Research Article

Partial Purification and Characterization of a Galactose Specific Lectin from *Chrysophyllum cainitol*, a Plant that Shows Hypoglycemic Activity

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ABSTRACT

Plant lectins are a heterogeneous group of carbohydrate binding proteins with agglutination properties. Lectins possess diagnostic and therapeutic value and are used as valuable tools in glycobiology. The objective of the study was to isolate and characterize the lectin from *Chrysophyllum cainitol* (CCL), a tropical tree of the family Sapotaceae. A lectin with high hemagglutination property has been partially purified by gel filtration chromatography on Sephadex G-75.Lectin activity was checked by hemagglutination assay and the sugar specificity was determined by hemagglutination inhibition assays. The lectin showed hemagglutination activity on all four blood groups such as A, B, AB and O. Galactose was the best inhibitor of *Chrysophyllum cainito* lectin and the minimum concentration of galactose needed to inhibit the hemagglutination activity of lectin was 25mM. SDS PAGE analysis of purified CCL showed a prominent band with approximate molecular weight of 29kDa. CCL was found to be stable at temperature of 40°C – 70°C. Maximum activity was observed at 37°C. The lectin was found to be stable at a pH range of 6.0 – 7.5. In the presence of reducing agents such as DTT, the activity of the lectin increased by 25% at a concentration up to 100 µM after which, its activity reduced by 50% while for 2-Mercaptoethanol the lectin activity was reduced by 25% at a concentration up to 200 µM. Agar disc diffusion assay was carried out and the purified CCL inhibited the growth of *E.coli*.

Keywords: *Chrysophyllum cainito* Lectin, Gel filtration chromatography, Hemagglutination, Sugar Inhibition.

INTRODUCTION

Lectins are a group of highly diverse, carbohydrate-binding proteins of non-immune origin that are ubiquitously distributed in plants, animals and fungi. They were initially found and described in plants, but later isolated from microorganisms and animals.[1]Generally lectins are observed as dimers or tetramers with hemagglutinating property. The subunits are made up of a single poly peptide chain that are encoded by different genes or by a multiple gene family of closely related genes. Lectins bind to carbohydrates and sugar containing substances in a specific and reversible way so that they can even precipitate glycoconjugates.[2]They vary, however in molecular size, amino acid composition, metal ion requirements and three dimensional structure.[3]

Chrysophyllum cainito L commonly known as Star apple or the tree with 'golden leaves' is an ornamental tree which bears edible fruits. It was native to the Greater Antilles and the West Indies but now it is grown throughout the tropics, including Southeast Asia, Carribean, West tropical Africa, Zanzibar, Brazil and India.[4] It is a tree with a spreading crown and numerous and slender branches.The leaves are leathery and ovate or oblong, pointed at the tip, blunt or rounded at the base and covered underneath with silky, golden-brown, soft hairs.

There are several reports from folk medicine indicating that the bark, leaves, fruits and seeds of the *Chrysophyllum* genus present pronounced biological activity. Different parts of *C. cainito*like fruits, bark have been used as an antitussive, astringent and antioxidant agent, in the treatment of diarrhea, fever, diabetes, gonorrhea, catarrh of the bladder and abscesses.[5-6]It is already reported that the aqueous decoction of *C.cainito* leaves showed hypoglycemic activity.[7] The hypoglycemic effect was mainly attributed to alkaloids, sterols or triterpenes. Recently, the leaves of *C. cainito* have been reported to possess anti-inflammatory and anti-hypersensitive activity.[8]The leaves of *C. cainito* are traditionally used in the treatment of diabetes and have been studied scientifically for their hypoglycemic activity in diabetic animal model. Traditionally, the leaves are also used as a poultice to wounds.[9]The present study aimed at purifying the lectin from the crude leaf extract. The methods such as ammonium sulphate precipitation, Gel filtration chromatography and SDS PAGE were done for isolation and separation of lectin.Blood group specificity and sugar inhibition studies were also carried out to characterize the lectin. pH and temperature stability studies were also performed. Effect of reducing agents and metal ions on the hemagglutination activity of CCL were also studied. Antimicrobial activity was also performed.

MATERIALS AND METHODS

Isolation and Purification of Lectin from Chrysophyllum cainito leaves

Leaves from the *C.cainito* tree were collected from the Calicut University campus. Collected leaves were washed with water and air dried for 2 days. Air dried leaves were chopped into small pieces and were soaked in 1X

Phosphate buffer saline (PBS) buffer (pH 7.4) in a ratio of 1:3 (100g in 300 ml) and kept overnight at 4°C.The leaves were homogenized and the homogenate obtained was stirred for 1 hour in ice bath and filtered through a cheese cloth. The filtrate obtained was centrifuged at 4500g in a cooling centrifuge. The crude extract thus obtained was subjected to ammonium sulphate precipitation to get a concentration of 20%, 40%, 60%, and 80% saturation of the salt by continuous stirring and the protein pellets were collected by centrifugation at 4,500g. The pellets were resuspended in PBS. The resuspended protein pellet was dialyzed against PBS for 24 hours at 4°C . After dialysis, each protein fraction was subjected to hemagglutination assay.

To purify the lectin, the dialyzed sample was subjected to Sephadex G-75 gel filtration chromatography using PBS as the mobile phase.The protein was eluted using the same buffer in 3ml aliquots and the flow rate was adjusted to 1ml/min. The absorbance was read at 280nm. SDS PAGE was carried out which helped in determining the molecular weight.

Protein Estimation

Protein concentrations were determined by the method of Lowry using BSA as the standard protein.[10]

Poly acrylamide Gel Electrophoresis

For poly acrylamide gel electrophoresis (PAGE) analysis in presence of SDS 12.5% resolving gel and 6% stacking gel were used. BSA (66kDa) was used as the protein marker. The gels were stained with Coomassie Brilliant Blue R-250.

Hemagglutination and Sugar specificity Assays for lectins

Blood from healthy donors was obtained from blood banks of Aster MIMS hospital, Calicut. The erythrocytes were washed thrice in PBS by centrifugation at 500 g(3,000rpm). Finally 2% R.B.C suspensions were prepared and used for determination of hemagglutination activity. The hemagglutination assay was performed in an U-bottom micro titer plates, wherein 50 µL of plant extract preparations were serially diluted in PBS. To this 50 µL of 2% R.B.C suspension was added and monitored for hemagglutination after 1 hour. Hemagglution Unit (HU) is defined as the reciprocal of the highest dilution of the sample that gives visible agglutination of the erythrocytes. Specific activity of the protein is expressed as the hemagglutination titre per mg of the protein and represented as Hemagglutination Unit per mg (HU/mg).

To test the sugar specificity of plant extract preparations, hemagglutination inhibition tests were performed using different sugars.25 µLstock solutions of various sugars such as arabinose, fructose, fucose, galactose, lactose, maltose, mannose, rhamnose, and xylose serially diluted in 25 µLPBS were used for this test. To this, 25 µL of purified lectin samples was added to the wells and incubated at room temperature for 1 hour. 50 µL of 2% erythrocyte suspension was added to all wells, incubated for another 30 minutes at room temperature and checked for hemagglutination inhibition activity.

Effect of pH on the hemagglutination activity of the lectin

Phosphate buffers of pH values 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared. 20 µL each of diluted buffer having an ionic strength double that of PBS (buffer: distilled water – 1:2) was mixed with an equal volume of lectin sample in PBS so as to maintain the same ionic strength of PBS in the reaction mixture. The samples were incubated overnight at 4°C. It was then brought back to room temperature and the hemagglutination assay was performed.

Effect of temperature on stability of lectin

The effect of temperature on the agglutinating activity of *C.cainito* lectin was determined by performing the assay at different temperatures.[11] The purified lectin was incubated in a water bath for 60 minutes at various temperatures 4°C, 37 °C, 40°C , 50 °C, 60°C, 70 °C , 80 °C and 100°C and then cooled to room temperature and the hemagglutination assay was performed.

Effect of denaturing agent on stability of lectin activity

To determine the effect of Guanidine hydrochloride on lectin activity, 25 µL of 4M guanidine hydrochloride solution was mixed with 25 µL of purified lectin. 50 µL of 2% erythrocyte suspension was added to all wells. The plate was visualized for agglutination after 30 minutes of incubation at room temperature against appropriate controls.

Effect of reducing agents on the stability of lectin activity

The effect of 2-Mercaptoethanol and DTT was determined by mixing 15 µL of each stock with 15 µL of the lectin to obtain a final concentration of the reducing agent as 50 µM, 100 µM, 150 µM, 200 µM and 250 µM. 30 µL of this mixture was serially diluted in equal volume of PBS. 50 µL of erythrocyte suspension was added and the agglutination pattern was visualized. The hemagglutination titres obtained were compared with appropriate controls.

Effect of EDTA and metal ions on the activity of lectin

Lectin sample was incubated with EDTA at a concentration of 100mM concentration overnight at 4°C (EDTA treated sample).Samples were dialyzed back to PBS and hemagglutination property of both samples was analyzed. Aliquots of dialyzed samples were treated with each of the metal ions- magnesium chloride ($MgCl_2$), Calcium chloride ($CaCl_2$), Manganese chloride ($MnCl_2$), Barium chloride ($BaCl_2$), and ferric chloride ($FeCl_3$) at a concentration of 100Mm, incubated at room temperature for 2 hours and hemagglutination activity was analyzed.[12]

Assay of antimicrobial activity

For assessment of antimicrobial activity all the glasswares and samples were sterilized in an autoclave.

Commercially available sterile discs (Hi-Media, Mumbai) were used for the present study. After preparation of the media, 10 ml of sterile nutrient broth was aseptically inoculated with the test culture organisms and incubated at 37 °C for 18 hours. After incubation, the test cultures were plated on air dried nutrient agar plates using a sterile glass spreader. Using a clean forceps, the sterile discs loaded with the lectin was plated on the surface of Muller Hinton Agar plates seeded with the test bacterial strains. Ciprofloxacin discs were used as the control. The plates were then incubated at 37 °C for 24 hours. The zone of bacterial growth inhibition was observed and its diameter was measured in millimeters.

RESULTS

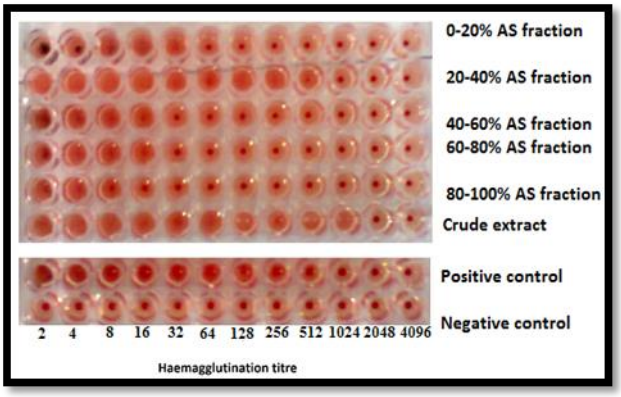

Figure 1: Hemagglutination activity of different fractions obtained through ammonium sulphate precipitation. Blood grouping sera was used as positive control and PBS without lectin extract was used as the negative control.

A lectin was isolated and partially purified from the leaves of *Chrysophyllumcainito* by Gel Filtration Chromatography on Sephadex G-75.Leaf extract of *C. cainito* was subjected to ammonium sulphate precipitation. 0 – 20%, 20 – 40%, 40 – 60%, 60 – 80% and 80 – 100% saturated fractions were collected and subjected to dialysis and concentrated. Hemagglutination titre of each fraction was determined as shown in Figure 1.

The fraction which gave maximum hemagglutination titre and specific activity i.e., 20 – 40% ammonium sulphate fraction as indicated in table 1 was selected.

It was then purified by gel filtration chromatography by using Sephadex G – 75 column. Each fraction was collected and absorbance measured at 280nm.

On the gel filtration column, the protein eluted as 2 peaks peak I and Peak II out of which only peak II showed haemagglutinating activity as shown in figure 2.

The fractions in Peak II were pooled together and stored at 4°C.Thefraction showing high lectin activity was used for further biochemical characterization. The purification profile of the *C.cainito* lectin is summarized in Table 2. The yield of lectin obtained was 0.54% and about a 5.47 fold purification was achieved.

Table 1: Specific activity of purified lectin

Sl No	Sample	Protein conc. (mg/ml)	Hemagglutination titre (HU)	Specific activity (HU/mg)
1.	Crude	18.4	1024	55.65
2.	0 – 20% AS fraction	1.87	32	17.11
3.	20 – 40% AS fraction	3.02	512	169.54
4.	40 – 60% AS fraction	3.71	16	4.312
5.	60 – 80% AS fraction	4.51	16	3.547
6.	80 – 100% AS fraction	4.89	8	1.635
7.	20 – 40 % Gel filtration Chromatography	0.168	32	190.476

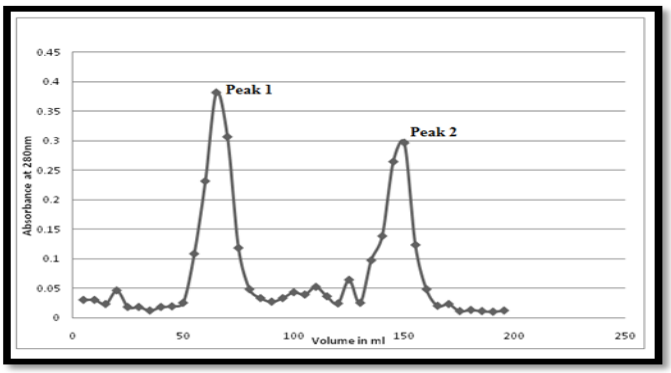

Figure 2: Elution profile of lectin on Gel Filtration,Sephadex G – 75 column (1.8 cm x 68 cm). About 15 milligram of protein was applied to the column, pre-equilibrated with 0.1X PBS, pH 7.4. The protein was eluted using the same buffer in 3ml fractions at a flow rate of 1ml/min.

Electrophoretic Analysis

SDS PAGE analysis of 20 - 40% gel filtration column fraction showed an approximate molecular weight of 29kDa as shown in the figure 3.

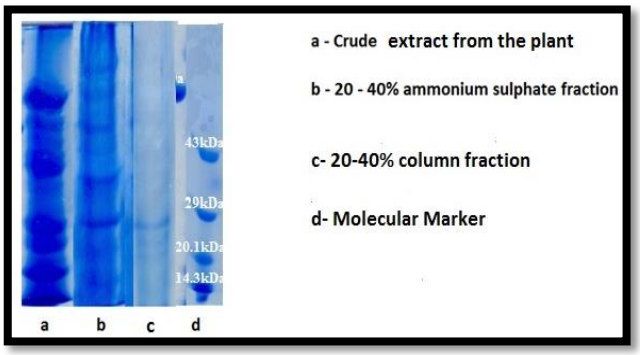

Figure 3: SDS PAGE analysis of purified lectin

Table 2: Purification profile of lectin from *Chrysophyllum cainito*

Sl No	Sample	Concentration ofprotein (mg/ml)	Titre	Specific activity (HU/mg)	Yield of protein (%)	Fold of Purification
1	Crude	18.4	1024	1113.04	100*	1*
2	20-40% AS fraction	3.02	512	3390.72	1.148	3.04
3	Sephadex Gel Filtration Chromatography	1.68	512	6095.23	0.547	5.47

*Values taken arbitrarily

$$\text{Yield of protein} = \frac{\text{Total protein content of purified fraction X 100}}{\text{Total protein content of the crude extract}}$$

$$\text{Fold of purification} = \frac{\text{Specific activity of the purified fraction X 100}}{\text{Specific activity of the crude extract}}$$

Sugar Specificity Assay and Blood group specificity of purified lectin

To determine the sugar specificity of the lectin, various sugars were tested at different concentration. It was found that galactose was the best inhibitor of purified lectin. Minimum concentration of galactose needed for hemagglutination inhibition was 25mM. No inhibition was found for the remaining sugars. Blood group specificity of purified lectin was checked by using 2% erythrocyte suspension prepared from different blood groups such as A, B, AB and O. The hemagglutination activity of purified lectin on RBCs of A, B and AB blood groups were found to be very similar and it gave the same hemagglutination titre i.e. 512HU. In the case of O group the titre value was 128HU, which was 4 times less than that of A, B and AB.

Effect of pH and temperature

On examination of *C.cainito*lectin in different pH range of phosphate buffer (6.0 – 8.0) it was found that the lectin was most stable in the range between pH 7.0 – 7.5. Below pH 7.0 the lectin activity was reduced. Similarly activity got reduced above pH 8.0 as shown in figure 4.

Lectin was found to be stable at temperature range from 4^0C – 70^0C.

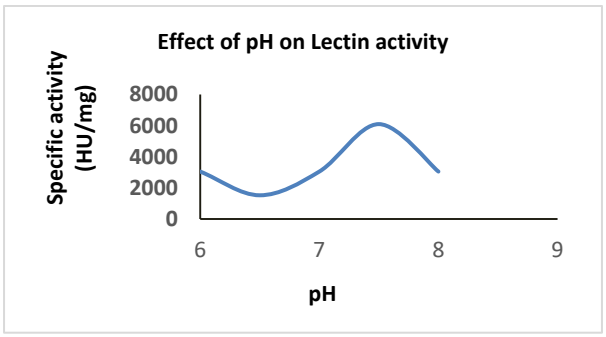

Figure 4: Effect of pH on lectin activity

Maximum specific activity (169.54HU/mg) was found at 37^0C. The activity was almost halved at 4^0C and 40^0C (84.76 HU/mg) and least (1.324HU/mg) activity was found at 80^0C. At 100^0C the specific activity was found to be zero (**figure 5**).

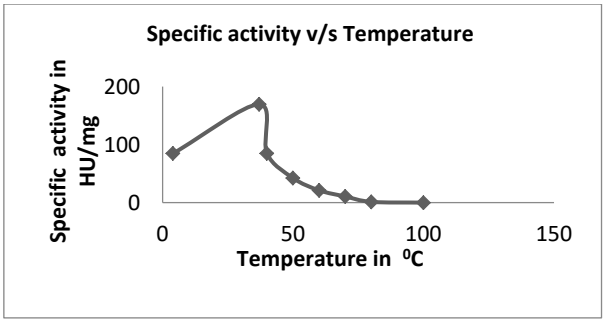

Figure 5: Stability of lectin at different temperature range.

Effect of denaturing agent on lectin activity

Stability of RBC in guanidine hydrochloride solution (of concentration 0.1M – 1M) was checked. RBC (2%) suspension was found to be stable in guanidine hydrochloride solution of concentration up to 1M. Then stability of purified lectin was checked in this concentration range. Lectin was found to be stable and showed hemagglutination activity in guanidine hydrochloride solution of concentration up to 1M.

Effect of reducing agents on the stability of lectin activity

It was found that the reducing agent 2-Mercaptoethanol improved the hemagglutination activity of the lectin by 25% at a concentration of 150 µM whereas DTT reduced the hemagglutination activity by 25% at a concentration up to 200 µM.

Effect of EDTA and metal ions on lectin activity

Ethylene Diamine Tetra Acetate (EDTA) is a chelator of metal ions. Hence a reduction in lectin activity was observed after incubation with EDTA. Hence it is observed that the ions in the buffer system play a role in hemagglutination activity of the lectin. But after dialysis of the treatment mixture back to the same buffer system could not restore the lectin activity it can be concluded that the ions present in the PBS buffer do not have a significant role in lectin activity.

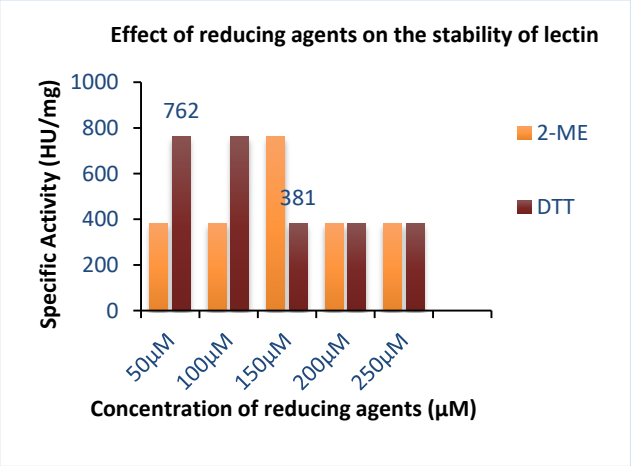

Figure 6: Effect of reducing agents on lectin activity

Aliquots of dialyzed samples were treated with different metal ions solutions - magnesium chloride ($MgCl_2$), Calcium chloride ($CaCl_2$), Manganese chloride ($Mn\ Cl_2$), Barium chloride ($BaCl_2$), and ferric chloride ($FeCl_3$) at a concentration of 100Mm. It was observed that none of the ions except $MgCl_2$, played a role in hemagglutination. Instead the ions themselves exhibited hemagglutination by themselves. But treatment with $MgCl_2$ reduced the specific activity of the lectin. Hence it can be concluded that $MgCl_2$ might be competing with lectin for RBC surface receptors or it is binding to the lectin domains thereby blocking carbohydrate recognition.

Assay of Antibacterial activity

Activity of purified lectin on growth of various microorganisms such as *E.coli* (gram negative) and *Staphylococcus aureus* (gram positive) were checked by disc diffusion method (Figure:7).

As shown in figure 7, growth of *E.coli* was found to be inhibited by purified lectin (100µl/disc) and produced a zone of inhibition of diameter 13mm. While purified lectin did not show any inhibitory action on the growth of *Staphylococcus aureus.*

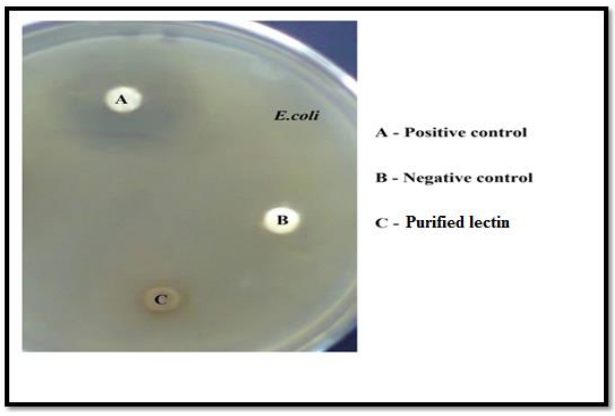

Figure 7: Antibacterial activity of purified lectin.

DISCUSSION

Lectins are highly specific sugar binding proteins of non-immune origin which bind to a soluble carbohydrate or to a carbohydrate moiety that is a part of a glycoprotein or glycolipid. Lectins are widely distributed in nature and are found in micro organisms, plants, animals and humans, have an important role in various cellular processes. A number of lectins belonging to Sapotaceae family have already been studied. However this is the first report on lectin activity being demonstrated by *Chrysophyllum cainito*.

A lectin was successfully isolated and purified from the leaf extracts of *Chrysophyllum cainito* by ammonium sulphate precipitation, dialysis and gel filtration chromatography. *Chrysophyllum cainito* leaf lectin was not blood group specific and it has hemagglutination activity on all four blood groups such as A, B, AB and O. Galactose was the best inhibitor of *Chrysophyllum cainito* leaf lectin. The minimum concentration of galactose needed to inhibit the hemagglutination activity of lectin was 25mM. A number of galactose bindinglectins have already been purified and characterized including the lectins such as *Arachis hypogaea, Abrus precatorius*, *Artocarpus integer.*[13-15]

SDS PAGE analysis of purified lectin showed a prominent band with approximate molecular weight of 29kDa.

Chrysophyllum cainito leaf lectin was found to be stable at a temperature of 4°C – 70°C. Maximum activity was observed at 37°C. However at a temperature above 70°C, due to thermal denaturation of protein, lectin activity was drastically reduced. These results seem to be in accordance with another study conducted by a group of scientists where the hemagglutination activity of MCJ lectin drastically reduced at a temperature above 65°C.[16]

In a similar study conducted by another group,where the treatment of denaturants like Guanidine hydrochloride, urea and thiourea on *Gonatanthus pumilus* lectin, the hemagglutination activity decreased.[17] These denaturing agents modify important amino acid residues in the protein thereby rendering it inactive and hence affecting the hemagglutinating activity. Furthermore, previous investigations carried out by other authors reveal that antimicrobial activity of *C.cainito* against selected pathogens and the potential application of *C.cainito* fruit as an alternative medicine in the treatment or control of enteric bacterial infections.[18]

CONCLUSION

In this work, we have tried to purify and characterize the lectin from *Chrysophyllum cainito.*In the future we plan to include, more purification steps to characterize this lectin further using affinity chromatography.This is the first report of a lectin been characterized from *C.cainito* which shows good hemagglutinating activity.

The purified lectin is a galactose specific lectin which shows inhibition at 25mM. The lectin inhibits the growth of *E.coli* but not *S.aureus.* Future work includes studying theanticancer activity of the lectin using different cell lines to test the potential of the purified lectin as a drug.

The results obtained are promising and more applications needs to be explored in the field of medical therapeutics.

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