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Isolation of Blood Group Non-specific Lectin from *Calotropis gigantean* Seeds

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Abstract

An altered expression of glycans on the cell surface can act as a marker of various diseases including cancer and AIDS. The identification of these altered glycans can be easily achieved by using glycan binding proteins, specifically antibodies and lectins. Identifying lectins, specific towards carbohydrates that are markers of various diseases will help towards an early diagnosis of diseases. The present study describes the isolation, carbohydrate specificity, and heat stability of lectin from the *Calotropis gigantea* seeds. *Calotropis gigantea* lectin (CGL), showed a blood group non-specificity strongly inhibited by glycans of mucin glycoprotein. The Ammonium sulphate precipitation of *Calotropis gigantean* crude extract results in the concentration of hemagglutination activity at 30-60 % saturation. Lectin retained its activity when exposed to as high as 50°C for one hour. Since *Calotropis gigantean* is commonly used as a medicinal plant, lectin from this plant may have haematological applications and can be used in the purification of glycoproteins.

Keywords: Lectins, Glycans, Hemagglutination, *Calotropis gigantean*, Hapten

1. Introduction

Various key biological processes including cell-cell interactions, cell migration, and induction of apoptosis, molecular trafficking, receptor activation, signal transduction and endocytosis are invariably mediated by carbohydrate ligands (Zeng *et al.,* 2012). Understanding the qualitative as well as quantitative expression of these glycans which tend to change at various conditions of cell, can provide useful information on whether the cell is normal or diseased along with their mechanisms. Among different molecules that recognize carbohydrates both in qualitative and quantitative manners are lectins (Sharon and Lis, 2004). Lectins are the carbohydrate binding proteins of a non-immune origin which recognize glycans that are specifically either expressed on cell surface or free in solutions. This glycan recognition property of lectins has been exploited in different fields of life sciences (Sharon and Lis, 2004). Some lectins bind specifically to tumor-associated carbohydrates, and therefore have the potential to serve as biomarkers to differentiate between normal and cancerous conditions of mammalian cells. Many of these specific glycans are considered as disease markers and are targets for diagnosis as well as for therapeutics (Brockhausen, 2006). Lectins from plant sources were the first proteins of this class to be studied, and to date most of the lectins studied so far are mainly from plant sources. Since the discovery of the first lectin from castor bean by Stillmark in 1888, many lectins from almost all parts of plants have been reported (Van Damme EJ, 2014). Although numerous plant lectins have been studied for their great structural detail, the physiological role of these proteins is still poorly understood. Recently, there are many speculated roles for plant lectins 'as storage proteins', and 'as defense molecules', in symbiosis that have been assigned. A number of lectins have been isolated from storage tissues in plants (seeds or vegetative storage tissues) where they make for a very large proportion of the total protein content in the tissue (Van Damme *et al.,* 1995). Some plant lectins have been implicated in the defense mechanism of plants (Bellande *et al.,* 2017). In contrast, some plant lectins are involved in the cell wall extension and recognition (Ghequire *et al.,* 2012).

Considering the application of lectins in various fields such as immunology (Ashraf and Khan, 2003), cancer biology (Gastman *et al.,* 2004), microbiology (Oppenheimer *et al*., 2008), insect biology (Fitches *et al*,. 2010), the current research is conducted to screen lectin activity in various plant sources. The study describes the, isolation and partial purification of lectin from *Calotropis gigantea* and its carbohydrate specificity.

2. Materials and Methods

Calotropis gigantea seeds were collected during March month from botanical garden, Karnatak University, Dharwad. Seeds were separated and used for lectin extraction, EDTA, Trypsin, Bovine serum albumin (BSA), Ammonium sulphate, Folin-Ciocalteau reagent, Sodium dodecyl sulfate, Acrylamide, N,N1-Methylene-bis-acrylamide, N,N,N1,N1-tetra methyl ethylene diamine (TEMED), and Commassie brilliant blue were from either

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Sisco Research Laboratory or from Himedia Laboratory, India. Sugars used for hapten inhibition studies were from Sigma Chemicals, USA. All other chemicals, plastic wares, glassware are of analytical grade unless they are specified with company names.

2.1. Extraction of Lectin from Calotropis gigantea Seeds

To extract lectin, *Calotropis gigantea* seeds were collected, washed with distilled water and dried. Next, the seeds were homogenized (5 gm in 25 mL) using mortar and pestle at room temperature with phosphate buffered saline (pH 7.2; 100 mM), containing 200 mM EDTA and 200 mM PMSF (Phenylmethylsulphonyl fluoride). The extraction procedure was carried out for overnight at 4ºC. The extract was filtered through muslin cloth and was clarified by centrifugation at 8000 rpm for fifteen minutes at 4ºC. The supernatant was stored at 4ºC till further analysis. A similar procedure was also adopted for other plant seeds.

2.2. Preparation of Trypsinized Erythrocytes

Human blood of different blood groups (A, B and O) was collected in 1 mL of 4 % sodium citrate solution. The erythrocytes were separated by centrifugation at 1500 rpm for five minutes. Erythrocytes were washed three times with saline and finally in PBS, and were adjusted to an OD of 2.5 at 660 nm. The total volume was measured, and a final concentration of 0.025 % trypsin was added and incubated at 37 ºC for one hour. The excess trypsin was removed by repeated washing in saline and was finally adjusted to OD 3.5 at 660 nm and used for hemagglutination assay and inhibition assays.

2.3. Hemagglutination Assay (HA)

To perform the hemagglutination assay, U-bottom 96-well micro titer plates were used. Initially, 50 µl of saline was added to all the wells of respective rows. Next, to the first well of each row, 50 µl of assay solution was added and 2-fold serial dilution was made up to the 11^{th} well. From the 11^{th} well, 50 µl was discarded. Trypsinized erythrocytes of each blood groups were added (50 µl per well) to each row in the plate. For each blood group and sample, the wells containing only saline and erythrocytes were included as negative controls. The plates were incubated at room temperature for one hour and visualized. The plates were photographed, and the geometric mean titers (GMTs) were calculated. The highest dilution of the extract causing visible agglutination was arbitrarily considered as the "titer", and the lowest concentration of the protein required for hemagglutination was considered as the "Minimum Concentration of Agglutinin" (MCA) which equals one unit of hemagglutinating activity (1 HAU) The specific activity of hemagglutination was expressed as activity in 1 mg of (unit mg -1) protein.

2.4. Hapten Inhibition Assay

Inhibition assays were carried out by incubating the lectin sample in serially diluted sugar/glycoproteins prior to the addition of erythrocytes in 25 µl of assay solution. The lowest concentration of the sugar/glycoprotein, which inhibited the agglutination, was taken as the inhibitory titre of the hapten. To the 10^{th} well, saline is added instead of sugar/glycoproteins solutions, while in 11^{th} well, saline is added instead of lectin. These wells served as both positive and negative controls respectively for inhibition studies. The 12^{th} well served as the regular control which had received only 50 µL of saline and erythrocyte suspension. The wells were mixed and incubated for one hour at room temperature, and then 50 µL of erythrocyte suspension was added and incubated further for one hour at room temperature. Finally, Inhibition of lectin activity was visualized and photographed as described earlier, and the minimum inhibitory concentration (MIC) which is defined as "the lowest concentration of the sugar/glycoprotein, which inhibited the agglutination" was determined for each sugars/glycoprotein.

2.5. Effect of pH

In order to know the optimum pH for lectin activity, lectin was extracted in different buffers with varied pH. For extraction, the same procedure was followed as described above containing appropriate protease inhibitors and sodium chloride. Various buffer systems used for obtaining the desired pH are sodium acetate (pH 4.0), phosphate buffer (pH 7.2), and carbonate buffer (pH 9.5). After extraction, the clear extract was used to determine the lectin activity using trypsinized erythrocytes.

2.6. Ammonium Sulphate Precipitation

The crude extract was subjected to 0-30, 30-60 and 60-90 % ammonium sulfate [$(NH_4)_2SO_4$] precipitation. Ammonium sulfate was added at room temperature and the precipitated proteins were separated by centrifugation at 8000 rpm for thirty minutes. The supernatant was saved while the precipitate (residue) was re–dissolved in 2 mL of PBS. Both the precipitate and the supernatant were extensively dialyzed against PBS, and the hemagglutination activity was determined in all fractions.

2.7. SDS-PAGE

Protein samples from the crude extract, and ammonium sulfate precipitations were separated on 15 % acrylamide gel. The protein sample was treated with 6x SDS buffer and boiled for five minutes at 100ºC. The cooled protein was loaded into the wells, and electrophoresed at 80 V for four hours. After completion of electrophoresis, the gels were stained with Coomassie brilliant blue R-250. A standard molecular weight protein ladder ranging from 14.3-97.4 kDa was also processed and electrophoresed under similar conditions.

2.8. Protein Estimation

The protein content in various steps including crude extracts was estimated according to the protocol described by Lowry et al., (Lowery *et al*., 1951).

3. Results

Among different plant seeds, , only seeds of *Calotropis gigantea* exhibited highest hemagglutination activity (Titre-16) as determined by serial two-fold dilution technique using rabbit erythrocytes (Table 1). Apart from *Calotropis gigantean*, seeds of *Lantana camara* has also exhibited hemagglutination activity but with lower titer (04). Since maximum hemagglutination activity was observed in *Calotropis gigantean* plant, further studies were carried out using this plant for lectin isolation, hapten inhibition assay etc.

Table 1. Hemagglutination activity in different plant seeds

Sl No.	Plant name	Activity titer*	Protein conc. (mg/mL)	Specific activity
1	*Chenopodium album*	-	1.18	
2	*Lantana camara*	04	2.76	029
3	*Barnyard grass*	-	1.09	-
4	*Calotropis gigantea*	16	4.10	312
5	*Parthenium hysterophorus*	-	0.98	-
6	*Chromolaena odarata*	-	0.66	-

*Hemagglutination activity was determined using rabbit erythrocytes

3.1. Calotropis gigantean lectin (CGL) did not discriminate human blood group erythrocytes.

Since lectin agglutinated rabbit erythrocytes, A, B, and O human blood group erythrocytes were used for the assay. It was found that CGL did not discriminate between A, B, and O blood group erythrocytes. However, lectin did bind with varied intensity, and recognized the "O" blood group erythrocytes with the maximum titer (64) and the "B" blood group erythrocytes with the least titer (08). These results are presented in Figure 1. For further studies, blood group O erythrocytes were used and maximum activity was obtained with these RBCs.

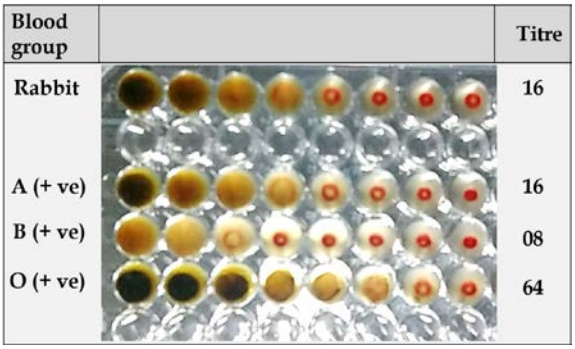

Figure 1. Hemagglutination activity of *Calotropis gigantean* lectin with different human blood group erythrocytes. CGL did not exhibited blood group specific agglutination of erythrocytes.

3.2. CGL lectin is strongly inhibited by glycans of mucin glycoproteins.

To determine the carbohydrate specificity of lectin, various monosaccharides, disaccharides and glycoproteins were used to perform hapten inhibition assay. The list of different sugars and glycoproteins used for this assay is given in table 2. As presented in Figure 2, hemagglutination activity of CGL lectin was strongly inhibited by mucin followed by fetuin. The lectin activity was not inhibited by any of the monosaccharides and disaccharides tested. These results indicate that lectin is not specific for simple sugars but recognizes complex sugars that are present in mucin or fetuin glycoproteins. This could be another reason why this lectin is blood group non-specific in nature.

Table 2. Sugars/glycoproteins used for hapten inhibition assay

Sl No.	Sugars/Glycoprotein	Inhibition	MIC*
1	Glucose (200 mM)	No	---
2	Galactose (200 mM)	No	---
3	Mannose (200 mM)	No	---
4	Xylose (200 mM)	No	---
5	Arabinose (200 mM)	No	---
6	D-Fucose (200 mM)	No	---
7	Lactose (200 mM)	No	---
8	Glucosamine (200 mM)	No	---
9	Mucin (1 mg/mL)	Yes	3.12 µg
10	Fetuin (1 mg/mL)	Yes	12.5 µg

Minimum inhibitory concentration (MIC)

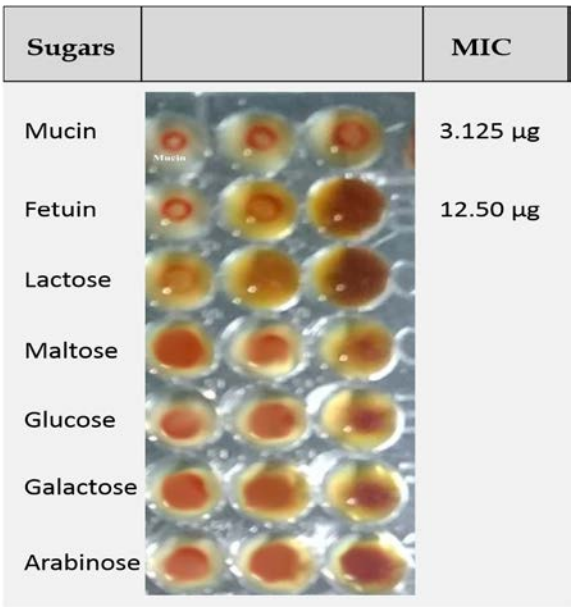

Figure 2. Hapten inhibition assay of CGL. Mucin and fetuin inhibited the lectin activity while other sugars did not show any inhibition. Mucin showed strong inhibition compared to fetuin.

3.3. Lectin activity is stable at 60°C temperature.

In order to determine the stability of lectin activity over different temperature, lectin was extracted and incubated at different temperature for one hour, and then, the hemagglutination activity was determined. As depicted in Figure. 3, lectin exhibited steady stability in its activity from 40°C-60°C. Although the titer decreased in 40°C-60°C treatments, but the same activity remained for several days. This may be attributed by the inactivation of proteases that are present in the extract. Furthermore, lectin activity was also stable for at least seven days, when it was kept at room temperature.

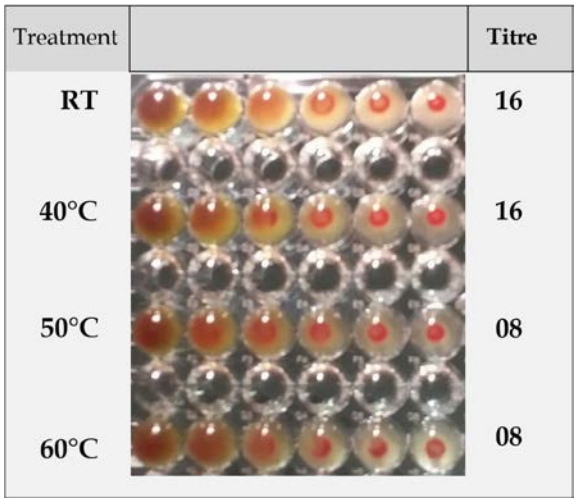

Figure 3. Lectin stability at different temperature. Hemagglutination activity of CGL remained constant after exposing lectin to different temperature for 1 h. Lectin also exhibited sustained stability at room temperature even after seven days.

3.4. Maximum hemagglutination activity of CGL was found in 30-60 % of ammonium sulphate saturation.

Next, ammonium sulphate precipitation of crude extract was performed to fractionate the proteins. The results of ammonium sulphate precipitation are presented in Figure 4. The results indicate that lectin concentration has increased in 30-60 % of ammonium sulphate precipitated fraction as evidenced by increased hemagglutination activity (titre-64). It is evident from Figure. 4 that some of the contaminated proteins can be removed by this step. Fraction 0-30 % showed some hemagglutination activity with titer 08. This may be attributed by the residual presence of lectin in this fraction. Although a good quantity of proteins was precipitated in 60-80 % fraction, but it did not show any hemagglutination activity.

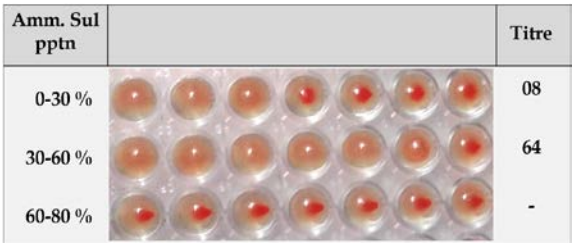

Figure 4. Ammonium sulphate precipitation of CGL. Crude extract of *Calotropis gigantean* seeds was subjected to 0-30, 30-60 and 60-80% saturation of ammonium sulphate. Lectin activity was mainly concentrated in 30-60% fraction.

3.5. SDS-PAGE analysis of partially purified lectin.

SDS-Polyacrylamide gel electrophoresis of crude and ammonium sulphate precipitated fractions was performed to analyze the number of proteins present in the samples. As shown in Figure 5, after 30-60 % of ammonium sulphate precipitation, the number of protein bands was reduced significantly (Lane-3) compared to the crude sample (Lane-1). The common protein bands that are present in all the fractions are near the molecular weight ranging from 40 to 50 kDa. These could be the protein bands which may be associated with lectin activity.

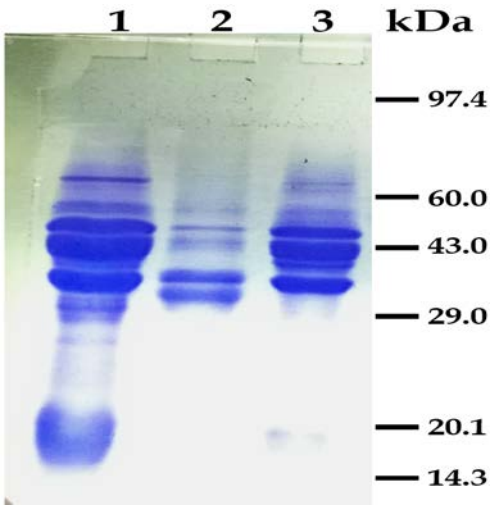

Figure 5. SDS-PAGE analysis of proteins precipitated from *Calotropis gigantean* crude extract. Common bands that are present in crude extract (lane-1), 0-30% (lane-2) and 30-60% (lane-3) ammonium sulphate precipitated proteins are closely associated with molecular weight from 40 to 50 kDa.

3.6. Fold purification of lectin.

Since lectin activity was increased in ammonium sulphate precipitated fraction, fold increase in purification of lectin was calculated based on a specific activity present in each step. Table 3 summarizes the fold purification of lectin in each step. In accordance with SDS-PAGE, it is clear from table 3 that there is a removal of some of the contaminated proteins in 30-60 % of ammonium sulphate fraction as evidenced by the increase in specific activity by 5.7-fold.

Table 3. Fold change in hemagglutination activity of CGL after ammonium sulphate fractionation.

Sl No.	Steps	Activity (titer)	Protein Conc. (mg/mL)	MCA (µg)	Specific Activity (HAU)	Fold change in activity
1	Crude	64	4.10	3.203	312	1.00
2	0-30%	08	1.07	6.687	149	0.47
3	30-60%	64	0.72	0.563	1776	5.70

4. Discussion

In the current study, the presence of lectin activity was screened in many plant sources. It was found that the seeds of *Calotropis gigantean* exhibited maximum lectin activity with all the human blood group erythrocytes. *Calotropis gigantean* plant is widely used as a medicinal plant (Kadiyala *et al.*, 2013), therefore, the researchers carried out a detailed study on the seeds of this plant to isolate and purify lectin.

Since the crude extract of *Calotropis gigantean* did not agglutinate any of the human blood group erythrocytes specifically, it was assumed that lectin probably recognizes complex sugars that are present on the cell surface of erythrocytes. This prediction was confirmed by hapten inhibition assay which revealed that lectin indeed exhibited specificity towards O-linked glycans of mucin and fetuin. These results are inconsistent with the blood group non-specificity of lectin.

In order to know the stability of lectin, the lectin extract was exposed to different temperatures and it was found that lectin is stable up to 60°C and when it was kept at room temperature for more than seven days. This result suggested that lectin is less prone to protease attack making it easy to operate during purification procedures. Many lectins are heat-liable (Devi *et al.*, 2011); however, CGL did not denatured at high temperature. Ammonium sulphate fractionation not only increased the lectin concentration. In addition, it has also helped to remove most of the contaminated proteins that are present in the crude extract. The electrophoretic pattern of crude and ammonium sulphate precipitated proteins revealed that bands below 30 kDa are effectively removed during the ammonium sulphate precipitation step.

5. Conclusion

The current study describes the isolation and partial purification of lectin from *Calotropis gigantean* seeds and its carbohydrate specificity. Given the fact that *Calotropis gigantean* is regularly used as a medicinal plant, the presence of lectin activity may have implication in its medicinal properties. Furthermore, the results of the ammonium sulphate precipitation and SDS-PAGE steps provide important information that this lectin could be purified to homogeneity by employing these steps coupled with other chromatographic techniques.

References

Ashraf MT and Khan RH. 2003. Mitogenic lectins. *Med Sci Monit.* **9(11)**: 265-269.

Bellande K, Bono J-J, Savelli B, Jamet E and Canut H. 2017. Plant lectins and lectin receptor-like kinases: How do they sense the outside? *Inter J Mol Sci.*, **18(6)**:1164

Brockhausen I. 2006. Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Reports*, **7(6)**: 599–604.

Devi SK, Singh SS, Singh SJ, Rully H and Singh LR. 2011. Purification and characterization of a magnesium ion requiring N-acetyl-D-glucosamine specific lectin from seeds of Quercus ilex L. *Biosci, Biotechnol, Biochem.*, **75(9)**: 1752–1757.

Fitches EC, Bell HA, Powell ME, Back E, Sargiotti C, Weaver RJ and Gatehouse JA. 2010. Insecticidal activity of scorpion toxin (ButaIT) and snowdrop lectin (GNA) containing fusion proteins towards pest species of different orders. *Pest Management Sci.*, **66(1)**: 74–83.

Gastman B, Wang K, Han J, Zhu Z, Huang X, Wang G-Q, Rabinowich H and Gorelik E. 2004. A novel apoptotic pathway as defined by lectin cellular initiation. *Biochem Biophys Res Commu.*, **316(1)**: 263–271.

Ghequire MGK, Loris R and De Mot R. 2012. MMBL proteins: from lectin to bacteriocin. *Biochem Soc Trans.*, **40(6)**: 1553–1559.

Kadiyala M, Ponnusankar S and Elango K. 2013. *Calotropis gigantiea* (L.) R. Br (Apocynaceae): a phytochemical and pharmacological review, *J Ethnopharmacol.*. **150(1)**: 32–50.

Lowry OH, Rosebrough NJ, Farr Al and Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem.*, **193(1)**: 265–275.

Oppenheimer SB, Alvarez M and Nnoli J. 2008. Carbohydrate-based experimental therapeutics for cancer, HIV/AIDS and other diseases. *Acta Histochemica*, **110(1)**: 6–13.

Sharon N and Lis H. 2004. History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiol.*, **14(11)**: 53R–62R.

Van Damme EJ, Goossens K, Smeets K, Van Leuven F, Verhaert P and Peumans WJ. 1995. The major tuber storage protein of araceae species is a lectin. Characterization and molecular cloning of the lectin from *Arum maculatum* L. *Plant Physiol.*, **107(4)**: 1147–1158.

Van Damme EJ. 2014. History of plant lectin research. *Methods Mol Biol.*, **1200**: 3–13.

Zeng X, Andrade CAS, Oliveira MDL and Sun X-L. 2012. Carbohydrate-protein interactions and their biosensing applications. *Anal Bioanal Chem*, **402(10)**: 3161–3176.