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Research Letter

Bacteriostatic activity of crude lectins

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ABSTRACT: Lectins are simply defined as glycoproteins which specifically bind to carbohydrates. Lectins were isolated from *Canavalia ensiformis, Phaseolus vulgaris* and *Ricinus communis* using the standard protein precipitation method. The extracted lectins have been characterized using physical and chemical methods including hemagglutination assay and gel electrophoresis for determination of molecular weight. Hemagglutination assay was performed on rat blood cells and human blood erythrocytes of A, B, AB and O positive blood groups. Gel electrophoresis SDS-PAGE analysis was performed and it was found that the *Phaseolus vulgaris lectins* (PHA-L) was 71.4 kDa, Canavalia lectin was 30.2 kDa and *Ricinus* communis lectin was 74.4 kDa. The lectins from *Canavalia ensiformis, Ricinus communis* and *Phaseolus vulgaris* show good bacteriostatic activity against gram positive bacteria, namely *S. aureus, B. subtilis* and *S. mutans* at concentrations of 500 µg/mL and 1 mg/mL when tested by agar-well diffusion method. The colony counting method was also performed which supported the results as with the agar-well diffusion method. A typical agglutination of *S. mutans* was observed in the presence of the lectins obtained from *Canavalia ensiformis and Phaseolus vulgaris.*

KEY WORDS: protein precipitation, hemagglutination, molecular weight

INTRODUCTION

Plants have been part of our lives since the beginning of time. We get numerous products from plants, most of them not only good and beneficial but also crucial to our existence. The use of plants to heal or combat illness is probably as old as humankind.

Lectins are defined in a simple way as glycoproteins which specifically bind to carbohydrates. Lectins show a good ability to agglutinate and immobilize the microorganisms. Each lectin molecule typically contains two or more carbohydrate-binding sites which are usually di- or polyvalent. When lectins interact with carbohydrates, they cause a cross-linking of the glycoproteins resulting in agglutination.[1] Lectins owing to their binding properties are target-specific and can be exploited by humans for their antiviral, antifungal, antidiabetic, antimicrobial and anticancer properties. Drug resistance to micro-organisms is a prevalent problem. Lectins have been reported to show potential antimicrobial activity.

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The amount of lectin concentration generally accounts for 1% to 3% of protein content of a food, and in the case of plants, the amount is dependent upon the degree of plant maturation.[2] Plants and animals do not grow in order to provide us with food. They grow in order to keep their species going. So, the foods we eat contain a mixture of what is necessary for us and other substances we should avoid. Same holds true with respect to lectins. Lectins are known to be toxic owing to their sugar-specificity and irreversible binding. But this same property can be explored and can be made use of to the benefit of humans. Appropriate extraction and isolation techniques should be performed to eliminate the toxic fractions and retain the non-toxic portions of lectins.

Canavalia ensiformis (Jack bean meal), *Phaseolus vulgaris beans, Ricinus communis* seeds, are a few beans which act as a source of nutrition in our daily life. Lectins are glycoproteins; hence protein precipitation method using organic solvent such as acetone and salts such as ammonium sulphate was used. The extracted lectins have been characterized using physical and chemical methods including hemagglutination assay and gel electrophoresis for determination of molecular weight. Crude lectins have been evaluated for bacteriostatic activity against bacteria namely, *E. coli, B. subtilus, S. aureus* and *S. mutans*. Hence these have been selected to explore the lectin-microorganism interaction and their effect on microorganisms.

MATERIALS AND METHODS

Lectins source

Seeds of *Phaseolus vulgaris* and *Ricinus communis* were purchased from the local market. The voucher specimen numbers have been deposited at Blatter Herbarium, St. Xaviers, Mumbai, India. *Canavalia ensiformis* (Jack bean meal) was obtained from Hi-Media.

Chemicals

All chemicals used for extraction were of analytical grade obtained from SD-fine chemicals. Molecular ladder for SDS-PAGE was obtained from SISCO Research Laboratories. All chemicals used for SDS-PAGE were of electrophoresis grade obtained from SD-fine chemicals.

Test microorganisms

E. coli (ATCC No. 8739), *B. subtilus* (ATCC No. 6633) and *S. aureus* (ATCC No. 6538p), were obtained from MKR laboratories, Bombay College of Pharmacy, Mumbai, India. *S. mutans* (MTCC No. 890) was obtained from Institute of Microbial Technology, Chandigrah, India.

Extraction of lectins

Lectins were extracted from *Canavalia ensiformis*, *Phaseolus vulgaris* and *Ricinus communis* by maceration technique at cool temperatures 4-8 °C

Canavalia ensiformis lectins were extracted from Jack bean meal. Maceration was done in distilled water for 4 hours. The filtrate was washed with hexane (4×100mL.) Crude lectin was precipitated out using acetone in the ratio 1:1. The obtained precipitate was further subjected to centrifugation at 6000 rpm for 20 mins. The pelleted material was then freeze-dried.[4]

Phaseolus vulgaris lectins were extracted by maceration in brine solution (sodium chloride solution). The pellet was discarded and the supernatant was subjected to fractional precipitation using ammonium sulphate (80% saturation). The pellet was dissolved in minimal amount of distilled water and was heavily dialyzed against distilled water at 4 °C until complete removal of ammonium sulphate. The dialyzed pellet was then freeze-dried.[5]

Lectins were extracted from Castor seeds (*Ricinus communis*). The seeds were decorticated and pulverized for size reduction. The pulverized mass was washed using diethyl ether (5×150 mL), and this step was repeated until complete removal of the lipids. The ether layer was decanted and the residue was air dried. To the air dried material 5 times the volume distilled water was added and pH was adjusted to pH 4.0 using glacial acetic acid. The suspension was centrifuged. The supernatant was subjected to precipitation using ammonium sulphate (60% saturation) and this was kept overnight at 4 °C. Later this was centrifuged in a cooling centrifuge at 7200 rpm for 20 mins. The resultant pellet was dissolved in minimal amounts of distilled water and dialyzed heavily against distilled water at 4 °C until complete removal of ammonium sulphate. One portion of the obtained lectin was stored at 4 °C. The other portion was subjected to freeze-drying and the obtained lectin was stored in vacuum dessicator at room temperature.[6]

Yield of the lectins is given in Table 1

Hemagglutination activity[7]

Hemagglutination assay was performed on rat blood cells and human blood erythrocytes. The erythrocytes were suspended in PBS (Phosphate Buffer Saline). The assay was performed in U-shaped 96 well titer plate. In the assay the 10% w/v lectin solution was added in the first well and serial dilution was done using saline till the 96th well, keeping wells 12, 24, 36, 48, 60, 72, 84, 96 as the control. To the well 25 µL erythrocytes were added. This plate was incubated at 37.5 ± 2 °C for 30 mins, followed by further incubation at room temperature. The hemagglutination activity of the lectin was expressed as the titer defined as the reciprocal of the highest dilution giving positive hemagglutination. Hemagglutination activity is given in Table 2

SDS-PAGE gel electrophoresis[8]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% resolving gel and 4% stacking gel. Electrophoresis was done in Banglore Genei apparatus. Amount of the lectins loaded in the Stacking gel for the electrophoresis were 25 µg/µL for lyophilized lectins and 200 µg/µL for crude lectins. After gel electrophoresis, the proteins are visualized by adding a dye such as Coomassie blue which bind to proteins but not to the gel itself. Further the gels were docked using Bio-Rad -XR Gel Doc.

Preparation of extracts

The crude lyophilized powders were weighed and suspended in sterile water to get concentration of 1 mg/mL and 500 µg/mL for testing their antimicrobial activity.

Agar well diffusion bioassay[3]

The *in vitro* growth inhibition assay against bacteria was carried out by agar well diffusion method. The autoclaved, cooled at 45 °C nutrient agar medium was seeded with microorganism (adjusted as of Mc Farland 0.5 standard). This seeded nutrient medium was poured into sterile petridishes to a depth of 3-5 mm. After solidification with the help of a borer, 3 wells were made equidistant from each other. With the help of micropipette, 50 µL of the two lectin concentration solutions were added in the 2 wells respectively. To the third well 50 µL, 500 µg/mL amoxicillin was added as positive. A control plate was also maintained in each case without any test material (lectins). A negative control plate was also maintained in each case. The plates

were incubated at 37.5 ± 2 °C for 24 hours to allow maximum growth of organisms. The antibacterial activity of lectins was determined by measuring the *mean diameter of zone of inhibition in millimeters* and *counting the colonies* after 24 and 48 hours. Each experiment was repeated thrice. All the results were compared with the standard antibacterial antibiotic amoxicillin.

In case of *S. mutans*, anaerobic bacteria, fluid thioglycollate medium was used following the procedure for aerobic bacteria.

Statistical analysis of data

The antimicrobial activity of lectins against bacteria was calculated using SEM (standard error of mean).

RESULTS AND DISCUSSION

Crude lectins were extracted from *Canavalia ensiformis, Phaseolus vulgaris* and *Ricinus communis*, It was observed that maintenance of cool temperatures throughout the extraction method capitulated into a better yield (Table 1).

The crude lectins were subjected to SDS-PAGE and crude lectins of *Canavalia ensiformis* 30.2 kDa and 12.5 kDa; *Phaseolus vulgaris* 71.4 kDa and 24.0kDa; *Ricinus communis* 74.4 kDa and 23.7 kDa were extracted (Figure 1). Lyophilized crude lectin were also subjected to SDS-PAGE which showed lectins of *Canavalia ensiformis* 34.5 kDa and 13.6 kDa; *Ricinus communis* 70.2 kDa and 25.2 kDa. There was no major difference in the band patterns of the crude extracts and freeze-dried samples which indicate that crude lectin extracts were not affected by the lyophilization process.

The crude lectin extract showed good hemagglutination activity against both rat and human blood erythrocytes (Table 2)

In vitro antimicrobial susceptibility was assayed against four pathogenic bacteria and compared to that of the antibacterial antibiotic amoxicillin. The results are presented in Table 3. Lectins frequently form complexes with oligosaccharides in glyco-proteins. Usually, the most important residues are the non-reducing terminals and their accompanying penultimate residue. The inhibitory effect of lectin on the

Table 1: Yield and description of extracts from *Canavalia ensiformis, Phaseolus vulgaris* and *Ricinuscommunis*

	Canavalia ensiformis	*Phaseolus vulgaris*	*Ricinus communis*
Extract description	Dark buff colored pasty mass	White colored dense colloidal suspension	White colored dense colloidal suspension
Yield (%w/w)	16.66	16.38	20.05
Lyophilized extract description	Dark buff colored powder	White colored powder	White colored powder
(Lyophilized) Yield(%w/w)	1.41	0.86	0.80

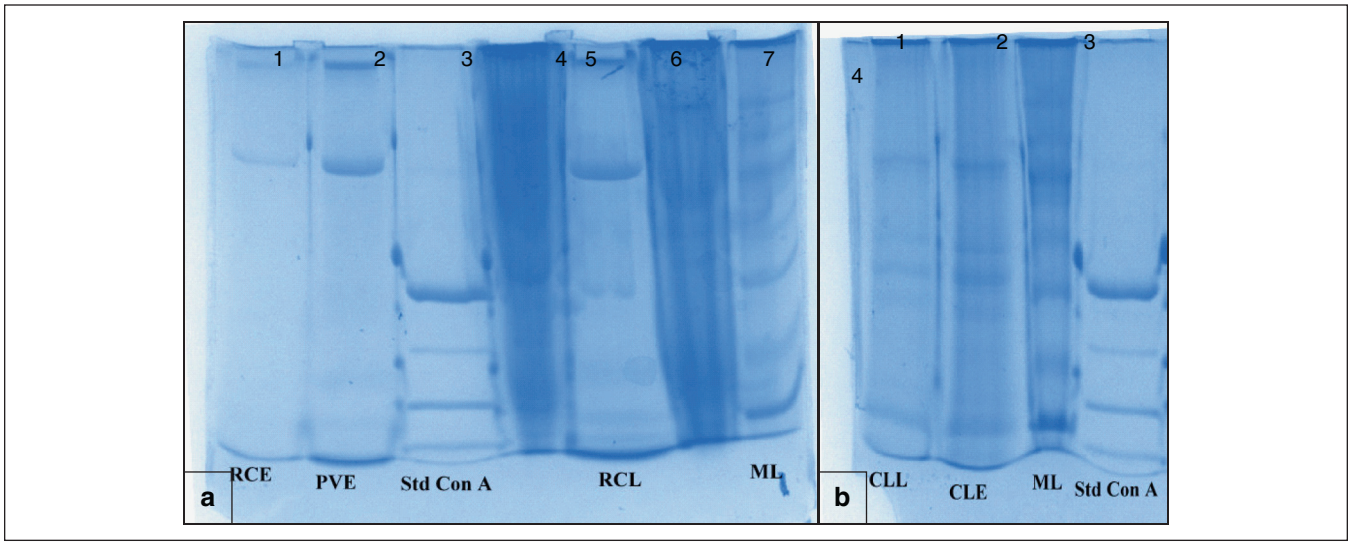

Figure 1: Determination of molecular mass by SDS-PAGE.
(A) SDS-PAGE, (12.5% poly-acrylamide gel) showing molecular mass of lectins. Lane 1, crude extract of Ricinus communis seeds (RCE); Lane 2, crude extract of Phaseolus vulgaris beans (PVE); Lane 3, Standard ConA; Lane 5, lyophilized extract of Ricinus communis seeds (RCL); Lane 7, molecular mass marker (ML). (B) SDS-PAGE, (12.5% poly-acrylamide gel) showing molecular mass of Canavalia ensiformis lectins. Lane 1, lyophilized extract of Canavalia ensiformis (CLL); Lane 2, crude extract of Canavalia ensiformis (CLE); Lane 3, molecular mass marker (ML); Lane 4, Standard Con A. The markers included (a) phosphorylase b (95-kDa), (b) bovine serum albumin (66-kDa), (c) ovalbumin (47-kDa), (d) Glyceraldehyde-3-P-Dehydrogenase (35-kDa) (e) carbonic anhydrase (25-kDa), (f) soybean trypsin inhibitor (20-kDa), and (g) Lysozyme (14-kDa). The molecular mass of PHA-L, RCA-L being 71 kDa and 74k Da respectively. Canavalia Lectins being 30 kDa.

bacteria could be due to the interaction with the substituted non-reducing α-D-glucose (Glc) or α-D-mannose (Man) residues present on the cell walls of bacteria. These interaction results in agglutination of the microorganism.[9]

The activity observed with crude lectins extract against the pathogenic bacteria tested was bacteriostatic and not bactericidal. The lectins tend to arrest the growth of the bacteria and not kill them (activity being static). The colony count after the 24 and 48 hours show that the numbers of the colonies are increasing after 24 h period but the 48 h observation shows no further increase in the number of colonies. Also agglutination of *S. mutans* was observed against *Canavalia ensiformis and Phaseolus vulgaris* (Table 4).

Table 2: Titre is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The initial amount of lectin used in these assays was 100 µg and was serially diluted 1:1 (v/v) for all subsequent dilutions. Data shown are the mean ± 1 SD and are derived from three repeats

Erythrocyte source	Agglutination titer		
	Canavalia ensiformis	*Phaseolus vulgaris*	*Ricinus communis*
Rat	2^8	2^{10}	2^7
Human A	2^{13}	2^{10}	2^6
Human B	2^6	2^6	2^{10}
Human O	2	2	2^{10}
Human AB	2^8	2^8	2^{10}

Table 3: Bacteriostatic activity of lectins by agar-well diffusion method

	Canavalia ensiformis	*Phaseolus vulgaris*	*Ricinus communis*
Bacillus subtilis	++	0	++++
Escherichia coli	+++	↑	++
Staphylococcus aureus	++++	+++	0
Streptococcus mutans	∞	∞	+++

Strong bacteriostatic activity: ++++; Mild bacteriostatic activity: +++; mildly deterrent bacteriostatic activity: ++
No activity: o; Enhanced growth: ↑; Agglutination and static: ∞

Bacteriostatic activity was observed (by agar-well diffusion method and colony counting method) against *S. aureus* for lectins from *Canavalia ensiformis* and against *B. subtilis* for lectins from *Ricinus communis*. Mild bacteriostatic activity was observed against *S. aureus* for lectins from *Phaseolus vulgaris* and against *E. coli* for lectins from *Canavalia ensiformis*. Mildly deterrent bacteriostatic activity was observed against *B. subtilis* for lectins from *Canavalia ensiformis* and against *E. coli* for lectins from *Ricinus communis*. The lectins extracted from *Phaseolus vulgaris* and *Ricinus communis* did not show activity against *B. subtilis* and *S. aureus,* respectively. In case of *E. coli,* microorganisms were observed to be multiplying utilizing the lectins from *Phaseolus vulgaris* lectins (PHA-L), as the source of nutrition. *Canavalia ensiformis* lectins, *Phaseolus vulgaris* lectins and *Ricinus communis* lectins showed strong bacteriostatic activity against *Streptococcus mutans*.

CONCLUSION

The extracted lectins were characterized by gel electrophoresis and were confirmed for their hemagglutination property. Lectins are glycoproteins and hence can affect the cell wall of the bacteria. In the current study, it has been observed that lectins from *Canavalia ensiformis*, *Ricinus communis and Phaseolus vulgaris* showed good bacteriostatic activity against gram positive bacteria, namely *S. aureus*, *B. subtilis, and S. mutans*. The lectins are typically mannose and galactose binding type. The bacterial cell wall has these or other similar carbohydrates. These carbohydrates (peptidoglycans) probably interact with the lectins causing their agglutination and leading to observed bacteriostatic activity.

It was observed that the lectins from *Phaseolus vulgaris lectin* (PHA-L) act as the growth promoter towards the gram negative microorganism *E. coli*. The gram negative bacteria have a different cell wall composition as compared to the gram positive bacteria. Hence, there might be a difficulty that PHA-L encounters in crossing the outer cell wall of the gram negative bacteria to reach the periplasmic space.

Table 4: Bacteriostatic activity of lectins by colony counting method

Microorganism	Number of colonies (Counted using Colony Counter)							
	Bacillus subtilis		*Escherichia coli*		*Staphylococcus aureus*		*Streptococcus mutans*	
Time	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
Canavalia ensiformis	120	127	129	131	140	140	Agglutination	Agglutination
Phaseolus vulgaris	220	232	291	305	137	140	Agglutination	Agglutination
Ricinus communis	105	106	125	132	200	215	97	101
Positive Control	220	225	210	215	200	210	200	215
Standard (Amoxicillin)	23	24	23	23	23	25	25	25
Control [Zone of Inhibition in mm]								

The lectins are glycoproteins and hence these can act as source of nutrition and most probably the gram negative bacteria were thriving on this nutrition source and hence, the lectins act as the growth promoter.

In the current study, it has been observed that lectins show a promising bacteriostatic activity against gram positive microorganism. They also showed a growth activity promoter feature for a few select microorganisms. Hence, the lectins need to be used judiciously for the bacteriostatic activity. Lectins could be good and promising candidates for treating cariogenic bacteria (*S. mutans)* in the oral cavity. Lectins can be used alone or in combination with other synthetic antimicrobials drugs.

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