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ISOLATION AND CHARACTERIZATION OF LECTIN FROM SEEDS OF *ZIZYPHUS MAURITIANA*

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ABSTRACT

The present paper deals with isolation and characterization of a novel galactose binding lectin from the seed cotyledons of *Zizyphus mauritiana*. The extracted lectin in PBS at pH 7.00 was fractionated by Ammonium sulphate precipitation. The fraction obtained between 0 – 35% ammonium sulphate precipitation was further purified by affinity chromatography on guar-gum followed by ion exchange chromatography on DEAE-cellulose. The isolated lectin was found to be tripeptide, molecular weight of three different subunits of 38 kD, 25 kD and 19.95 kD on SDS - PAGE. Isolated lectin agglutinated rabbit erythrocytes showing maximum agglutination with human B^{+} erythrocytes. It was active between pH 4.0 - 9.0 with an optimum activity at pH 7.0 and was found fairly stable from pH 4.5 to 8.0. The lectin optimally active at 37°C was stable up to 80°C. Presence of metal ions such as Mg^{++} and Mn^{++} positively influenced the activity of lectin but the activity was inhibited in the presence of Cd^{++}, Hg^{++} and Pb^{++}. The lectin showed maximum enzymatic activity towards the substrate β – oNPGal.

KEYWORDS: Lectin, Zizyphus, erythrocyts, affinity chromatography, agglutinin.

Abbrevation used

ZMSL, *ZIzyphus mauritiana* seed lectin; PBS, 0.02M sodium phosphate buffered saline, pH 7; oNPG, o-nitrophenyl-β-D galactopyranoside; α – pNPG, p-nitrophenyl- α -D galactopyranoside; SDS–PAGE, sodium dodecyl sulphate– polyacrylamide gel electrophoresis.

INTRODUCTION

Lectins are glycoproteins in nature which bind specifically and reversibly to carbohydrate moieties of complex glycoconjugates on cell surfaces resulting in their agglutination.[1] They are widely found to be present in viruses, bacteria, fungi, plants, and animals.[2] Owing to the property of carbohydrate binding they participate in a variety of cellular processes like, cell to cell recognition,[3] cell adhesion,[4] cell signaling,[5] fertilization,[6] opsonisation,[7] mitogenesis,[8] and apoptosis.[9] Participation of lectins in biological processes also includes host pathogen interaction, serum glycoprotein turn over and innate immune response.[1] Majority of the lectins bind to mono and oligosaccharides as observed by their inhibition of hemagglutination; but some are specific to complex carbohydrates and their activity are inhibited by glycoproteins as found in *Scilla campanulata* bulb lectin[10], *Acacia constricta* seed lectin,[11] and *Arisaema flavum* tuber lectin.[12] Lectins find tremendous applications in analytical chemistry as well as preparative biochemistry, for example, purification and characterization of glyco-complex as well as in bio-medical fields such as fractionation of cells and their use in bone marrow transplantation.[13] Over the last few decades, lectins have created interest in a large spectrum of researchers, owing to their potential biological properties including anti-HIV potential,[14] mitogenic,[15] antitumor,[16] immunomodulatory and anti-insect,[17] antifungal,[14] antibacterial,[18] activities.

A large number of plants have been reported to promote the physical, mental and defense mechanism of the body as reported in the Indian medicinal literature. Also a large number of medicinal plants included in Rasayanas have been claimed to possess immunomodulatory activities.[19] The use of plant products like polysaccharides, lectins, proteins and phytochemicals like flavonoids and tannins has been reported for the immune response in various *in-vitro* models.[20] There are various medicinal plants being used in different branches of medicine throughout the world to improve and cure various diseases. In India use of plants as medicinal remedy can be traced back to 6000 BC.[21] Ayurveda is an ancient science of life, has been believed to be prevalent in use of medicinal plants for last 5000 years in India.[22,23] Now a day's modulation of immune response to cure different diseases has been very interesting concept and the concept of *rasayana* in Ayurveda deals with the same.

Ziziphus mauritiana, belongs to family *Rhamnaceae*. Many parts of this plants are used to cure and prevent various diseases like jaundice,[24] it is reported to have antimalarial,[25] antihelminthic,[26] antibacterial, phytotoxic and hemagglutination activities etc.[27]

The present study was designed to isolate the lectin from the seeds of *Zizyphus mauritiana* with some conventional protocols including salting out, affinity chromatography and ion-exchange chromatography and characterization of isolated lectin.

MATERIALS AND METHODS

Materials

Plant material

Fruits of *Zizyphus mauritiana* were collected in fruiting season from single tree growing in the forest of Gadchiroli district in Maharashtra (India). The plant was authenticated by the taxonomist of the region (Voucher No.: 9351). Seeds were dried and cotyledons were removed at the time of experiment.

Blood Samples

Human blood samples of groups A^+, B^+ and O^+ were collected from the healthy donor from Clinical Biochemistry Laboratory. Rabbit blood samples were collected from Departmental Animal and Breeding House, University Department of Biochemistry; RTM Nagpur University, Nagpur (MS), India.

Chemicals

Bovine serum albumin, Guar-gum, D-Arabinose, D-Xylose, α-D-Glucose, D-fructose, D-Mannose, D-Maltose, D-Galactose, D-Mannitol, Sucrose, Lactose, Raffinose, N-Acetyl D-Glucosamine, N-Acetyl D-galactosamine, SDS, Acrylamide, N-N-Methylene bisacrylamide, ammonium persulphate, Tetramethylendiamine, Glycine, Tris-hydroxy-methyl aminomethylene diamine, Bromophenol blue, Glycerol, Coomassie brilliant blue, Glacial acetic acid, Methanol, Sodium carbonate, Copper sulphate, were obtained from Sigma chemicals, St Louis M. O., USA. Native (PMWN 623110600011730) and SDS-low molecular weight protein markers wer purchased from Genei, Bangalore and all other reagents were of analytical grade.

Isolation of lectin

Extraction

Cotyledons of *Zizyphus mauritiana* were crushed in mortar pestle using liquid nitrogen treatment. Ten g powdered cotyledons homogenized in a blender with 100 ml of 0.02M sodium phosphate buffered saline (PBS) pH 7, kept on the shaker at 4^{o}C for 1h, filtered

through folds of cheese cloth, centrifuged at 10,000 rpm for 10 min (Remi C24). The resultant supernatant, designated as crude extract was used for further purification of lectin.

Salting out by ammonium sulphate

The crude extract was fractionated and precipitated by ammonium sulphate at 4^{o}C. The precipitate obtained between 0 to 35% saturation was collected by centrifugation at 12000 rpm for 30 min. The precipitate was dissolved PBS having 35% ammonium sulphate and again centrifuged as same. The resultant precipitate was dissolved in PBS and dialyzed against the same with three changes at 4^{o}C and designated as ammonium sulphate fraction (ASF).[28]

Affinity Chromatography on Guar-gum

Three ml ASF (0 – 35%) was loaded on 3 g of cross linked guar-gum equilibrated with 0.02M PBS pH 7 and kept it to bind at 4^{o}C. The unbound proteins were washed with PBS and the fractions were collected until it showed 0.02 readings at 280 nm and checked for agglutination. The bound proteins were eluted from the column by the extraction solution containing 0.4 M galactose. The flow rate was adjusted to 5ml/10 min using fraction collector and peristaltic pump, (LKB Pharmacia). Fractions of 5 ml each were collected and read at 280 nm.[28] The fractions with higher optical density at 280 nm were subjected to dialysis against extraction solutions until the galactose was removed and checked for protein concentration and agglutination activity. The proteins in the fraction showing agglutination were precipitated to 100% saturation by ammonium sulphate. The precipitates were solubilized in small volume of extraction solutions, dialyzed against the same for removal of ammonium sulphate and purity of samples detected on native polyacrylamide gel electrophoresis (PAGE). The affinity chromatography eluted fractions (ACF) did not show purity on native PAGE therefore subjected to Ion-Exchange chromatography on DEAE-Cellulose.

Ion-Exchange Chromatogaphy on DEAE-cellulose

The ACF showing agglutination was further purified through an ion exchange chromatography which was equilibrated with 0.02 M PBS after the washing with 0.1M NaOH and 0.1M HCl and distilled water, respectively.[29] The column was eluted with a NaCl gradient of 0.05 - 0.25M and 1.5 ml fractions of each molar solution were collected.[30] The absorbance of elutes was measured at 280 nm spectrophotometrically (Eppendorf AG 2331). The elutes of Ion-Exchange Chromatography fraction (IEF) were analyzed for protein

estimation by the method of Lowry *et al*., (1951)[31] and agglutination activity. Elute shows maximum agglutination was then subjected for homogeneity and molecular weight determination by Native and SDS – PAGE.

Protein estimation

Protein concentration in the purified fractions was determined by the method of Lowry *et al*., (1951).[31]

Phenol sulphuric acid test: presence of Carbohydrate in purified fractions was determined by phenol sulphuric acid test using D-glucose as standard (Dubois *et al.,* 1956).[32]

Characterization of isolated lectin

2.3.1. Homogeniety and molecular weight determination

Homogeneity and molecular weight of the isolated lectin was determined on discontinuous 10% native polyacrylamide gel electrophoresis while subunits of lectin were separated on 12% SDS-polyacrylamide gel electrophoresis.[33]

Agglutination assay and blood group specificity

Human blood group A^+, B^+, O^+ and rabbit erythrocytes were prepared and used by the method of Dhingra *et al*., 1995. Human blood group A^+, B^+, O^+ and rabbit erythrocytes suspension were used for hemagglutination assay by the method of Suseelan *et al*.,(1997).[34]

Agglutination inhibition assay/ Carbohydrate specificity

Haemagglutination inhibition assay was performed by mixing 50 µl of lectin with an equal volume of 0.1 M different carbohydrates (α-D-Glucose, D-fructose, D-Galactose, Lactose, Sucrose, Raffinose, D-Mannose, D-Arrabinose, D- Maltose, Stachyose, p-Nitrophenyl-β-D-Galactopyranoside, o-Nitrophenyl-α-D-Galactopyranoside), using the method suggested by Kurokawa *et al.,* (1976).[35]

pH Stability

The buffers used to study the stability of lectin under different condition of pH 1 to 13 were as follows, for pH 1 – 0.01 N HCl, for pH 2 and 3 – 0.2 M glycine – HCL buffer, for pH 4 and 5, 0.2 M sodium acetate buffer, for pH 6 and 7 – 0.2 M sodium phosphate buffer, for pH 8 – 0.2 M Tris –HCL buffer, for pH 9 – 0.2 M glycine – NaOH buffer, for pH 10, 11, 12 and 13 – 0.2 M carbonate – bicarbonate buffer. One hundred µl lectin solutions and one hundred

µl of buffer solutions were incubated for 1 h at ambient temperature. Aliquots were withdrawn and assayed for agglutination as mentioned previously.[34, 36]

Effect of Temperature and Thermal Inactivation

The heat stability of hemagglutinating activity of lectin was determined by incubation of aliquots of lectin solutions at different temperatures.[34] To examine the thermostability, thirty µl of lectin solution was added to one ml 0.006 M sodium phosphate buffer pH 7 and were incubated for various periods at 20, 40, 60, 80 and 100^{o}C for 1 h. Aliquots were withdrawn and assayed for agglutination at ambient temperature after cooling as mentioned previously.

Effect of metal ions on agglutination activity

The metal ion requirement for lectin activity was examined by demetalizing the lectin and then treating with different metal ions. Lectin solutions (100 µg of 4.5µg/µl) were taken in an eppendorf tube and incubated with four hundred µl of 10 mM EDTA at pH 5.0 for 20 h at 4^{o}C. The samples was then dialyzed against 25 mM citrate buffer pH 5 and 50 µl aliquots were transferred to eppendorf tubes containing fifty µl of 1 mM $CaCl_2$, $MnCl_2$, $MgCl_2$, $HgCl_2$, $ZnCl_2$, $FeCl_2$, $ZnSO_4$, $PbCl_2$, 1,10-phenanthrolene and incubated for 2 h. Activity of samples were then examined by agglutination assay as described.[37]

Effect of inhibitor on agglutination activity

The effect of inhibitor on the agglutination activity of purified lectin was determined by the method given by the Deshpande and Patil (2003).[38] The purified fractions were mixed with 1 mM inhibitors solutions namely 1, 10-phenanthrolene, reduced glutathione, meso-inositol, SDS, cystein hydrochloride, phenyl methyl sulphonylfluoride, 2-mercaptoethenol, para – chloromercuribenzoate, n-bromosuccinamide and EDTA and incubated at 37^{o}C for 1 h and tested for hemagglutination with rabbit erythrocytes. Each of the purified lectin solution without treatment with any inhibitor was used as a positive control and normal saline with erythrocytes was used as a negative control.

α and β- galactosidase activity

The substrates used in the study were α – p NPGal (3 mM) and β – oNPGal (3 mM). α and β-galactosidase assays were carried out by the method of Murrey, (1983).[39]

Data were expressed in mean of at least six independent experiments with standard deviation calculated by MedCalc 10 and Graphs were plotted by Sigma Plot 10.0 and Microsoft Excel.

RESULTS AND DISCUSSION

Isolation and purification

The lectin was purified from 0-35% ASF on guar gum was principally based on affinity binding towards the substrate D-galactose. The F-1 peak (Figure 1) gave maximum agglutination activity than F-2 and F-3 but gave multiple bands over the non denaturing PAGE therefore the F-1 fraction loaded on DEAE cellulose for ion exchange chromatography. The 0.20M concentration was suitable for elution of lectin and gives major peak (Figure 2) which gives maximum agglutination and single band on non denaturing PAGE. Table 1 shows that the lectin was purified in the good yield (22%) with specific activity 17066 and 20480 HAU/ml. The positive phenol sulphuric acid test of the isolated lectin shows attached carbohydrate moiety in the structure of the lectin indicating glycoprotein nature of the lectin.

Table 1: Summary of purification procedure of seed lectin isolated from *Zizyphus mauritiana*.

Purification step	Volume (ml)	Proteins (mg/ml)	HAU[a]	SA[b]	Purification fold	Yield %
ASP	100	15.4	5120	332.46	1	100
ACF	23	7.76	10240	1319.58	3.96	46
IEF	10	2.16	20480	9941.74	29.90	40
ASP	6	1.2	20480	17066.6	51.33	22

a)HAU: Hemagglutination Unit, b) SA: Specific Activity

ASP: Ammonium Sulphate Precipitated Fraction

ACF: Affinity Chromatography Eluted Fraction

IEF: Ion Exchange Chromatography Eluted Fraction

HAU: Receprocal of last dilution of lectin which will give visible agglutination with rabbit erythrocytes

Affinity Chromatography on Guar-gum

The elutes of ASP from the column of cross linked guar-gum were collected and absorbance was measured at 280 nm. In the figure 1, three peaks were observed (F-1, F-2 and F-3) having maximum absorption at 280 nm from which in the third eluted sample (F-1) shows highest protein concentration and hemagglutination activity. Three peaks indicate that in the seed of *Zizyphus mauritiana* (0 – 35% ASP) there are three different proteins which shows specificity towards substrate galactose and has a sugar binding domain.

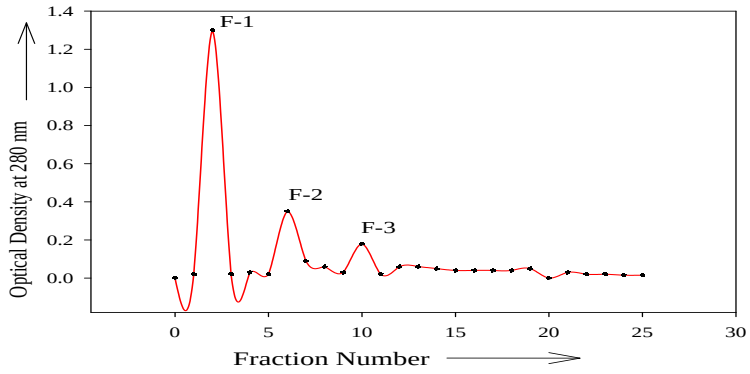

Figure 1: Affinity chromatography elution profile of ZMSL on cross linked guar gum.

Ion-Exchange chromatography on DEAE cellulose

F-1 of ACF was eluted from the DEAE-Cellulose column with 0.02M phosphate buffered saline containing concentration gradient of NaCl. In figure 2, elute 16 shows a single peak at 280 nm in the 0.20 M concentration of NaCl. The peak was found to be lectin and gave maximum agglutination with rabbit erythrocytes and later found to be homogeneous on non denaturing PAGE. The single peak on DEAE-cellulose and single protein band on non denaturing PAGE of IEF confirms anionic nature of purified lectin.

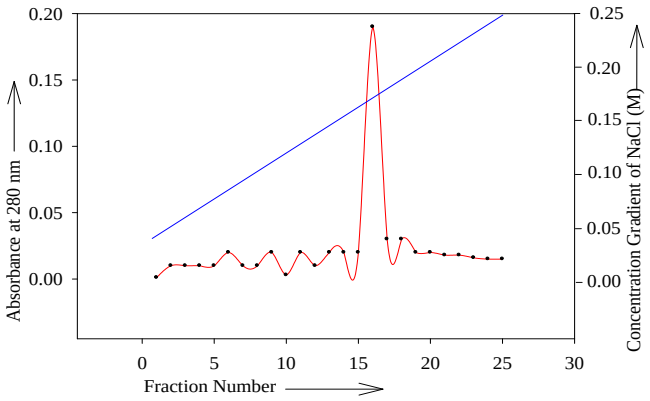

Figure 2: Ion-exchange chromatography elution profile of ZMSL on DEAE-cellulose.

Polyacrylamide Gel Electrophoresis

In Figure 3, A-1The homogeneity of isolated lectin (IEF) was checked on 10% Non denaturing (Native) PAGE. Figure A-1 showed native gel electrophoresis of some standard proteins (Native PAGE Molecular Weight Maker, Genei, Lane 1- 4) and purified lectin (lane 5) shows single band. The single band on the non denaturing PAGE confirms homogeneity of the isolated lectin. The three bands of IEF on 10% SDS-PAGE in figure 3, A-2 of lane 1 indicates the isolated lectin was a tripeptide having molecular weigh 38 kD, 25 kD and 19.95 kD.

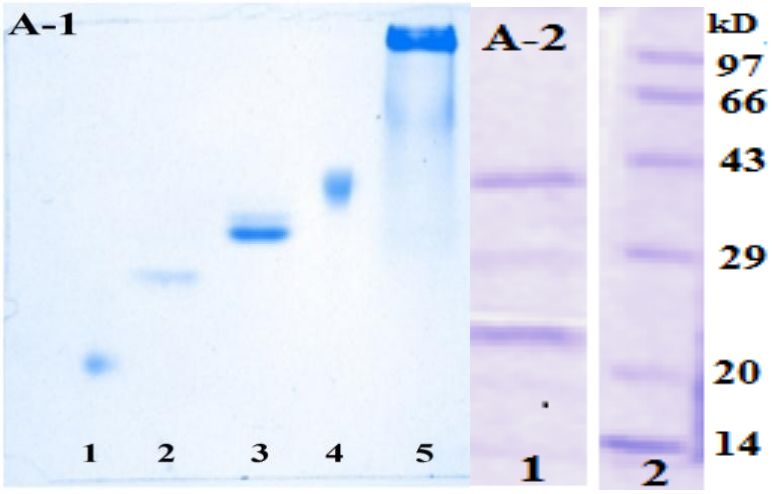

Figure 3: Native and SDS polyacrylamide gel electrophoretic pattern of ZMSL.

(Figure, A-1; Lane1: Lactoglobulin, Lane 2: Trypsin Soybean Inhibitor, Lane 3: Egg Albumin, Lane 4: Bovine Albumin, Lane 5: Isolated Lectin. In figure A-2; lane 1:isolated lectin, lane 2: SDS - Molecular weight marker).

Characterization of isolated lectin:

Agglutination Assay and Blood Group Specificity

The purified fraction had major binding capacity with rabbit (59%) and human B^+ (30%) erythrocytes as compared to human A^+ (7%) and O^+ (4%) erythrocytes (Figure 4). Human B^+ erythrocytes has galactose moiety on their surface and the purified lectin shows maximum affinity towards the galactose by showing hemagglutination.

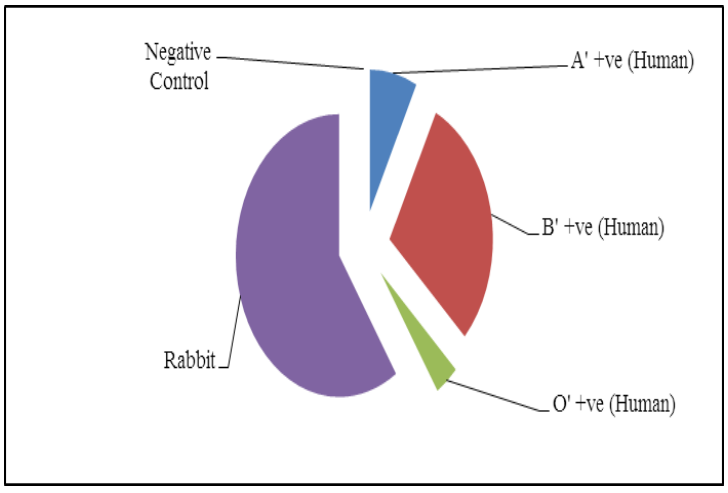

Figure 4: Blood group specificity of ZMSL with human A^+, B^+, O^+ and rabbit erythrocytes.

Hemagglutination Inhibition assay/Carbohydrate specificity

Purified lectin agglutinated human B^+ as well as rabbit erythrocyte as shows in figure, 2. It was found from the results that when rabbit erythrocytes incubated with lectins, D-galactose and its derivative such as para- nitrophenyl-α- D- galactopyranoside, ortho- nitrophenyl-β-D-galactopyranoside, lactose and D-raffinose are the most potent inhibitor for the purified lectin (Figure 5). These results indicated that the purified lectin was galactose specific.

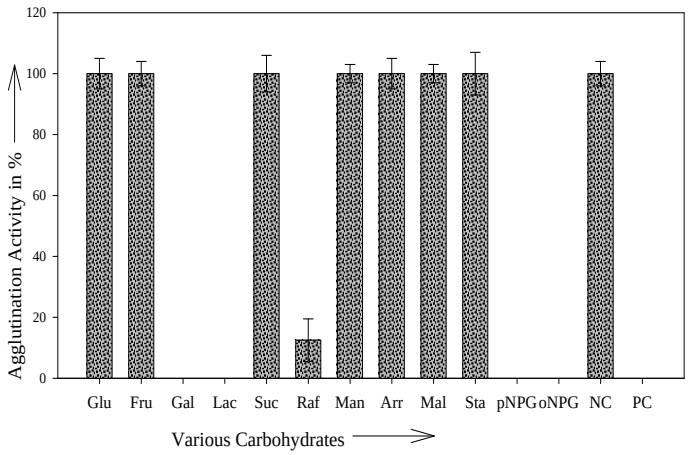

Figure 5: Effect of carbohydrates on agglutination activity of ZMSL.

(Glu: α-D-Glucose, Fru: D-fructose, Gal: D-Galactose, Lac: Lactose, Suc: Sucrose, Raf: Raffinose, Man: D-Mannose, Arr: D-Arabinose, Mal: D- Maltose, Sta: Stachyose, pNPG: p-Nitrophenyl-β-D-Galactopyranoside, oNPG: o-Nitrophenyl-α-D-Galactopyranoside, NC: Negative Control, PC: Positive Control).

pH Stability

The pH dependence of lectin was determined by incubating in buffers of varying pH. The optimum pH for agglutination activity was found to be pH 7. The purified ZMSL lost 50% activity in pH 4 and 9 and was completely inactive at pH 2, 3, 11, 12 and 13 (Figure 6). The isolated ZMSL was found to be active at wide pH range 4 – 9.

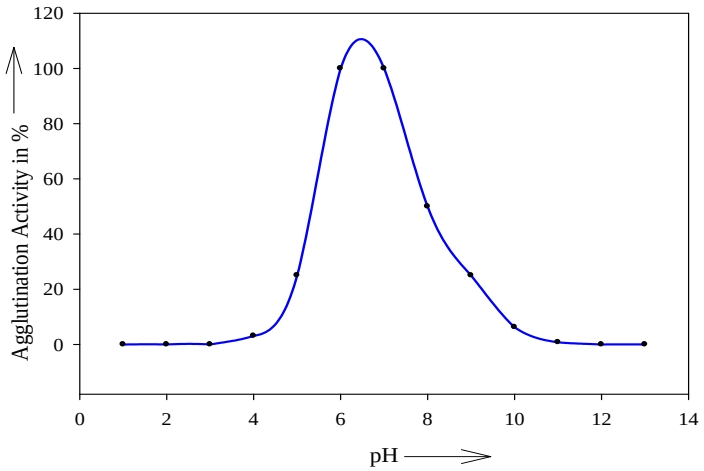

Figure 6: Effect of pH on agglutination activity of ZMSL.

Thermostability and thermal inactivation

When ZMSL was heated at room temperature to 80^{o}C at interval of 20^{o}C, a linear relationship was observed for percent residual activity vs. temperature. ZMSL when heated at several temperatures ranging from 20 – 80^{o}C for 1 h showed 100% activity indicating the lectin to be thermostable. The lectins did not lose its agglutination activity when incubated at temperature from 20 – 140 min (Figure 7).

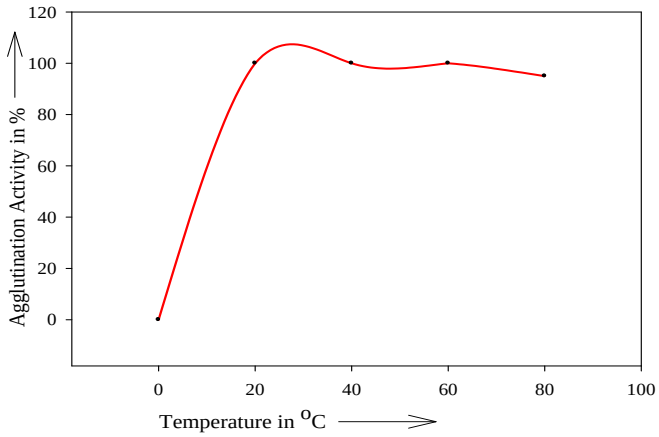

Figure 7. Effect of temperature on agglutination activity of ZMSL.

Effect of metal ions (Activators) on agglutination activity

Results in the figure 8 represent the effect of metal ions on hemagglutination activity by lectin. It is found that heavy metal ions like Hg^{++}, Sb^{++}, Pb^{++} inhibit activity of lectin, while Mg^{++} and Mn^{++} completely restored the activity as compared to other metal ions and controls.

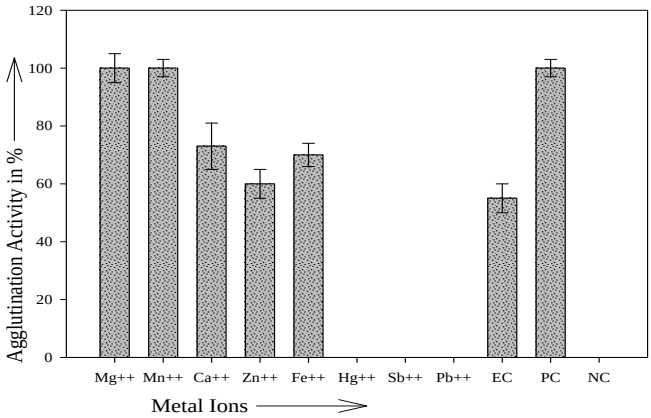

Figure 8. Effect of metal ions on agglutination activity of ZMSL.

Effect of Inhibitors on agglutination activity

Treatment of purified lectins with different inhibitor solution such as SDS and 2-mercaptoethenol gave complete inhibition of agglutinating activity as compared to other inhibitors. Figure 9 shows that inhibitors used in this experiment inhibit the activity of the purified lectins.

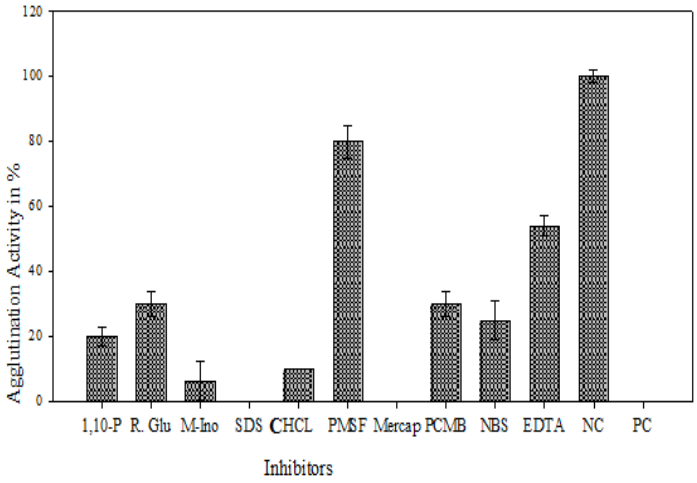

Figure 9: Effect of inhibitors on agglutination activity of ZMSL

(1,10-P: 1,10-phenanthrolene, R. Glu: reduced glutathione, M-Ino: meso-inositol, SDS: sodium dodecyl sulphate, CHCL: cysteine hydrochloric acid, PMSF: phenyl methyl sulphonyl fluoride, Mercap: β-mercaptoethanol, PCMB: para – chloro mercuric benzoate, NBS: n-bromo succinimide, EDTA: ethylene diamine tetra acetate, NC: negative control, PC: positive control).

α-and β- galactosidase activity

The lectin exhibited α-and β-galactosidase activities with substrates α-PNPGal, 13 U/ml and β-ONPGal, 29 U/ml respectively. From the results it was found that the purified proteins have major β- activity as compared to α. From the result it has been clear that the lectin was bifunctional having agglutination and enzymatic activity.

CONCLUSION

It has been proven that lectins had great biological properties and the researchers have great attraction towards these molecules due to their novel properties. Isolation of galactose specific lectin might be a step ahead in the contemporary lectionology. The isolated lectin was found to be tripeptide - glycoprotein, having thermostability and active over the wide pH range. Activity was restored with Mg^{++} and Mn^{++} ions, Lectin shows agglutination as well as enzymatic activity. The medicinal properties of *Zizyphus mauritiana* might be due to the presence of lectin (further work is going on).

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