WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 7, 977-987. Research Article ISSN 2277– 7105

ISOLATION AND PARTIAL PURIFICATION OF LECTIN FROM SEEDS OF *CUCURBITA PEPO*

Ravichandran P.[1], Dr. Gowri S.[1]* and Sundara Prasath S.[2]

[1]Dept. of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore.

[2]Dept. of Biochemistry, NMSSVN College, Madurai.

Article Received on
05 May 2016,
Revised on 25 May 2016,
Accepted on 15 June 2016

DOI: 10.20959/wjpr20167-6498

***Corresponding Author**
Dr. Gowri S.
Dept. of Biochemistry,
Dr.N.G.P. Arts and Science College, Coimbatore.

ABSTRACT

Crude plant lectins were isolated from the seeds of Pumpkin seed (*Cucurbita pepo).* Lectins isolated were purified by ammonium sulphate precipitation and dialysis. The amount of proteins and carbohydrates present in crude extract and purified samples were estimated. Lectin was characterized by hemagglutination assay using human erythrocytes of A, B, AB and O groups and the specific activities were determined in crude and purified samples. The stability of the purified lectin samples was determined at various pH and temperature values. The ability of the lectins to bind the bacterial strains were analyzed with strains namely *E.coli*, *Klebsiella pneumoniae* and *S.aureus*.

KEYWORDS: *Cucurbita pepo*, Lectin, Isolation, Purification, Hemagluntination, Antibacterial activity.

INTRODUCTION

Lectins are proteins/glycoproteins, which have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharide's or oligosaccharides.[1] "Lectin" has been derived from the Latin word "legere", which means "to select", by William Boyd.[2] This term was generalized to embrace all sugar-specific agglutinins of non immune origin, irrespective of source and blood type specificity.[3]

Lectins have the ability to bind carbohydrates and the name "hemagglutinins" is used when the sugar specificity is unknown. Lectins have been powerful tools in preparative and analytical purposes in Biochemistry, cell biology, immunology, molecular biology, pharmacology and clinical chemistry. Lectins are present in a wide range of organisms from

bacteria to animals, being present in all classes and families, although not in all the kinds and species.[4] The first report on the occurrence of lectins in marine algae is relatively recent.[5]

The lectin contents in some parts of plants are higher, e.g., 390 and 75 mg of the purified lectin was recovered from 100 g *Remusatia vivipara* tubers[6] and *Astragalus mongholicus* roots[7], respectively. Lectins are also found in seeds. The lectin content in non legume plants is low, e.g., 3.3 mg lectin from 100 g *Hibiscus mutabilis* seeds.[8] Lectins are found in abundance in legume seeds.

Many plant lectins are found in storage tissues where they are highly abundant. These plant lectins are often referred to as the 'classical lectins' and this group comprises most lectins that have been characterized so far. Most classical plant lectins are present in seeds but a lot of them are also found in different vegetative tissues.[9]

Yields of lectins from fresh mushrooms are low, e.g., 2.6 mg from 100 g of fresh fruiting bodies of *Pleurocybella porrigens*.[10] In fact, the water content in fresh mushrooms is very high. Dried fruiting bodies of the mushrooms *Russula lepida*, *Pholiota adiposa* and *Inocybe umbrinella* yielded 39, 70 and 15 mg lectin per 100 g fruiting bodies, respectively.[11,12] Therefore, production from fresh mushroom is also unpractical.

Lectins are found in different animals. However, the yields are usually extremely low. Mass purification of animal lectins necessitates bulk quantities of raw materials which make it not feasible.[13]

Isolation and purification of lectins may be done through a variety of protein purification methods.[14] Methods for purifying lectins vary due to lectin sources (*i.e.* plant or animal). Methods also will depend on lectins' structure, specificity, physiochemical properties and biological activity.[15]

The pumpkin is an angiosperm belonging to the cucurbitaceae family. *Cucurbita pepo* is more tolerant to harsh environmental conditions than other cucurbitaceae species.[16] Pumpkin seeds have a high nutritional value, provides good quality oil and excellent source of protein.[17] In addition to good health benefits, pumpkin seeds are less expensive and are widely distributed. In the traditional medicine in North America and Mexico, pumpkin seeds have been used as an antihelmintic agent and for supportive treatment in functional disorders of the bladder. The healing powers of plants have been used for hundreds of years; about

80% of the available therapeutic substances are originated from medicinal plants.[18, 19] The seed of pumpkin has pharmacological activities such as anti-diabetic, antifungal, antibacterial and antiinflammatory and antioxidant effect.[20]

So the present study aimed at extraction, purification and characterization of lectin from the *cucurbita pepo* seeds.

MATERIALS AND METHODS

Sample Collection

The Pumpkin (*Cucurbita pepo*) seeds were collected from a local area at Avinashi market, Tirupur. The chemicals used in the study are supplied by Hi-Media.

Preparation of Crude Extract

The dried seeds were dehulled manually and ground using mortar and pestle, then defatted using n-hexane at ratio of 1:5 w/v for 15 min. Defatting was done twice until the hexane was clear of fat. 0.02 M PBS (pH 7.2) was added to the sample at a ratio of 5:1 (v/w) then subjected to continuous stirring using a magnetic stirrer for 16 h. After stirring, the sample was filtered. The liquid portion was clarified by centrifugation at 9000xg for 45 min at 4°C. After centrifugation, the residue was discarded and the clarified crude extract was stored at 0°C for further use. Before the purification process, the crude extract was subjected to hemagglutination assay and protein determination.

Ammonium Sulfate Precipitation

The crude extract was subjected to 90% ammonium sulfate precipitation using Cooper's nomogram (1977). For every 100 mL crude extract, 61.1 g of ammonium sulfate was added in three lots (with stirring) for 30 min. After standing for 1 h, the mixture was subjected to 9000xg centrifugation and the precipitate collected were dissolved in minimum amount of buffer and stored at 0°C for further use. Addition of ammonium sulfate, standing and centrifugation procedures were performed at 4°C to prevent protein denaturation.

Desalting

The ammonium sulfate in the previous procedure was removed using a Sigma membrane dialysis tubing with molecular weight cut off of 12 kDa. The tubing was pre-treated by subjecting it to running water for 3-4 h to remove the glycerin which acts as humectant. The tube was sealed at one end with a cotton string then the ammonium sulfate fraction was

introduced through the other opening with the use of a pipette to ensure no formation of bubbles. After sealing the other end, the tube was placed in a beaker filled with distilled water and stirred for 6 h at 4°C with three changes of water. The desalted sample was collected by punching a hole to one end, allowing the liquid to flow in a container. The dialyzed sample was stored at 0°C for further use.

Preparation of Red Blood Cells

A 200µl blood of different types (A, B, AB, O) was mixed with 10 ml Phosphate Buffer Saline (PBS) with pH 7.2 in a centrifuge tube. The mixture was then subjected to a 5 min centrifugation at 1000xg. The centrifugate was removed and discarded using a Pasteur pipette. The compacted red blood cells were diluted with PBS until the volume reaches the 10 mL mark. Dilution and centrifugation was repeated until the supernatant is clear. The erythrocytes were diluted to 10 mL mark using PBS to make a 2% blood suspension.

Hemaggluntination Assay

Fifty microliters of samples (crude extract and ammonium sulfate fractions) were serially two-fold diluted using PBS in plastic multi-well microtiter plates. Then 50 µL of 2% (v/v) erythrocyte suspension were added in each well.

After an incubation period of one hour, the plates were examined visually. A uniform layer of the erythrocytes over the surface of the well indicates positive agglutination. Negative result was indicated by the formation of a distinct button at the bottom of the well. The reciprocal of the maximal dilution of extract that exhibited positive reaction is the agglutination titer. Specific activity of the lectin was calculated by dividing the titer by the protein content, which is expressed in mg per mL extract.

Carbohydrate Content Determination

Neutral sugar content of crude and purified lectin was estimated by the phenol sulphuric acid method with glucose as standard.

Protein Content Determination

Protein was estimated by the method of Lowry *et al* (1951) with bovine serum albumin was used as the standard protein.

Evaluation of Antibacterial Activity

Human pathogenic bacteria *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* obtained from Department of Microbiology, Dr. N.G.P Arts and Science College, Coimbatore were used to check the antibacterial activity. The Agar disc diffusion method was used to determine the antibacterial activity. This test was done according to the method of Edward and Lansing. The microbial (bacterial/fungal) colony was picked by the inoculating wire and the media plates were subsequently inoculated with specific microbial strains and labeled accordingly. Standard antibiotic discs of Amphicillin (positive control) were also impregnated on plates at previously labeled positions. Loaded plates were kept as such for some time under Laminar hood and then incubated at 37°C for 24 hours for bacterial strains. A transparent ring around the sample loaded well signified antimicrobial activity. Zone diameters (mm) around each of the well were measured to the nearest mm.

RESULT AND DISCUSSION

Mature seeds of *cucurbita pepo* were found to contain hemagluntinating protein and an aggluntination inhibitor. Lubag and Pahm previously reported the presence of aggluntination factor in the seeds.[21] Aggluntination of normal red blood cells did not occur in the more concentrated crude extract but was observed only when the lectin extract was diluted 2-fold.

The dehulled, ground seeds were first extracted with n-hexane to remove fats or lipids that may interfere with agglutination. In the preliminary studies, there were no observed differences in lectin activity in samples extracted with n-hexane compared to those not treated with the solvent. After extraction with n-hexane, the solvent remains clear. This indicates that the sample contains very little amount of lipid from the 60.5 g sample and 370 mL PBS, 335 mL of crude extract was obtained.

Ammonium Sulfate Precipitation

Proteins were separated from other substances co-extracted by PBS in the crude extract by precipitating it with ammonium sulfate. Zero to ninety percent (0-90%) ammonium sulfate saturation was employed to ensure that most proteins were precipitated.

Dialysis

Ammonium sulfate was removed from the protein since it may cause false positive agglutination. Desalting was done by extensive dialysis with distilled water. Dialysis was performed in the cold room to prevent possible denaturation of the protein. The membrane

used has a molecular cut-off of 12 kDa. This allows the proteins with molecular weight greater than 12 kDa to remain inside the dialysis bag.

The amount of proteins present in crude extract and purified samples were found to be 45 mg/ dl and 38mg/dl respectively. The amount of carbohydrate present in crude extract and purified samples were found to be 60 mg/dl and 44mg/dl respectively.

Purification of the Lectin from *Cucurbita pepo*

Table. 1: Purification of lectin from *Cucurbita pepo*

Purification stage	Volume/ml	Protein mg/ml	Total protein mg/ml	Type Of blood	Total lectin (titer*ml)	Specific activity (HU/mg)	Purification fold	Total Carbohydrate mg/ml
Alcoholic crude extract	100	0.45	45	A	3200	71.11	1	60
				B	1600	35.55	1	
				AB	6400	142.22	1	
				O	6400	142.22	1	
Ammonium sulphate precipitation and Dialysis	50	0.38	38	A	6400	168.42	2.36	44
				B	3200	84.21	2.36	
				AB	12800	336.84	2.36	
				O	12800	336.84	2.36	

Hemagglutination assay

The isolated lectin from *Cucurbita pepo* is considered to be non-blood type specific since it agglutinates all human blood types. which is also non-blood type specific. However, it is a blood group specific lectin since it agglutinated blood from human.

The human blood types have different sugar moieties on the surface of the cell. Type A has N-acetyl-D-galactosamine, D-galactose for type B and L-fucose in type O. Blood type AB contains the sugar determinants for both A and B. Agglutination occurs when the lectin interacts with these sugar moieties. Non-blood type specificity of the lectin may be due to the presence of multiple binding sites where it can recognize all the determinants for each blood type.[22]

Figure.1: Hemagluntination assay

Lectin was characterized by hemagglutination assay using carbohydrates of human erythrocytes of A, B, AB and O and the specific activities were found to be 71.11, 35.55, 142.22, 142.22 and 168.42, 84.21, 336.84, 336.84 for crude and purified samples respectively. Purification steps increased the purity from 1 to 2.36 fold.

The lectin interaction with carbohydrates are performed through a combination of hydrogen bonds between sugar hydroxyl groups and protein main-chain, as well as side-chain groups, water-mediated contacts, vander Waals packing of the hydrophobic sugar ring face against an aromatic residue and hydrophobic interactions.

Effect of temperature on the activity of lectin

The effect of temperature on agglutination was shown in Figure 2. The lectin was stable from 10-30°C and has a consistent titre value decreased at 100°C, it is devoid of lectin activity. This may be brought about by the denaturation of the lectin that removes its aggluntinating capacity.

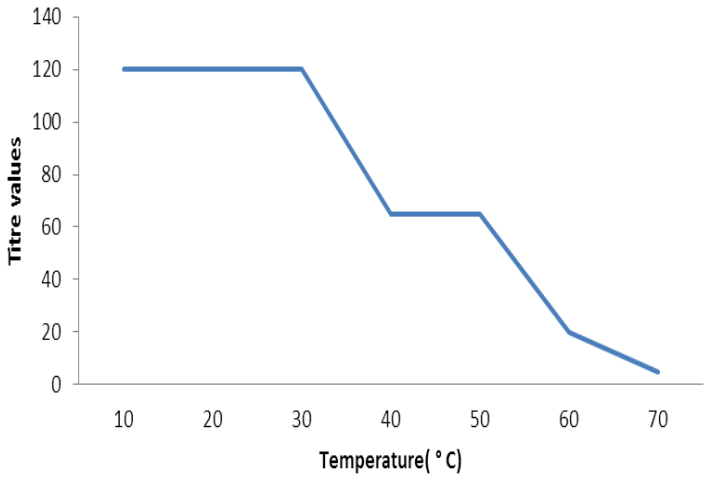


Figure 2 Effect of temperature on the activity of lectin isolated from *Cucurbita pepo*

Effect of pH on the activity of lectin isolated from *Cucurbita pepo*

The effect of pH on agglutination at different pH values is shown in Figure 3. The values showed that the lectin was stable in the pH range of 1-6, indicating that the amino acid residue involved in carbohydrate binding are not affected by changes in pH in this range.

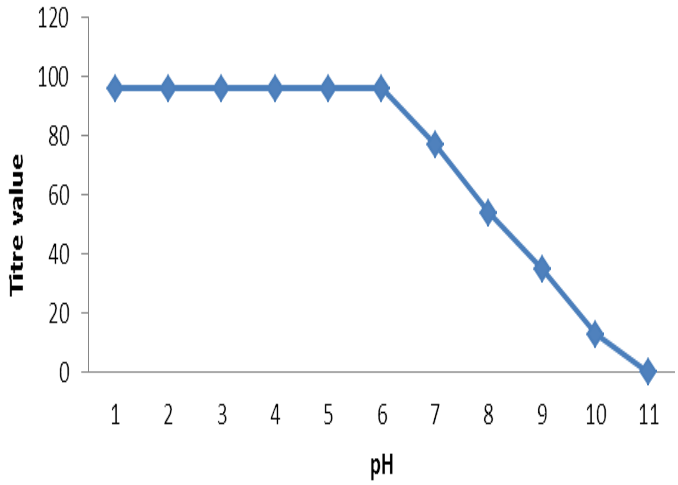
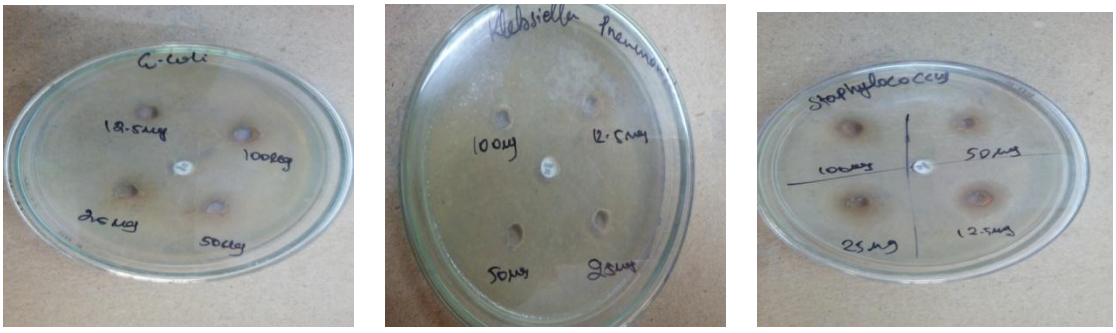


Figure 3. Effect of pH on the activity of lectin isolated from *Cucurbita pepo*

Antibacterial activity of lectin from *Cucurbita pepo*

The Purified lectin were tested against different bacterial strains and compared to that of standard antibiotic, Amphicillin. The result of the sensitivity test was shown in Figure 4. Purified *Cucurbita pepo* exhibited a significant antibacterial effect on three strains namely *E.Coli, Klebsiella pneumoniae* and *Stapylococcus aureus.* The diameters of the zones of inhibition by the addition of *Cucurbita pepo* were 11mm, 05mm, 12mm respectively.



(A) **(B)** **(C)**

Figure. 4: Antibacterial activity of purified letin sample in various bacterial strains.
(A) *E.Coli,* (B) *Klebsiella pnumoniae,* (C) *Staphylococcus aureus*

Table 2: Antibacterial activity of purified lectin sample in various bacterial strains

NAME OF BACTERIA	Zone of Inhibition(in mm)				
	100µg/ml	50µg/ml	25µg/ml	12.5µg/ml	Amphicilin 25µg/disc
E.Coli	-	05	08	11	07
Klebsiella pneumoniae	-	-	03	05	04
Staphylococcus aureus	04	07	09	12	05

CONCLUSION

Crude plant lectins were isolated from the seeds of Pumpkin seed (*Cucurbita pepo).* Lectins isolated were purified by ammonium sulphate precipitation method and dialysis. The amount of proteins present in crude extract and purified samples were found to be 45 mg/ dl and 38mg/dl respectively. The amount of carbohydrate present in crude extract and purified samples were found to be 60 mg/dl and 44mg/dl respectively.

Lectin was characterized by hemagglutination assay using carbohydrates of human erythrocytes of A, B, AB and O and the specific activities were found to be 71.11, 35.55, 142.22, 142.22 and 168.42, 84.21, 336.84, 336.84 for crude and purified samples respectively. Purification steps increased the purity from 1 to 2.36 fold.

The purified lectin samples were found to be stable in the pH range 1 to 6 and in the stable in the temperature range of 10-30 degrees. Every surface-exposed carbohydrate of microorganism is a potential lectin-reactive site. Ability of lectins to form complexes with microbial glycoconjugates can be exploited as potential antimicrobial drug targets.

The ability to bind the bacterial strains were analyzed with strains namely *E.coli, Klebsiella pneumoniae* and *S.aureus*. The zone of inhibitions was found to 11, 5 and 12 mm at 100 µg/dl respectively and highly effective than standard antibiotic amphicilin. In conclusion, the isolated lectins from *cucurbita pepo* can be used as a potential drug targeting agents for cancer and antimicrobial therapy because of their ability to bind sugars specifically.

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