 1: DLL-II (Galactose specific lectin antibody available in lab), Well 2: Crude extraction of *Moringa oleifera* seeds, Well 3: Purified MoSL, Well 4: DLL-II (positive control) and Well C: PBS buffer (negative control). Arrows indicates precipitin line (Arc). **(B)** MoSL lectin blot using DLL-II antiserum as the primary antibody. Arrow indicates the lectin band in the blot developed with BCIP/NBT.

TABLES

Table: 3.1. Purification of *Moringa oleifera* Seed Lectin (MoSL)

Fraction	Total protein Volume(mL)	Total protein (mg)	Total Activity (HU) [b]	Specific Activity (HU/mg)	Purification fold	Yield (%)
Saline extract [a]	500	800	1000	1.25	1	100
Galactose-Sepharose eluate	30	40	865	21	16.8	86.5
G-150 Gel filtration	22	33	730	22.12	17.6	73

[a] 100 g of the seeds were used.

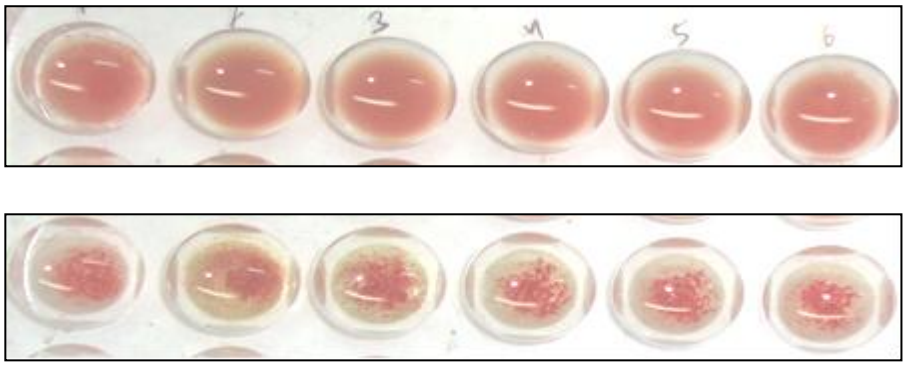
[b] Hemagglutination unit (HU) is defined as the minimal concentration of protein required to cause visible agglutination of a 4% pronase treated rabbit erythrocytes.

Table: 3.2. Hemagglutinating activity of *Moringa oleifera* seed lectin (MoSL) against native, trypsin and pronase treated rabbit erythrocytes.

Source	Type	Agglutination (titer)
Rabbit	**Native**	**ND**
	Trypsin treated (1%)	**ND**
	Pronase treated (0.05%)	**2^7**

2^7 denotes the dilution factor causing visible agglutination of pronase treated rabbit erythrocytes.

ND: Not Detected



Control

Agglutination pattern

Table: 3.3. Influence of simple and synthetic sugars on the purified MoSL.

Simple Sugar	MIC (mM)	Synthetic Sugar	MIC (mM)
D-glucose	NI	Galactosyl C_1 – Amine	1.56
D-mannose	200	Lactosyl C_1 – Amine	1.56
D-galactose	3.12	Galactosyl Salicilyl Imine	0.39
D-fucose	4.1	Galactosyl Naphthyl Imine	0.04
Lactose	12.5	Mannosyl Naphthyl Imine	NI
Melibiose	12.5	Lactose Salicilyl Imine	1.56
Arabinose	NI	Lactose Naphthyl Imine	1.1
L-rhamnose	9.8	Glucosyl Salicilyl Imine	NI
Stachyose	8.2	Glucosyl Naphthyl Imine	NI

MIC: Minimum Inhibitory Concentration,

NI: No Inhibition at 400 mM Concentration for simple sugars and 10 mM for synthetic sugars.

3.4.0. Discussion

The *M.oleifera* occurs widely in the tropical and subtropical regions of India. In the present study, we isolated and characterized a new D-galactose-binding lectin, MoSL from the seeds of *M.oleifera*. Though lectins were identified from *M.oleifera* seeds by other researchers, the lectin we isolated exhibits properties distinct from those reported [Santos *et al.,* 2005; Katre *et al.,* 2008]. The agglutination activity was observed in saline extracted samples. Hemagglutination activity test showed that MoSL was able to agglutinate only pronase treated rabbit erythrocytes and the hemagglutinating activity could be inhibited by galactose and its derivatives. This nature of agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin. Iglesisa *et al.,* [1982] reported that purified lectin from *Erythrina cristagalli* did not agglutinate native mouse erythrocytes upto a concentration of 1 mg/mL. These properties of the lectin were also seen in *Cicer arietinum* (chickpea) lectin which showed agglutination only with the pronase-treated rabbit or human erythrocytes [Katre *et al.,* 2005]. WSMoL showed agglutination with glutaraldehyde-treated rabbit erythrocytes [Santos *et al.,* 2005]. Thus different lectins seem to show distinct agglutination patterns with different animal erythrocytes.

The Seralose-galactose gel used to affinity purify the MoSL yields the lectin in a pure form and the fact that the lectin can be eluted using 0.3 M galactose signifies its sugar specificity. From 100 g of *Moringa* seeds 33 mg of purified MoSL were obtained [Table: 3.1] (lectin was purified in batch wise). Seralose-galactose gel has earlier been tested in our laboratory for the purification of the *Dolichos lablab* galactose specific lectin (DLL-II) [Latha *et al.,* 2006; Rao and Nadimpalli, 2007].

Gel filtration experiments showed that MoSL was approximately 27 kDa monomeric and non-glycosylated protein. However, the molecular weight of WSMoL and MoL reported in literature were 20 kDa and 14 kDa respectively. The non glycoprotein nature is common in some lectins. cMOL is also a non-glycoprotein hemagglutinin where as WSMoL is a glycopolypeptide. The hemagglutination activity of MoSL was inhibited by D-galactose and its derivatives. This inhibitory property is similar to that of other D-galactose-binding lectins [Yan *et al.,* 2005]. Katre *et al.,* [2008] isolated a *Moringa oleifera* seed hemagglutinin (MoL) whose activity was inhibited by complex

carbohydrates like thyroglobulin, fetuin and holotransferin. In this respect the lectin we isolated appears different in its properties compared to already published reports.

Different glycoses were modified either at their C1- (Gal and Lac) or at their C2- (Glc) positions to result in glycoconjugates possessing amino (GC1A and LC1A) and aromatic-imino moieties (GSI, GNI, LSI, LNI, Glu2SI and Glu2NI) as shown in Figure: 3.4 by adopting the synthetic methodologies described by Kumar *et al.* [2009]. All the aromatic-glyco-imino-conjugates and appropriate controls have been used in the present study. It is interesting to note that napthyl imine derivatives of galactose and lactose showed 10 times more inhibition than Salicilyl imine derivatives of the same sugars, whereas, 79 times more than the simple galactose. These results suggest that the napthyl imine at C1 position posses more inhibitory effect by readily binding to the active sites of the lectin and preventing it from exhibiting hemagglutination. The better inhibition of synthetic sugars over the galactose indicated that MoSL may possess a hydrophobic –binding pocket in the proximity to the lectin sugar affinity site. Synthetic sugars which are derived from the natural sugars showed greater inhibition. Similar results were also observed for the galactose specific jacalin lectin [Kumar *et al.,* 2010]. Availability of large concentrations of the synthetic sugars should be very useful for large scale isolation of the MoSL. The biological activities of this lectin shown here highlight the importance of sugar–protein interactions which can possibly have applications in biological research.

The optimal pH of the lectin was slightly in the acidic range as has been seen for WSMoL [Santos *et al.,* 2005]. The optimal pH of WSMoL is around pH 4.5 and agglutinating property is completely abolished at pH 7.0. Whereas, MoSL activity is not abolished at pH 7.0. The lectin exhibited thermo stability until 70°C and is similar to other lectin isolated from this plant [Katre *et al.,* 2008; Santos *et al.,* 2009]

The MS/MS data generated from the peptide mass fingerprinting of the purified MoSL did not show any similarity with any known lectin. This implies that this is a new type of lectin. However, it is necessary to obtain complete sequence information of MoSL to support this statement and to correlate with other lectins.

The degree of immunological relatedness between MoSL and DLL-II was assessed by Ouchterlony's double diffusion method [Ouchterlony, 1948]. Figure 3.7 show the typical results for the study. The antibodies raised against DLL-II a known galactose-

specific lectin, formed a single precipitin line with MoSL. These results are interesting that lectins from different families that have similar sugar specificity share unique immunological relatedness. Many legume lectins show high degree of sequence identity and immunological relatedness [Rao and Nadimpalli, 2007].

Lectins are thought to be involved in plant defense system by being resistant against insects, bacteria and fungi. In particular chitin binding lectins seems to have a role in defending plant against insects. Some of the proven insecticidal lectins are GNA (*Galanthus nivalis* agglutinin, GNA) ConA (Mannose/glucose specifies), PNA (galactose specifies), wheat germ agglutinin (WGA) [Macedo *et al.,* 2002]. Recent finding showed that cMoL a seed lectin of *M.oleifera* showed insecticidal activity on the survival and growth of the Mediterranean flour moth, Anagasta kuehniella (Zeller) (Lepidoptera: Pyralidae) [Oliveira *et al.,* 2010]. Another lectin from *M.oleifera*, WSMoL (**W**ater **S**oluble ***M****oringa* ***o****leifera* **L**ectin), showed larvicidal activity to *Aedes aegypti*, the vector of dengue [Coelho *et al.,* 2009].

Furthermore, Water soluble lectin (WSMoL) from this source is mainly observed in the seed coat, it is released upon seed imbibition at pH 6.0. This property of releasing the lectin into the buffer upon imbibition suggests the protective function in the early stage of germination when the seeds are particularly vulnerable to pathogen attacks. It would be interesting to carryout studies with the purified MoSL to analyze its insecticidal activity and to understand its specific *in vivo* role in causing protection against any pathogens.

In the present study, we have isolated and extensively characterized a galactose-binding lectin from the seeds of *M.oleifera*. Though MoSL shares some degree of similarity with other lectins isolated from the same source in terms of molecular weight and physicochemical parameters, it showed distinct variations from other members in its carbohydrate binding ability. It is interesting from a comparative biochemical point of view that lectins with different sugar-binding specificity occur in the same plant. It is noteworthy that homologous lectins are expressed in several different tissues of the same plant. In addition, MoSL showed considerably different characteristics including molecular mass and sugar-recognition specificity, and we conclude that at least, four different kinds of lectins (WSMoL, MoL, cMoL and MoSL) are present in *M.oleifera* seeds. These differences may be attributed to the variation in the variety of seeds used in each study, there origin and storage

conditions. To obtain a clear information about the relation between these lectins a complete primary sequencing should be carried out which is beyond the scope of present investigation. Our studies reveal that the *Moringa oleifera* seed lectin (MoSL) we isolated and characterized is a novel lectin with potent and potentially exploitable activities.