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Isolation, Characterization of Lectin from *Cucumis Melo Var Agrestis*

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ABSTRACT

Lectin was isolated from *Cucumis melo Var Agrestis*. Protein and carbohydrate present were identified by thin layer chromatography and also quantified. The lectin obtained showed its ability to bind with mannose and demonstrated thermostability. Hemeagglutination assay showed its human blood group specificity to O+ and AB+ and among the animal blood tested, none of them showed specificity to *Cucumis melo Var Agrestis* lectin. . Hemeagglutination inhibition assay was observed with Glucoise, Ribose, Sucrose, Maltose. The protein content assessed by lowry's method (77.03±2.86mg/g) and specific activity 583. 47 HAU/mg was found to be high in *Cucumis melo Var Agrestis* lectin when compared to dye method. Lectin from *Cucumis melo Var Agrestis* possessed moderate erythrocyte membrane stability confirming its *invitro* anti-inflammatory and also good antioxidant potential showing IC50 value of 25.04µg/ml. Also showed stability to denaturing agent urea at 2M on heating, salts such as $FeCl_3$ at 100mM, Na_2So_4 at 25mM. SDS PAGE result showed band near 36KDa, 55KDa.

Key words: Anti-inflammatory, Antioxidant, Thin layer chromatography, SDS PAGE.

1.0 Introduction

Lectin is a carbohydrate binding protein of non immune origin, having the ability to agglutinate, glycoconjucates[1], which allows scientist to use lectin as a molecular tool for the study of glycoconjugates as well as in targeted drug delivery[2-3].Lectin having varied amino acid sequences givingdivergent structure and functions are found in microbes, animals, viruses, in higher plants[4]especially in seed cotyledons, cytoplasm/protein bodies,[5]stem, leaves, roots, bark, rhizomes, tubers, cereals. Lectin, similar to antibodies but differ in molecular weight, aminoacids, metals involvement, structure. Lectin binding exclusively to carbohydrate on the cell surface is reversible, non covalent, and exhibit more than two binding site, for agglutination reaction with carbohydrates of complex nature allowing attraction for different di, oligosaccharides, offering enormous beneficial properties such as anti-bacterial, anti-fungal, anti-tumor, anti-nematode, anti-viral, mitogenic activity. Lectin due to its adhesion, agglutination ability it plays a role in symbiotic, pathogenic interaction between microorganism-host and they escape acid denaturation, proteolysis [4]. Hence, for the present study lectin was extracted from *Cucumis melo Var Agrestis* seed in order to study its therapeutic property as it is commonly available weed in gardens.

2.0 METHODS

2.0.1 Sample collection

100gm of sample *Cucumis melo Var Agrestis* seed was purchased from the local market at Salem, Tamil Nadu, India. The seeds were separated and then washed and dried under shade which was ground to a powder and used for further experiment.

2.0.2 Lectin isolation

The *Cucumis melo Var Agrestis* seed lectin was isolated by soaking it overnight in 50mM phosphate buffered saline (pH 7.4). Next day homogenized in a homogenizer, filtered through double layered cotton cloth, centrifuged at 16,000 rpm for 30min.[6] Stored the supernatant for further work.

2.0.3 Protein assay

The lectin extracted was assessed for its protein content by Lowry and Dye method [7-8].To 10µl sample added 1ml alkaline copper reagent, incubated for 10 minutes, then added 0.1ml Folinciocalteu reagent, incubated at room temperature for 30-60min. and the color obtained was read at 660nm. For dye method, to 10µl sample added 5ml Coomassie brilliant blue dye, mix well and incubated for 5 minutes. Read the color developed at 595nm using spectrophotometer. BSA was used as a standard. From the calculated protein, specific activity was calculated.

2.0.4 Carbohydrate analysis

The total sugar content was performed by anthrone method[9]. The crude lectin was converted into its monomers on boiling (3hr) with 5ml 2.5N HCl, neutralized with alkali, centrifuged, supernatant was used for the analysis. To 10µl lectin added 3ml 0.2% Anthrone reagent, heated at 100°C for 7min., cooled the tubes and read at 630nm using spectrophotometer schimadzu UV 1800.Glucose was used as a standard.

2.0.5 Thin Layer Chromatogaphy

Thin layer chromatography was performed to study the protein and carbohydrate present in the isolated lectin. [10-11]Silica gel prepared was activated by heating at 110°C, then lectin extracted was loaded on the gel leaving 1cm from the bottom and allowed to run in a suitable solvent system for protein [Butanol (8): Glacial acetic acid(2): water(2)] and for carbohydrate [Butanol (4): Acetone(5): Phosphate buffer (1)] and after 30 minutes the plates were taken out and sprayed with ethanolic ninhydin for protein and with anisaldehyde for carbohydrate, dried, observed the spots obtained and calculated its Rf value by dividing the distance travelled by the sample with solvent front.

2.0.6 Red Blood Cell (RBC) Suspension Preparation

Human blood samples (2ml) from volunteer was collected in a tube,washed repeatedly using normal saline (0.9%) and prepared 2-5% erythrocyte suspension [12].Only freshly prepared suspension was used for the experiments. And animal blood sample was collected from the slaughter house early in the morning freshly on the day of experiment and processed similarly. Hemeagglutination assay was conducted with all human and animal blood samples and other parameters were done with human blood group O^+ RBC suspension alone.

2.0.7 Hemeagglutination assay

The hemeaggultination assay was performed by serially diluting lectin samples and agglutination was observed visually with carpet and button pattern after 1hr using 2-5% RBC suspension. Hemagglutination unit is defined as the last dilution that mediated agglutination. Specific activity is HAU per mg protein.

2.0.8 Hemeagglutination inhibition assay

Agglutination inhibition assay[13]was performed with sugars like glucose, sucrose, ribose, fructose, xylose, lactose, maltose, mannose. To equal volume lectin sample, added various sugar solutions at different concentrations, 2-5% RBC suspension was added and incubated for 1hr and examined for agglutination as well as its inhibition.

2.0.9 Effect of pH

The effect of pH was assessed by selecting pH range from 1 to 10 [14] using buffer solutions such as 0.1N HCL(pH 1), 0.2M Glycine HCL, sodium acetate and sodium phosphate, Tris HCL, glycine sodium hydroxide, carbonate- bicarbonate buffer (pH 2-10).To equal volume lectin sample added buffer solutions of varying pH range and tested for agglutination using 2-5% RBC suspension.

2.0.10 Effect of Temperature

The effect of temperature was assessed at different temperature ranging from 10° to 100°. The samples were subjected to heat at each particular temperature for 3hrs, and each sample was collected at regular intervals i.e every 30min. (data not shown) and centrifuged, supernatant was used for the agglutination assay using 2-5% RBC suspension.

2.0.11 Effect of urea

The effect of denaturing agent urea on lectin hemeagglutination was assessed in 2, 5, 8, 10M solution. Lectin extracted were assessed with and without heat. Equal volume of two different variants of samples were treated with respective concentration of urea, assessed for its hemeagglutination activity using 2-5% RBC suspension.

2.0.12 Effect of salt

Effect of salt [15] was assessed using $FeCl_3$, Na_2So_4, $CaCl_2$ at two different concentration 25 and 100mM. The test was performed similar to hemeagglutination test. Minimum concentration required for hemeagglutination was observed.

2.0.13 Anti-inflammatory Activity

The anti-inflammatory activity[16] was studied with O+ RBC suspension. To 10µl sample, 1ml phosphate buffer, 2ml hyposaline, 0.5ml RBC suspension was added, incubated for 15-30minutes, centrifuged for 5min. The supernatant was measured at 560nm using spectrophotometer. Diclofenac sodium served as a standard drug. Control was also developed simultaneously without sample. The RBC membrane stabilization in percentage was calculated as follows:Membrane stabilization (%)=100–[(O.D of drug treated sample/O.D of control) *100].

2.0.14 Antioxidant activity

Antioxidant activity was studied using DPPH[17].To various concentrations of lectin ranging from 5 to 500µg/ml, added 100µl of 0.01mM methanolic DPPH solution, incubated for 30 min. in dark at ambient temperature and the absorbance was recorded at 517nm using spectrophotometer. Ascorbic acid was used as a standard. Control was also maintained. The percentage of free radical scavenging activity was calculated by the formulae:[(Ac–As)/Ac]× 100.

2.0.15 SDS PAGE

SDS-PAGE was performed according to Laemmli [22] to determine the molecular weight of the protein samples. 50µl of protein sample was mixed with 2:1 ratio of sample buffer (Tris–HCl, pH 6.8 containing 2-mercaptoethanol, glycerol, bromophenol blue, 5% SDS) and heated at 90°C for 5min. Then, 25µl sample was loaded along with high molecular weight protein markers. Perform electrophoresis at 50mA in the beginning, later at 100mA. Stain the bands with Coomassie brilliant blue R250. The molecular weights were determined by comparison with standard protein markers.

Statistical tool

All Quantitative experiments were done thrice, and measurements were taken using Schimadzu UV spectrophotometer. Control was maintained for all experiments. Standard deviation was calculated.

2.1 RESULTS AND DISCUSSION

2.1.1 Nutrient assay

Table.1 Protein, Carbohydrate by TLC, Quantitative analysis of carbohydrate in *Cucumis melo Var Agrestis* seed lectin

Lectin Source	Nutrient	Results
Cucumis melo Var Agrestis seed lectin	Protein (TLC) Rf Value	0.77±0.13
	Carbohydrate(TLC) Rf Value	0.73±0.14
	Carbohydrate(Anthrone method) mg/g	18.37±2.69

Values are Mean ± SD for Three experiments

Protein and carbohydrate content in *Cucumis melo Var Agrestis* seed lectin by thin layer chromatography (Table.1) showed the presence of protein and carbohydrate in the extracted lectin. Rf value reported was 0.77±0.13 for protein, for carbohydrate 0.73±0.14. The quantitative estimation of carbohydrate by anthrone method was found to be 18.37±2.69.

Table.2 Protein profile of *Cucumis melo Var Agrestis* seed lectin

Lectin Source	Total protein (mg/g)		Total HAU	Specific activity (HAU/mg protein)		Purification fold/ Yield(%)
	Lowry method	Dye method		Lowry method	Dye method	
Cucumis melo Var Agrestis seed lectin	7.03±2.86	11.81±1.41	512	583.47	310.30	1/100

Values are Mean ± SD for Three experiments

Table.2 shows the protein profile in *Cucumis melo Var Agrestis* seed lectin. The protein content tested by lowry method was found to be 7.03±2.86, while by dye method it was 11.81±1.41, 11.2.The specific activity calculated was 583.47/310.30HAU/mg. The purification fold and yield is 1%.

2.1.2 In vitro Erythrocyte membrane stabilization test

Table.3 Anti-inflammatory activity of *Cucumis melo Var Agrestis* seed lectin

Lectin Source /Standard	**Antiinflammatory activity (%)**
Cucumis melo Var Agrestis seed lectin	37.14 ±0.00
Diclofenac sodium (Standard)	100.00±0.00

Values are Mean ± SD for Three experiments

The lysosomal enzyme released on inflammation bring out several acute or chronic inflammatory disorders. Anti-inflammatory drugs act both by inhibiting lysosomal enzymes as well as by stabilizing the lysosomal membrane. Since human red blood cell membrane is comparable to lysosomal membrane, this study was performed to test the human red blood cell membrane stability by *Cucumis melo Var Agrestis* seed lectin. The anti – inflammatory activity observed was 37.14±0.00%. (Table.3).

2.1.3 Hemeaagglutination assay

Table.4 Hemeagglutination assay of *Cucumis melo Var Agrestis* seed lectin in human and animal erythrocytes

Lectin Source	**Blood group**	**2^1**	**2^2**	**2^3**	**2^4**	**2^5**	**2^6**	**2^7**	**2^8**	**2^9**	**2^{10}**	**2^{11}**	**2^{12}**	**2^{13}**	**2^{14}**	**2^{15}**	**2^{16}**
Cucumis melo Var Agrestis seed lectin	O^+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
	A+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AB+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	Cow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Goat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ Hemeagglutination - No Hemeagglutination

Table 4 shows the results of blood group specificity of *Cucumis melo Var Agrestis* seed lectin by hemagglutination assay. The hemagglutination activity assay was assessed using human O^+,A^+,B^+,AB^+as well as using Cow, Goat, Pig red blood cell suspension. *Cucumis melo Var Agrestis* seed lectin was found to be specific to O+ and AB+ blood group and showed non-specificity to human as well as to cow, goat, pig blood. The non specificity observed with the lectin might be due to non treatment of erythrocytes with proteases.

2.1.4 Hemeagglutination Inhibition assay

Table.5 Hemeagglutination inhibition assay of *Cucumis melo Var Agrestis* seed lectin

Lectin Source	**Sugars**	**10mM**	**20mM**	**50mM**	**100mM**	**200mM**
Cucumis melo Var Agrestis seed lectin	Glucose	+	+	+	+	+
	Sucrose	-	+	-	-	-
	Ribose	+	-	-	-	-
	Fructose	-	-	-	-	-
	Xylose	-	-	-	-	-
	Lactose	-	-	-	-	-
	Maltose	-	-	-	-	+
	Mannose	-	-	-	-	-

+Hemeagglutination, - Hemeagglutination Inhibition

Table. 5 shows the results of sugar binding specificity of *Cucumis melo Var Agrestis* seed lectin through hemeagglutination inhibition assay with sugars. Among the sugars tested, hemeagglutination inhibition was observed with glucose at 10, 20, 50, 100, 200mM concentration, and for sucrose at 20mM concentration and for ribose at 10mM, for maltose at 200mM concentration respectively. All the remaining sugars were not showing hemeagglutination inhibition at the concentration studied. The reduced sugar binding ability of lectin might be due to demetalization.

2.1.5 Effect of pH

Table.6 pH stability of *Cucumis melo Var Agrestis* seed lectin

Lectin Source	**pH**									
Cucumis melo Var Agrestis seed lectin	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
+	-	-	-	-	-	-	-	+	-	-

Hemeagglutination Inhibition, + Hemeagglutination

Table.6 shows the pH stability of *Cucumis melo Var Agrestis* seed lectin. The pH range selected for the study was pH 1 to 10: pH 1- 0.1N HCL and 0.2M for the remaining pH 2 to 10. Except pH 8 all the pH showed hemeagglutination inhibition at the tested molarity by *Cucumis melo Var Agrestis* seed lectin. The absence of hemeagglutination at the studied pH might be due to the less favorable condition either more acidic or more alkaline.

2.1.6 Effect of Temperature

Table.7 Effect of Temperature on *Cucumis melo Var Agrestis* seed lectin

Lectin Source	**Temperature in °**								
Cucumis melo Var Agrestis seed lectin	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**100**
	+	-	-	-	-	-	-	-	+

-Hemeagglutination Inhibition, + Hemeagglutination

Table.7 shows the effect of temperature on *Cucumis melo Var Agrestis* seed lectin . It was studied by heating at various temperature for and the results were recorded. The results depicts that *Cucumis melo Var Agrestis* seed lectin showed hemeagglutination at 10°C, 100°C and hence found to be thermostable. Absence of hemeagglutination was mainly due to its sensitivity.

2.1.7 Effect of urea

Table. 8 Effect of Denaturing agent Urea on *Cucumis melo Var Agrestis* seed lectin

Lectin Source	**Crude lectin heated**				**Crude lectin Not Heated**			
	2M	**5M**	**8M**	**10M**	**2M**	**5M**	**8M**	**10M**
Cucumis melo Var Agrestis seed lectin	+	-	-	-	-	-	-	-

- Hemeagglutination Inhibition, +Hemeagglutination

Table.8 shows the results of effect of denaturing agent urea on *Cucumis melo Var Agrestis* seed lectin by heating as well as by not heating for hemeagglutination. *Cucumis melo Var Agrestis* seed lectins showed hemeagglutination at 2M urea on heating. Whereas, lectins not subjected to heat showed no hemeagglutination at studied concentration.

2.1.8 Effect of salt

Table.9 Effect of various salts on *Cucumis melo Var Agrestis* seed lectin

- Hemeagglutination Inhibition, + Hemeagglutination

Lectin Source	**Salt**	**2^1**	**2^2**	**2^3**	**2^4**	**2^5**	**2^6**	**2^7**	**2^8**	**2^9**	**2^{10}**	**2^{11}**	**2^{12}**	**Lectin activity HAU**
Cucumis melo Var Agrestis seed lectin	$FeCl_3$ (25mM)	-	-	-	-	-	-	-	-	-	-	-	-	
	$FeCl_3$ 100mM	-	-	-	-	-	+	-	-	-	-	-	+	64/4096
	Na_2SO_4 25mM	-	-	-	-	-	-	-	-	-	-	-	+	4096
	Na_2SO_4 100mM	-	-	-	-	-	-	-	-	-	-	-	-	-
	$CaCl_2$ 25mM	-	-	-	-	-	-	-	-	-	-	-	-	-
	$CaCl_2$ 100mM	-	-	-	-	-	-	-	-	-	-	-	-	-

Table. 9 shows the result of $FeCl_3$, Na_2SO_4, $CaCl_2$ on *Cucumis melo Var Agrestis* seed lectin at two different concentration i.e 25, 100mM. $FeCl_3$ at 100mM showed lectin activity at 64/4096 HAU. While for Na_2SO_4 hemeagglutination was observed at 25mM and lectin activity was found to be 4096 HAU. All the remaining concentration of the respective salt showed no hemeagglutination.

2.1.9 Antioxidant activity

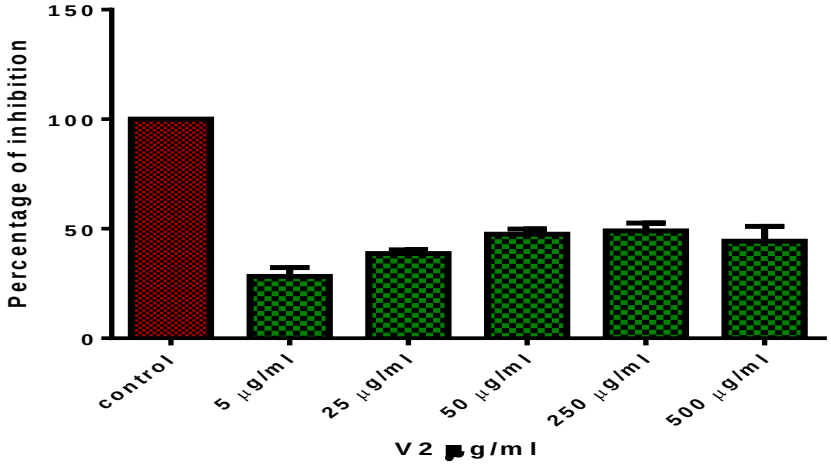

Fig. 1 DPPH antioxidant activity of *Cucumis melo Var Agrestis* seed lectin

Fig.1 shows the free radical scavenging ability of *Cucumis melo Var Agrestis* seed lectin (labelled as V2) at 5- 500µg/ml against methanolic DPPH. The percentage of inhibition observed for ascorbic acid was 100% for *Cucumis melo Var Agrestis* seed lectin it was found to be 28.28, 38.63, 47.50, 49.04, 44.29. IC50 for *Cucumis melo Var Agrestis* seed lectin was 25.04 µg/ml. DPPH becomes stable by accepting an electron/hydrogen radical, its oxidization is not easy and an irreversible process. The strong absorption at 517nm is due to its odd electron 1,1-diphenyl-2-picryl hydrazyl group maintains a deep violet color. On pairing of this odd electron, the color changes to yellow and the color change depends on the number of electrons taken up. This confirms the antioxidant reaction/scavenging efficacy of the lectin with DPPH, its conversion into 1,1-diphenyl-2-picryl hydrazine. This antioxidant activity helps in the damage reversal.

2.1.10 SDS PAGE

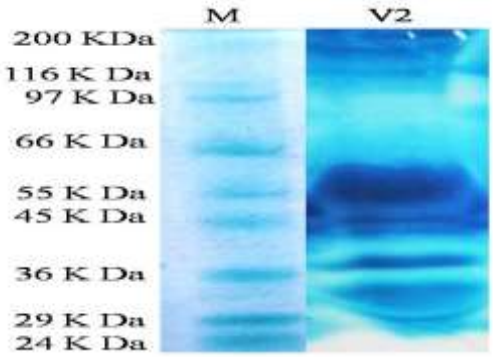

Fig. 2 SDS PAGE of *Cucumis melo Var Agrestis* seed lectin

Fig.2 shows the SDS PAGE results of *Cucumis melo Var Agrestis* seed lectin. The Band was observed near 36KDa, 55KDa. This confirms the presence of lectin protein.

3.0 CONCLUSION

The results of present study showed, that *Cucumis melo Var Agrestis* seed lectin could agglutinate type O+, AB+ human erythrocytes. It exhibited specificity to glucose, ribose maltose. pH stability was noticed with pH8, resistant to temperature, urea might be due the presence of divalent cation present in it and showed stability to $FeCl_3$, Na_2SO_4 salts. Possess good antioxidant, moderate membrane stabilization. SDS PAGE showed protein comprising of 36KDa, 55KDa. Hence, it is concluded that it could be studied futher for its specific therapeutic application related to anticancer activity.

Conflict of interest

The author declares no conflict of interest

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