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Purification and properties of three novel monocot lectins from the family Zingiberaceae

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ABSTRACT

Three new monocot lectins from the members of Zingiberaceae, namely, Alpinia galangal L. (AGTL), Curcuma amada Roxb. (CARL) and Elettaria cardamomum M. (ECSL) have been purified by sepharose and agarose affinity chromatography. These lectins possess similar physicochemical and biological properties. In SDS-PAGE, all the lectins gave a single band corresponding to a subunit of 16 and 17kDa and yielded single band peak on PAGE (pH 4.5). The lectins agglutinate rabbit and sheep red blood cells (RBCs) but are inactive towards chicken and human ABO erythrocytes. The haemagglutination activity of these lectins is inhibited by mannose and their derivatives while other simple sugars D-galactose, D-maltose, D-fructose, sucrose, lactose, D- sorbitol, D-mannitol did not react. The lectins activity were enhanced at acidic but stable at physiological pH range of 6- 8. Lectins were stable at 50°- 60° C but able to agglutinate for a short period when it increases.

Keywords— *Monocot lectins, Rhizome, Seeds, Tubers, Zingiberaceae*

1. INTRODUCTION

In recent years lectins were gaining more attention due to their wide range of resistance towards pests, fungal, bacterial and viral pathogens besides nematodes [1]. Lectins were discovered at the end of the nineteenth century by the invention of ricin from the *Ricinus communis* [2]. Later there is a number of lectins have been isolated from a variety of plant species and from other organisms including humans, their biochemical and sugar binding properties were documented. Lectins represent a various group of oligomeric proteins varying in size, structure, molecular organization and their carbohydrate-binding sites. Structural analysis of lectins showed slight variations in primary structure and carbohydrate-binding specificity but differences in their biological activities [3]. For a long period, plant lectins have been considered as typical seed proteins, but advances in science, the occurrence of plant lectins in vegetative plant tissues is now very well documented. Seed lectins generally account for 0.1%–5.0% of the total seed protein. Non-seed lectins have been detected in almost all vegetative tissues ranging from roots to flowers and even nectar. Lectin concentrations in vegetative storage tissues can amount to 50% of the total protein, whereas other tissues in the listing is a minor protein (e.g., in the leaves of leek).

On the basis of structural analysis and sequence, data lectins were classified into seven families of structurally evolutionary related proteins [4]. Plant lectins have been subdivided on the basis of their carbohydrate-binding specificity into mannose-, mannose/glucose-, mannose/maltose-, Gal/GalNAc-, GlcNAcc/ (GlcNAc)n-fucose-, and Sialic acid- binding lectins [4]. The monocot mannose-binding lectins (MMBLs) have received much interest and become an important tool in plant protection and plant biotechnology since their genes confer resistance against sucking insects and nematodes [5]. Unlike all other plant lectins, MMBLs are encoded by large families of closely related genes [6]. MMB's are found to be very similar at the protein level but differ in the processing and post-translational modifications of the primary translation products of their genes [7].

Till date, seven monocot plant families were well documented for their insecticidal activity and their structure analysis revealed that they are structurally and functionally related ones and potential mannose – recognizers [8]. The wide distribution of lectins in all tissues of the plant kingdom put forward important roles for these proteins. The insecticidal activity of lectins is associated to the sugar-binding properties. The mechanism of action could be the binding of the lectin to the midgut epithelium causing disruption of the epithelial cells, leading to impaired nutrient assimilation by cells and allowing absorption of potentially harmful substances [9]. Zingiberaceae is one among the monocots constitutes medicinally a vital group of rhizomatous medicinal and aromatic plants characterized by the presence of volatile oils and oleoresins which is not explored. In addition, this family includes important genera namely, *Curcuma*, *Kaempferia*, *Hedychium*, *Amomum*, *Zingiber*, *Alpinia*, *Elettaria* and *Costus* are reported as potential medicinal plants. A number of investigations have been done on medicinal aspects of zingiberous plant species by means of leaf extracts and their important volatile compounds. This study aims at extracting and purifying a lectin from the tissues of *Alpinia galanga*, *Curcuma Amada* and *Elettaria cardamomum*, characterize the protein activities.

2. MATERIALS AND METHODS

All the experiments were carried out at the United Planters Association of Southern India (UPASI) Tea Research Foundation, Tea Research Institute, Valparai, Tamil Nadu, India (latitude 10°30'N, longitude 27°0'S and altitude 1050 M). Fresh rhizomes of *Alpinia galanga* and *Curcuma Amada* were collected from the local fields of Valparai. Peeled rhizomes and washed with tap water at least for thrice then washed with distilled water. *Elettaria cardamom* dry seeds were purchased from the local market for protein purification.

Affinity matrix (D-Mannose Agarose in saline suspension) was purchased from Sigma Chemicals Bangalore. Chemicals which are used for matrix preparation were purchased from Hi-media Chemicals (MB grade) Mumbai. ConA - sepharose 4B was purchased from GE Healthcare Bangalore. The Chromatography matrix used for the lectin purification was pre-equilibrated with corresponding extraction buffer after the wash with plenty of distilled water as per the manufacturer's instructions.

2.1. Purification of lectins

Fresh tubers of *Alpinia* were washed to purify the AGTL, then cut into small pieces and homogenized in a blender in 0.02M Tris HCl buffer at pH 7.4, containing 0.15M NaCl at (1:5, w/v) ratio then left to extract overnight at 4 °C. Initially, the suspension was filtered and then clarified by centrifugation at 10000 rpm for 20 min at 4°C (Sigma 3-18K). The clear supernatant was 50% saturated dialyzed against distilled water, then with extraction buffer. The crude was centrifuged once to remove any precipitates and incubated with pre-equilibrated D-Mannose Agarose (DM Agarose) with occasional shaking at 4°C. The column was packed with 20 ml of DM agarose and washed with TBS at a flow rate of 2ml/min as well monitored for its absorbance at 280nm fell to <0.05. After elution of the unbound proteins in the equilibrium buffer, the adsorbed proteins were washed out with an increasing gradient of 0.2M methyl-α-D-mannopyranoside as the competitor to desorb the lectin from the column. The same protocol was followed for mango ginger rhizomes but 0.07 M phosphate buffered saline (PBS) with pH 7.4 (1:2, w/v) used as a homogenizing buffer and 0.2M mannose gradient was used to desorb lectin from the column. Whereas Cardamom seeds were de-husked and powdered using pre-chilled mortar and pestle, then defatted using 5 volumes of pre-chilled n-Hexane. The fat-free powder (1:5, w/v) was mixed uniformly with 0.2M Tris HCl buffer containing 0.5M of NaCl, pH-7.4 by stirring overnight at 4 ºC. The suspension was clarified by centrifugation at 10000 rpm for 15 min at 4°C. The collected supernatant was adjusted to sixty per cent saturation by adding solid ammonium sulfate. The precipitate was again collected by centrifugation, dissolved in a minimum volume of extraction buffer and dialyzed against distilled water and then with extraction buffer for 24 h at 4°C. The dialysate was applied to Con A-Sepharose column, which was previously equilibrated with the Tris–HCl buffer. After washing the column with the buffer, the protein absorbed was eluted by the buffer containing 0.1 M methyl-α-D-mannopyranoside. Active fractions on agglutination assay were pooled for further use.

2.2. Hemagglutination assay

Single band purified lectins were subjected to agglutination assay, which is the preliminary characterization of lectins. Purified lectins were finally dissolved in phosphate buffer saline for easy handling to all characterization steps. For this reaction serially diluted solution of the purified lectins incubated with 100µL of 4% rabbit erythrocyte suspension in 96-well U-shaped microtiter plates and the agglutination was scored after 1 hour at room temperature. The Hemagglutination unit (HU) was expressed as the reciprocal of highest lectin dilution showing detectable visible erythrocyte agglutination, and the specific activity was calculated as HU/mg protein. The Hemagglutination activity was assayed separately, in the same manner as above, against the sheep, chicken and 4 human ABO and AB blood groups. Unless or otherwise mentioned the assay was carried out as duplicates with PBS as a control in each assay.

2.3. Inhibition of various carbohydrates on lectin activity

To investigate the inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a way similar to the hemagglutination test. Serial dilutions of sugar samples were prepared in phosphate buffer saline (0.2 M initial concentration). All the dilutions were mixed with an equal volume (25-50 µl) of the lectin solution of known hemagglutination units. The mixture was standing for 1 hour at room temperature and then mixed with 50 µl of a 4% rabbit erythrocyte suspension. The obtained hemagglutination titres were compared with a non-sugar containing a blank. The sugars used were: D-glucose, D-mannose, D-galactose, D-maltose, D-fructose, sucrose, lactose, D- sorbitol, D-mannitol, methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside. The minimum concentration of the sugar in the final reaction mixture which completely inhibited hemagglutination units of the lectin sample was calculated.

2.4. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed by the method of Laemmli, (1970).Glass plates of size 100 x 100 mm and 4.0 mm thick were used. The plates were assembled with side spacers in gel casting setup and 15% separating gel solutions were prepared, poured into the assembly. After polymerization, 4% stacking gel was poured on the separating gel into the gel cast. Lectin samples to be analyzed were treated with reducing sample buffer and boiled for 2 min prior to application on the gel. Electrophoresis was carried out at 20 mA, once the protein entered the separating gel the voltage was increased to 25 mA and it was carried out for 3 hrs at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co resolved in each gel alongside the samples to determine the subunit molecular weight of the purified proteins. The gel was visualized by massive blue -250 staining.

2.5. Total Sugar determination

Carbohydrate content of the purified lectin was determined by the phenol-sulfuric method using D-mannose as standard [9].

2.6. Effect of pH on lectins stability

Incubating the lectin in buffers of broadly similar salinity levels, but variations in pH from 2-12 were used to assess the pH stability of the lectin. The buffers used were 20 mM glycine-HCl (pH 2-4), 20 mM sodium acetate (pH 4-6), 20 mM potassium phosphate (pH 6-8), 20 mM Tris-HCl (pH 8-10), and 20 mM glycine-NaOH (pH 10-12). The purified lectin was mixed in each of the different buffer-pH compositions (control was just PBS) and left for 1 hour at room temperature. Then the samples were adjusted back to PBS and assayed for agglutinating activity as above with 4% rabbit erythrocytes.

2.7. Assay for thermal stability

To determine the thermal stability of the purified lectins, 50µl of the purified lectins was incubated at temperatures ranging between 30-100ºC with a 10ºC interval for 20 minutes at each temperature in the water bath. The samples were cooled at

room temperature and centrifuged to eliminate precipitated material and evaluated for agglutination activity.

2.8. Metal ion requirements of proteins

To find the dependency of haemagglutinating activity of metal ions the purified lectins were incubated in 0.1M EDTA (pH 8.0) for 2 hrs and were dialyzed against 0.07M PBS, pH 7.4 for 12 hrs at 4 ºC and then subjected for agglutination assay in the presence and absence of ions in the Hemagglutination buffers. The following ions were used Ca2+, Mg 2+, Cu 2+, K+, Na+ and NH4+.

2.9. Stability of lectins on denaturing agents

The effect of two denaturing agents, urea and guanidine- HCl (Gdn.HCl), at a concentration ranging from 0.5−8M, was tested on lectin activity by incubating 25µl of each denaturant solution with an equal volume of lectins at 37 ºC for 2h.

3. RESULTS AND DISCUSSION

The present study reports the purification and characterization of a lectin from rhizomes and seeds of zingibeorus plants. Preliminary experiments with crude extracts indicated the presence of a lectin that strongly agglutinated rabbit erythrocytes but was inactive with human erythrocytes irrespective of blood group (figure 1). Crude, as well as eluted fractions of proteins, were tested for their agglutinating ability with different erythrocytes of sheep, chicken, rabbit and four human blood types. Among tested all the four proteins were efficiently agglutinated rabbit erythrocytes and they were not acted upon chicken, A, B, O and AB types of humans. This was in accordance with previous reported mannose-specific lectins [10, 11]. This behaviour of these lectins is showing that they are specific towards mannose. Mannose-specific lectins which are reported to date have exclusively agglutinated rabbit erythrocytes. [12] Reported araceous lectins being all capable of agglutinating rabbit, guinea pig, rat and sheep erythrocytes but not human erythrocytes. Additionally, in this study, *Alpinia* lectin agglutinated sheep erythrocytes. This nature was seen in a 34.2kDa lectin from *Curcuma amarissima* does not reactive against all four type of humans and mouse, pig, goose, guinea erythrocytes [13]. The agglutination behaviour is because of various glycans exposed on a different type of erythrocytes, for example, human blood group an expresses non-reducing terminal α-N-acetylgalactosamine, group B, terminal α-galactose and group O, fucosyl-galactose [14].

Two hundred and fifty gram of galanga rhizome (sitharathai) was used for initial crude protein preparation with the respective buffer. 50% saturated dialysate was added to the mannose coupled agarose column and allowed to complete absorb the protein then flow through was collected. The nonbinding proteins were washed with extraction buffer and the bound lectin was eluted from the column using 0.2M methyl-α-D-mannopyranoside as the competitor. In initial preparation of crude from the rhizome showed about 33 units of specific activity.

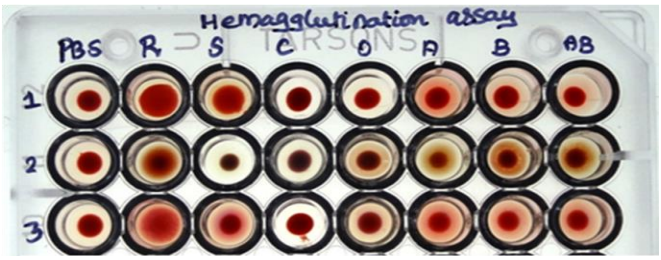

Fig. 1: Agglutination assay with different erythrocytes 1-AGTL, 2-CARL, and 3-ECSL

Hemagglutination activity titer value 4 corresponded to the concentration of 40µg/ml of AGTL required for agglutination as represented in table 1. In initial preparation of crude from the *Curcuma amada* (mango ginger) rhizome showed about 120mg of total protein with a high level of specific activity. Two hundred grams of mango ginger rhizome was used an initial material and crude was extracted in PBS buffer. The dialyzed crude was loaded on the DM agarose column then flow through was collected after allowing it for some time to complete absorption. Lectin from mango ginger was purified using 0.2M D-mannose as a competitor. Table 2 represents the purification steps.

Table 1: Purification table of *Alpinia galanga* tuber lectin

Step	Vol (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mp)	Purification (fold)	Yield (%)
Homogenate	1250	3500	6.6	33	1	100
$(NH_4)_2 SO_4$ ppt	200	40	250	1250	37.9	5.7
After elution	44	8.8	1136	5681.9	5	0.75

Table. 2: Purification table of *Curucma amada* rhizome lectin

Step	Vol (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mp)	Purification (fold)	Yield (%)
Homogenate	400	120	833	2777.8	1	100
$(NH_4)_2 SO_4$ ppt	50	15	6666.7	22222	7.9	8
After elution	35	9.45	10582	39192.6	14	12.7

Dried capsules of cardamom were used for lectin purification. Dehusked, Powered powder was treated with non-polar solvent n-hexane. In 2004 [15] tested n-hexane, hexanes and light petroleum to eliminate the possible inhibitory effect caused by fat and n-hexane were chosen as the defatting solvent since the other two solvents were each observed to form a stable film, which was difficult to separate from the extract. About fifty grams of the powdered meal was incubated at overnight then ground for crude extraction. Crude was loaded on con A sepharose column and flow through was collected repeatedly by circulating it twice for proper binding. The column washed to remove unwanted protein and lectin was desorbed from a column by eluting with 0.1M methyl-α-D-mannopyranoside in extraction buffer, Hemagglutination activity titer value 3 corresponding to the concentration of 38µg/ml of ECSL required for agglutination. The final purified lectin (6.3mg) amounted to 11.15% of the initial protein table 3.

Table 3: **Purification table of *Eletteria cardamom* seed lectin**

Step	Vol (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mp)	Purification (fold)	Yield (%)
Homogenate	250	100	40	100	1	100
$(NH_4)_2 SO_4$ ppt	40	16	625	1562.5	15.62	15
After elution	30	6.3	1587	7558.6	75.58	11.15

Choice of buffer and elutor placed an important role in the efficient isolation as well purification of interested protein hence it was chosen by previous researchers' experience. Rhizomatous lectin from *Curcuma longa* was purified using 0.5M methyl-α-D-glucopyranoside by [16]. Zingiber rhizome lectin from *Kaempferia parviflora* was eluted with 0.5M methyl-α-D-glucopyranoside in TBS buffer pH 7.4 [17]. A neutrophil migration-inducing *Artocarpus integrifolia* seeds lectin was purified through 100mM D- mannose in 10mM PBS buffer [18]. The mannose-specific lectin was isolated from leaves of an ornamental plant (*Neoregelia flandria*) belongs to Bromeliaceae using 0.2M D-mannose in PBS [19]. Other than plant lectins, a lectin from coral (*Acropora millepora*) was purified using 0.2M D-mannose [20] and [21] purified the lectin from the green marine alga, *Bryopsis plumosa* with 0.1M D-mannose in PBS buffer.

Desorption of lectin by changing the conditions to extremes of ionic strength/pH depends on the chemical stability of the matrix, ligand, and adsorbed substances. However, carbohydrates and lectins are usually stable molecule scale must be taken not to damage them irreversibly. Therefore, fractions containing the proteins should be neutralized immediately, usually with 2M or 1M Tris-HCl buffer, pH 7.5 as well the column also has to be equilibrated with the binding buffer.

The initial discovery of a lectin with an exclusive specificity towards mannose in snowdrop bulbs [22] similar proteins have been found in species of the monocot families Amaryllidaceae, Alliaceae, Araceae, Orchidaceae, Liliaceae, and Bromeliaceae [23]. Monocot mannose-binding lectins are built up of 4 subunits of about 12kDa and sequence comparisons showed that they belong to similar super-family of evolutionarily related proteins [24]. Gramineae or Poaceae plant family lectins are found to be dimers of about 18kDa subunits [25]. Amaryllidaceae lectins are of dimers or tetramers composed of 13kDa subunits. In 1995 [26] and their crew have purified four lectins from araceous plants and they are composed of 13kDa subunits. Two lectins from roots and leaves of garlic were found to be dimers of 12kDa and 15kDa respectively [27]. As well in our study, we could able to isolate lectins in a single band at the molecular weight between 16–17kDa as like that of previous lectins from monocots. Purified proteins were showed single band both presence and absence of β-mercaptoethanol, this indicates that these lectins are not linked by di-sulfide bonds. This condition was observed in many lectins [28, 29, 30].

There are other Zingiberous lectins have shown variation in their molecular weights, for instance, [16] reported 17.3kDa lectin from *Curcuma longa* with α-glucosidase inhibitory. A 34.2kDa lectin isolated from *C.amarissima* lectin by [15]. In 2007 [31] reported *C. zedoaria* rhizome lectin of molecular size13 kDa. *Kaempferia rotunda* is another tuberous lectin from this family is about 29kDa molecular weight and it also proved its anti-proliferative activity against carcinoma cells [30]. This condition was observed the other family lectins like seed lectin of *Trigonella foenumgraecum* reported as 27.3kDa by [32]; horseradish seed lectin of 12.2kDa [33]; rhizome lectin of *Ophiopogon japonicas* in molecular weight 12kDa [34]; seed lectin of *Capsicum annum* of 25kDa [35].

In addition, since only a single band of the same apparent size was seen under non-reducing condition this suggests that the purified lectins could be a monomeric protein, or at least if a multimeric one that dissociates into subunits under these conditions, then this subunit has able to show hemagglutination activity alone. The apparent size of these lectins approximately 16kDa, 17kDa and 17kDa (figure 2) is in agreement with the previously published sizes of the other plant lectins which ranged from 12 to 34.2kDa. Same lectins in native PAGE at the acidic condition of pH 4.5 gave single band shows homogeneity of their purity showed in figure 3.

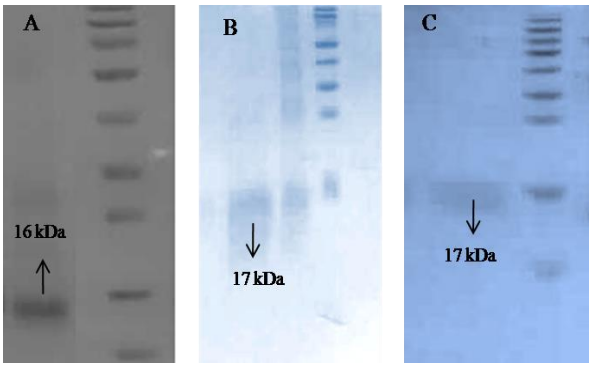

Fig. 2: Co-massive blue stained non-denaturing PAGE of purified lectations (A) Alpinia (B) mango Giner and (c) Cardamom

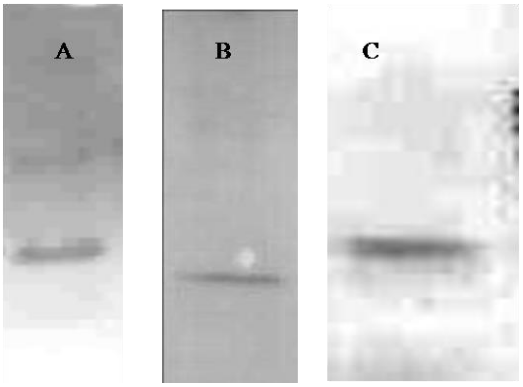

Fig. 3: Native PAGE homogeneity of purified lectins at pH 4.5 in tris-cl (A) Alpinia (B) mango Giner and (c) Cardamom

Protein estimation of lectins resulted for mango ginger, cardamom seed and sitharathi of 270µg, 215µg, and 150µg /ml respectively. In carbohydrates estimation lectin solutions were produced characteristics yellowish color indicates these are glycoproteins and having a neutral sugar content of 7.2%, 4.4%, and 6% for sitharathai, mango ginger and cardamom respectively. These results were correlating with other lectins which have purified from different sources have estimated for carbohydrates by this method. For instance *Caladium bicolor* tuber lectin has 2.2% sugar content [35]; *Arundo donax* rhizome lectin has 2.1% [36]; *Sambucus nigra* L. bark lectin has 5.5% [37]; *Curcuma longa* rhizome lectin has 5.2% [16] and *Kaempferia rounda* rhizome lectin has 4% [30]. There are few lectins have reported for high sugar content such as *Typhonium divaricatum* L. tuber lectin has 12% [38]; *K.parviflora* rhizome lectin has 14.1% [17] and as high as seeds of *Mimosa invisa* L. reported for 21% sugar content by [39].

Hapten sugar specificity assay gives the idea of about the specificity of the isolated protein. Inhibition assay is that of agglutination assay but involves "sugar inhibitors" to interfere with the lectin protein and red blood cells. Serial two-fold dilutions of sugar solutions were prepared from 0.2M to 1.56mM for each sugar then they were eventually mixed with lectin solutions, the mixture was allowed to standard for 30mins at room temperature without disturbing. After that agglutination ability of lectins were tested by adding 4% erythrocytes and incubating them for another 1 hour. "Buttons" like cells appeared in mannose incubated lectins. Sugar

specificity of purified lectins in the presence of various mono saccharides was assayed by inhibition of hemagglutination.

Serial dilutions of sugars were prepared and the initial concentration is 0.2M. Mannose showed maximum inhibition followed by methyl-α-D-mannopyranoside, glucose and methyl-α-D-glucopyranoside was weakly inhibitory. D-galactose, D-maltose, D-fructose, sucrose, lactose, D- sorbitol, D-mannitol were all non-inhibitory (tables 4-6 in that + denotes hemagglutinating activity; - denotes no hemagglutinating activity; PBS, phosphate-buffered saline).

Table 4: Sugar specificity of *Alpinia galanga* Tuber Lectin

Sugars in mm/l	200	100	50	25	12.5	6.25	3.12	1.56	PBS
Mannose	-	-	-	-	+	+	+	+	-
Glucose	-	-	+	+	+	+	+	+	-
Man-α-p	-	-	-	-	-	-	+	+	-
Glu-α-p	-	-	-	+	+	+	+	+	-

Table 5: Sugar specificity of Curcuma amada rhzome lectin

Sugars in mm/l	200	100	50	25	12.5	6.25	3.12	1.56	PBS
Mannose	-	-	-	-	-	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	-
Man-α-p	-	-	+	+	+	+	+	+	-
Glu-α-p	-	+	+	+	+	+	+	+	-

Table 6: Sugar specificity of *Eletteria cardamom* seed lectin

Sugars in mm/l	200	100	50	25	12.5	6.25	3.12	1.56	PBS
Mannose	-	-	-	+	+	+	+	+	-
Glucose	-	-	+	+	+	+	+	+	-
Man-α-p	-	-	-	-	-	+	+	+	-
Glu-α-p	-	+	+	+	+	+	+	+	-

In sugar specificity assay the lectins have inhibited by mannose (C-2 epimer), and by its isomers mannopyranoside, glucopyranoside and also by glucose at different concentrations. *C.amada* rhizome lectin inhibited by D-mannose at a minimum concentration of 6.25mM, hence mannose used for the purification. *Alpinia* tuber lectin and cardamom seed lectin were inhibited by mannose at 25mM and 50mM concentrations respectively. Additionally, Alpinia tuber lectin incubated by glucose at the concentration of 50mM as well as the isomers of mannose. Mannopyranoside is inhibited at the minimum concentration of 6.25mM hence taken as a competitor for the purification of Alpinia lectin. In the same way, mannopyranoside was chosen for purification of cardamom seed lectin since it's inhibited at 12.5mM than mannose. Purified Zingiberaceae lectins from K. rotunda [30]; *C. longa* [16] and *C.zedoaria* [31] were reported to have specificity for mannose and isomers also as reported in the present study. This behavior has consonance with many of the lectins which are specifically purified with carbohydrate affinity chromatography. Mannose-specific *Aspidistra elatior* B. rhizome lectin is inhibited by mannose at 50mM, however, thyroglobulin inhibited at a minimum of 12.5µg/ml [41]. Whereas inhibition assay of series of sugars with seed lectin T. *foenumgraecum* showed maximum inhibition with D-mannose and glucose only [32].This kind of behavior has reported by the previous purification of *cajanas cajan* seed and root lectins, in which they inhibited by d-mannose followed by glucose [40].

Ophiopogon japonicus rhizome lectin strongly inhibited by Man-α (1,3:1,6)-mannotriose, Man- α (1,6)-Man, Man- α (1,3)-Man and Man- α (1,2)- at concentration of 2.5mM, 5mM, 10 mM and 30mM, respectively and also inhibited by methyl a-D-mannopyranoside, D-mannose, and D-fructose [33]. In our experiments, the activity of *Alpinia*, mango ginger and cardamom seed lectins were inhibited by mannose, isomers of mannose and glucose at different minimum concentration than by single sugar. The other sugars which are tested along with the above mentioned were not able to inhibit even after one-hour incubation with lectins solutions individually.

Studies were carried out to know protein carbohydrate recognition [41, 42]. This recognition is a major form of inter-cellular communication and plays a role in many biologically important processes such as viral, bacterial, mycoplasmal and parasitic infections, targeting of cells and soluble components, fertilization, cancer metastasis and growth and differentiation [43]. The specific recognition of an oligo/saccharide by a protein is a much more complex problem than other biologically relevant recognition processes such as protein-protein or protein–DNA interactions. The monosaccharide building blocks of a glycan are difficult to distinguish from each other due to the limited repertoire of functional groups involved. Apart from the occasional N-acetyl group or rarely a carboxylate group, one finds invariantly a large abundance of hydroxyl groups interspersed with small aliphatic patches. In addition, the glycosidic bonds between two mono-saccharides are rather flexible, especially the 1-6 linkage. As a result, a high entropic cost limits the binding affinities that can be obtained [43].

Legume lectin family covers the widest possible range of carbohydrate specificities among all known lectin families hence considered as a model system to study such recognition. The Mannose-binding Pterocarpusangolensis seed lectin (PAL) has been previously studied in detail by [47] through x-ray crystallography and a variety of biophysical techniques. This resulted in a clear picture of how this lectin recognizes mono-, di-, trisaccharides and complex-type oligosaccharides also how its structure, stability, and activity respond to demetallization. They described the carbohydrate binding site of PAL as one with a primary binding site recognizes glucose and mannose. This primary binding site lies in the center of a shallow groove on the surface of the protein. Extensions to the sugar bound in the primary binding site (M) can be made in two directions, followingO1 or O2. Independent of the exact linkage that is present, these additional sugar residues occupy two particular regions of the lectin, which noticed as -1 (attached to O2) and +1 (attached to O1)subsites. Where as in the case of longer oligosaccharides, subsites extends to additional -2, -3,…or +2, +3,….and so on depending on their position relative to the primary site.

There are other plant family lectins having specificity towards different sugars/glycoprotein were reported by many researchers [26, 36]. Based on the similarity with available lectins; lectins purified from zingiberous members assumes significance as a biological tool because earlier it has been reported in the literature that mannose-specific lectins are one of the important proteins in biological applications as well as plant disease management.

A detailed characterization of protein through its pH, temperature stability, and their biological applications make them to use as a promising source for a number of applications. Hemagglutination assay allows the assessment of lectin stability to pH and temperature values and thus can determine the conditions to be used in the application of lectin. Initially, lectins were incubated with a different pH range of buffer then brought back to neutral by adding pH 7, PBS buffer then assay was carried out. Lectin at pH 7.0 was taken as control. 50ml of each lectin were treated with different buffers ranging from pH 2.0-12 and they were showed stability at a range between pH 5.0-8.0. *Alpinia* tuber lectin was able to maintain its agglutination ability up to 40% it gradually followed in higher pH till 8 then it was declined at there no activity at higher alkaline conditions. *C.amada* rhizome showed 60% agglutination ability at the basic pH condition, it increased pH 7-8 and retains its activity till pH 9 then it dropped suddenly pH 10 and there is no agglutination till pH 12 (figure 4 A-C). *E.cardamom* seed has higher activity at pH 5 comparatively than pH 4 but it retained its agglutination ability till pH 8 then gradually decreases in the subsequent pH range. In the same way, the other lectins which are eluted with a competitor have eluted in neutral condition itself behaved the same manner, for example, *Allium ascalonicum* bulb lectin [48]; *Caladium bicolor* tuber lectin [32] and *Trigonella foenumgraecum* seed lectin [36]. In this work, all the purified lectins were found to be stable in the range of pH 5-8.

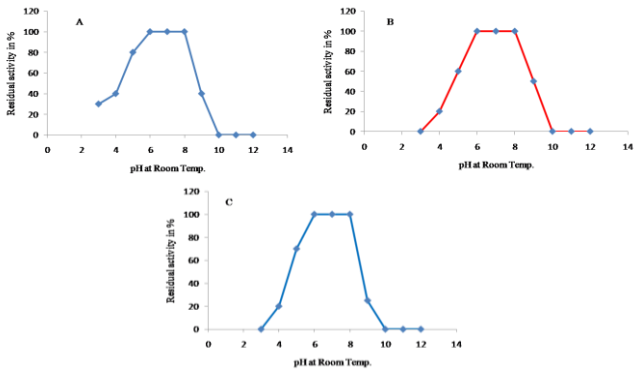

Fig. 4: (A)-(C), pH dependence of purified lectins

The results of the temperature stability with respect to haemagglutination ability of the lectins were found to be stable at a higher temperature with considerable visible agglutination. All lectin solutions were individually heated from 30°C for 20mins then cooled at room temperature and taken for assay. Lectins were agglutinated the blood erythrocytes when they heated at a low temperature of 30°C. Whereas lectins were readily agglutinated at 40-70°C showed their optimum temperature level, however, their activity gets delayed at higher levels till 100°C. All the lectins Alpinia, mango ginger and cardamom showed an almost similar pattern of agglutination. The assay was shown in figure 5.

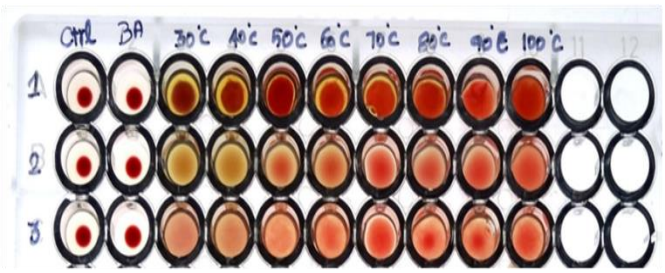

Fig. 5: Temperature stability of lections (1) AGTL, (2) CARL, (3) ECSL

This is accord with purified lectins of different plant sources were tested for temperature tolerance by other researchers. For example, the lectin from the rhizomes of *Smilax glabra* was stable at temperatures up to 50°C; although above 80°C very little haemagglutination activity was noted [46]. *Phaseolus vulgaris* is another example of extremely thermostable temperatures around 82°C [47]. Surprisingly above lectins were overtaken by two seed lectins *Artocarpus integrifolia* and *Canavalia gladiate* are found to be 100°C [29]. Most of the lectins have its maximum activity at temperatures 40-60°C, for example, *Arundo donax*, *C.amarissima*, and *A. elatior* Blume rhizome lectins were stable up to 55°C and showed only 80% activity at 60°C and no activity at 85°C higher temperatures [37, 12, 10]. Additionally, *Psophocarpus palustris* and *C.longa* lectins were their activity lost at 70°C [48, 16]. Together all these reports suggesting that the haem agglutination activity depends on the native conformation of the protein [46].

The requirement of metal ions was evaluated by incubating the lectin solution in 0.1M EDTA for 2 hrs followed by, agglutination assay. Lectin solutions without EDTA treatment kept as control. AGTL and CARL were failed to agglutinate but ECSL agglutinated as usual (figure 6). Hence, the lectins were incubated with 0.025M salts of $Ca^{2+,}$ Mg^{2+}, Cu^{2+}, K^{+}, Na^{+} and $NH4^{+}$ for half an hour then the assay was repeated. AGTL and CARL retained their activity in the presence of Ca^{2+}, Mg^{2+}. This condition had been observed in many of the lectins and the following are some of the examples. Atomic absorption spectroscopy analysis on *A. jacquemontii* lectin identified the presence of Co^{2+} and Zn^{2+} at a conc. of 0.003 and 0.002 moles per mole in their subunit [49]. *Archidendron jiringa* N seed is reported to require divalent ions Ca^{2+}, Mg^{2+}, and Mn^{2+} [50]. Other Zingiberous lectins were tested for metal ion requirements and *C.longa* rhizome lectin needs less than 50mM conc. of $Ca^{2+}$and $Mn^{2+}$for their effective agglutination [16]. Correspondingly, *K. rotunda* tuber lectin retains their 100% activity from EDTA treatment when they treated with 10mM of Ca^{2+}, Mg^{2+} as well as 50% activity $Ba^{2+}$and Mn^{2+}. *Dioclea violacea*, *D.grandiflora*, *D.guianensis*, *D.rostrata* and *D.virgata* lectins were restored their activity in the presence of Ca^{2+} and Mn^{2+} [30].

There are some other lectins like ECRL that do not requires metal ions for active agglutination. *Arisaema favum* and some other araceous lectins, *Amaranthus hypochondriacus* var. Mexico seed lectin, *Smilax glabra* rhizome lectin, *Erythrina cristagalli* lectin are some of the examples need not metal ions for agglutination [51,52,46,53]. The requirement of metal ions is based on the structure and activity of the lectins and this is agreement with statements given by [54] that "Metal ions play important roles in many biological systems, In particular, metal ions are required for the activity of a large number of enzymes and proteins.

Metal ions are well suited for these functions because of the following properties: (1) Metal ions are almost always positively charged and, hence, electrophilic. They can act as Lewis acids for binding and activating substrates. (2) Many metals can exist stably in a number of different oxidation states differing by one or by several units. This allows these metals to participate in various types of oxidation-reduction processes. (3) Metal ions generally bind four or more ligands. By binding several protein side chains, metals can act as multi dentate cross-linking agents".

Fig. 6: Metal ion requirements of proteins

Denaturants urea and guanidine- HCl (Gdn.Hcl), at a concentration range of 0.5-8 M was tested on lectin activity by incubating 25µl of each denaturant solution with an equal volume of lectins at 37ºC for 1h. In different characterization assays, our purified lectins have a more or less similar pattern of behaviors whereas they showed the various stage of activity when exposed to denaturing agents. The denaturant-treated samples with 0.5-8M denaturants were showed in figure 7. Erythrocytes added at a higher concentration from 5-8M were dispersing immediately hence, the activity of lectins was noticed till 5M conc. On two denaturants *Alpinia* lectin was able to show its activity at 4M and at 5M activity lost in case of urea whereas erythrocytes got dispersed in Gdn-HCl. Mango ginger lectin cannot withstand its higher conc. of both denaturants than 0.5M. Cardamom seed lectin was influenced by urea however it can withstand till 1M cons of Gdn-HCl then gradually it lost activity further (figure 7).

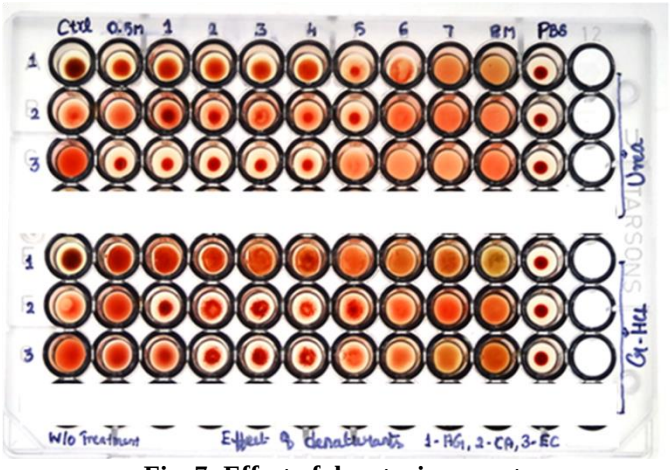

Fig. 7: Effect of denaturing agents

On three different denaturing agents namely urea, thiourea and Gdn-HCl *Caladium bicolor* Vent tuber lectin was stable up to 3M [35]. *K. rotunda* rhizome lectin lost its activity 0.5M and 1M conc. of urea and Gdn-HCl respectively [30]. These denaturants are known to disturb the three-dimensional conformation and binding sites of lectins by affecting the hydrophobic interaction that plays a crucial role [55]. Hence, the decrease in activity of our purified lectins due to denaturants indicating that they are mainly stabilized by hydrogen bonding and hydrophobic interaction.

4. CONCLUSION

Experiments and literature that are presented here related to purification of mannose specific lectins from the family zingiberaceae give an idea their potent. Moreover their biochemical properties and their wide range of pH, temperature tolerance may useful to field level application. Hence, it's concluded that these lectins may be a one among the powerful proteins can be suggested for biological applications.

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