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Identification and Characterization of Naturally Occurring Agglutinin from the Flower of *Ipomoea pes-caprae*

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ABSTRACT

Naturally occurring agglutinin was detected in the crude extract ofthe *Ipomoeabiloba* (*Ipomoea pes-caprae*) flower. The agglutinin agglutinated different mammalian erythrocytes but showed high specificity towards rabbit erythrocyte. Physico-chemical analysis of flower agglutinin revealed that it was stable to a wide range of pH and temperature, dependant on calcium and sensitive to calcium chelators. Enzyme treatment of erythrocytes showed an increased HAtitre with trypsin, slight decrease with neuraminidase and remained unaffected with neutral protease. Hem agglutination inhibition assay document edlac to ferrin among glycoprotein's and D-mannose among sugars as the potent inhibitor. The cross adsorption assay with pre-adsorbed erythrocytes suggested the presence of a single agglutinin. Thus, this preliminary characterization of agglutinin of *Ipomoea pes-caprae* flower would provide strategy for the purification of a lectin and assess its therapeutic value.

KEY WORDS: Agglutinin, calcium chelators, erythrocytes, glycoprotein's, *Ipomoea pes-caprae.*

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INTRODUCTION

Agglutinin/Lectins are proteins that are found in all living organisms and have the ability to bind carbohydrates[1]. These agglutinins have mono/multivalent binding sites that agglutinated red blood cells and have high specificity to particular carbohydrate. Lectins recognize glycoconjugates like glycoproteins or glycolipids and mono-oligo or poly saccharrides on the cell surface[2]. Hence, they play an important role in identification of different blood groups[3].

Lectins are diversified group of proteins based on their size, aminoacid composition and structure with many biological properties[4]. Lectins were isolated from plants and their various tissues[5]. Investigation of lectin activities on various plant tissue or organs like seeds, leaves, stems, flowers are reported by Kauss (1981). Lectins are abundant in vegetative plants and these plant lectins are mostly secretory proteins[6]. Biological function of plant lectins are divided as internal and external. External functions as protection of plants from insects and fungi where as internally storage of carbohydrate and enzymatic activity[7]. The specific physiological role of plant lectin is providing defense to the plant and they are a heterogeneous group of protein that show variation in biological activities, carbohydrate binding specificity and their molecular structure[8].

*Ipomoea pes-caprae*belongs to Convolvulaceaefamily which have been recorded with plants containing various medicinal properties. *Ipomoea nil*possesseshepato protective[9], the leaves of *Ipomoea batatas*– anticancer[10], anti-inflammatory and anti hyperycomia[11]properties. *Ipomoea pes-caprae* is widely used in traditional medicine. The plant is mucilaginous and is considered astringent, tonic, alterative, diuretic and purgative[12], to cure tumours and rheumatic pains[13], digestive disorders, kidney ailments, internal pains, insulinogenic, and possess hypoglycaemic activity[14].Many of the medicinal herbs are proved to possess agglutinin[15]. It is reported thatlectins have been isolated from *Convolvulus arvensis, Ipomoea batatas* and *Calystegiasepium*[8]. Among the Convolvulaceae family genus *Ipomea* has been less studied for lectins. The *Ipomoea pes-caprae* plant was previously studied by different investigators on its phytochemical composition but the presence of agglutinins has not been investigated. Hence, the purpose of this study was to identify and characterize agglutinin/lectin present in the flower extract of *Ipomoea pes-caprae*.

MATERIALS AND METHODS

Collection of Ipomoea pes-caprae

Plants were collected from the Anjugramam sea shore. The plant was authenticated by Dr. P. Nagerndra Prasad, Head, Department of Biotechnology, Sri Paramakalyani College, Alwarkurichi, Tirunelveli.

Preparation of samples

Stem, leaves and flowers were washed with distilled water, ground and homogenized using a mortar and pestle. Then, the homogenate was filtered using Whatmanfilter paper and centrifuged at 3000 rpm for 10 minutes. The crude extract was analyzed for the presence of hem agglutinin either immediately or stored at -20^{o}C for further study.

Preparation of erythrocyte suspension

Blood samples of human A, B, AB, O were collected from Thangam Blood Bank. Rabbit blood were collected by vein puncture method, rat by heart puncture,pig, buffalo, cow and goat were collected from slaughter house directly in modified Alseivier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulfate (100 µg/ml) and chloramphenicol (330 µg/ml). They were suspended and washed three times with ten volumes of Tris-buffered saline (TBS) pH 7.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM) and suspended in the same as 1.5% suspension[16].

Hem agglutination assay

The crude extract of the flower *Ipomoea pes-caprae* was assayed for the presence of agglutinins using TARSON 96 well U-bottom microtitre plates. The sample was diluted by two-fold dilution in TBS (25 µl) and incubated with 1.5% suspension of RBCs (25 µl) at room temperature (30±2ºC) for an hour or until the negative control showed a red button formation. Agglutination activity was detected based on the RBCs appearance on the well: a positive result appear as a red-carpet layer, while negative results appear as a red button in the bottom of the well[16].

Biochemical Assay

Water[17], calcium[18], lipid and protein[18, 19] content of the extract were estimated.

Effect of pH and temperature on hemagglutinin activity

pH dependence of flower agglutinin was tested at different pH levels (5 to 11) and the effect of temperature on agglutinin was studied by incubating the crude extract at different temperature (0ºC to 100ºC) for an hour before adding erythrocytes.

Effect of divalent cations and chelators on hemagglutination activity

To assess the effect of cations/chelators on HA activity of the crude extract, the extract was serially diluted with 25 µl of TBS with different concentration of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) and

chelators (EDTA and trisodium citrate) and was incubated at room temperature (30±2ºC) for an hour prior to the addition of rabbit erythrocytes and the hemagglutinationtitre was determined.

Effect of enzyme treated rabbit erythrocytes on heamagglutination of crude flower extract of Ipomoea pes-caprae

Protease treated erythrocytes were prepared following the method of[20]and, asialo-erythrocytes were prepared following the method of[21].

Hemagglutination of flower extract of Ipomoea pes-caprae after adsorption with different erythrocytes

To 1 ml each of washed and packed rabbit, rat, human B, Human O and rat erythrocytes, 1 ml of crude flower extract of *Ipomoea pes-caprae* was added and mixed well. This crude flower extract erythrocyte mixture was incubated at 10ºC overnight (18 hours) with gentle occasional shaking. After centrifugation, the supernatant was tested against the selected erythrocytes for hem agglutination assay.

Hem agglutination Inhibition assay

To a known concentration of serially diluted inhibitor (sugars/glycoproteins) solution (25 µl), 25 µl of the extract of the whole body diluted to sub agglutination concentration was added, mixed and the plate was incubated for 1 hour at room temperature. Finally 25 µl of 1.5% rabbit erythrocytes suspension was added and incubated for 1 hour at room temperature (30±2ºC). The minimum concentration of the inhibitors required to completely block the agglutination after 1 hour of incubation at room temperature (30±2ºC) was reported as the HAI titre.

RESULTS

Hem agglutinability of the crude extract

Survey of crude agglutinin of various parts of plant *Ipomoea pes-caprae*like stem, leaf and flower were carried out with different mammalian erythrocytes and given in Table 1. The rabbit erythrocytes gave maximum HA titre of 1024 in flower extract followed by rat erythrocytes showed HA titre 512. Erythrocytes like Buffalo, Goat, Cow and Mice show HAtitre 2. The agglutinins failed to agglutinate Human A, B and AB erythrocytes.

Table 1: Hem agglutinationtitre of naturally occurring extract of *Ipomoea pes-caprae*with different mammalian erythrocytes

Erythrocytes(n = 10)	HA Titre		
	Flower	Stem	Leaf
Rabbit	1024	512	512
Rat	512	128	256
Dog	128	32	32
Human O	64	8	16
Mice	16	4	4
Human A	2	2	2
Human B	2	2	2
Buffalo	2	2	2
Goat	2	0	0
Cow	2	0	0
Human AB	0	0	2

Analysis of Biochemical factors

Bio-chemical factor like protein, water, calcium and lipid were examined. The crude flower extract contains 91.3% of water, 27.1 mg/ml of protein and 0.41 mM of calcium. The total lipid present is trace amount nearly 0.027 mg/ml (Table 2).

Table :2 Biochemical content of flower agglutinin of *Ipomoea pes-caprae*

Characteristics analysed	Quantity
Water (%)	**91.3±0.43**
Total Protein (mg/ml)	**27.1±0.32**
Total Lipid	**0.027±0.34**
Total Calcium (mM)	**0.41±0.26**
HA titre	**1024**

Effect of pH and Temperature on HA

The pH activity of flower agglutinin is maximum between pH 6.5 and 9.5 and it gradually decreased above 10 and below 6.5. The hemagglutination activity was stable from 20^{o}C to 50^{o}C and it decreased gradually above 60^{o}C and completely loss above 100^{o}C (Table 3).

Table 3: Hemagglutinationtitreof flower agglutinin of Ipomoea pes-caprae in relation to change in pH and Temperature

pH(n= 5)	HA Titre	Temperature	HA Titre
5.0	512	0	1024
5.5	512	10	1024
6.0	512	20	1024
6.5	1024	30	1024
7.0	1024	40	1024
7.5	1024	50	1024
8.0	1024	60	1024
8.5	1024	70	512
9.0	1024	80	256
9.5	1024	90	128
10.0	512	100	32
10.5	512	-	-

Effect of cations and calcium chelators on HA

Addition of divalent cations (Mg^{2+} and Mn^{2+}) slightly reduces the HA activity and Ca^{2+} showed an increase in HA titre. A decrease in HA titre is observed with increasing in concentration of all three cations (Table 4). Maximum hemagglutination was observed in the presence of 1-5mM of Di and tetra sodium EDTA and trisodium citrate. Whereas an increase in concentration of Disodium EDTA showed a sudden decrease in HA titre and in higher concentration similar impact of decrease is observed in all the three chelators (Table 4).

Table 4: Effect of divalent cations and Calcium Chelators on the hemagglutination of flower agglutinin of *Ipomoea pes-caprae*

Concentration (mM) (n=5)	HA Titre					
	Cation			Chelator		
	Ca^{2+}	Mg^{2+}	Mn^{2+}	EDTA		Trisodium Citrate
				Disodium	Tetrasodium	
0	512	512	512	512	512	512
0.01	512	512	512	1024	512	512
0.1	1024	256	256	1024	1024	512
1	1024	256	256	1024	1024	1024
5	1024	128	256	1024	1024	1024
10	1024	64	128	256	1024	1024
20	1024	32	128	128	1024	512
30	512	32	64	16	512	128
40	256	32	64	8	256	128
50	128	32	32	2	2	4
100	64	32	32	0	0	0

Haemagglutination Inhibition assay

Heamagglutination inhibition of *Ipomoea pes-caprae* flower agglutinin with rabbit erythrocytes was highly inhibited by glycoprotein lactoferrin, moderately inhibited apotransferrin and weakly inhibited by fetuin and transferrin(Table 5). Among the sugars tested D-mannose extremely inhibited the *Ipomoea pes-caprae* flower agglutinin followed by N-acetyl mannosamine, sucrose and D-glucosamine and weakly inhibited by L-fucose (Table 5).

Table 5: Hemagglutination inhibition (HAI) titre of flower agglutinin of *Ipomoea pes-caprae* by various Sugars and Glycoproteins

Inhibitors (Sugars/Glycoproteins) (n=3)		HAI titer	Minimum Conc. Required (mM) / (mg/ml)	Relative inhibitory potency (%)
Sugars	D-Mannose	1024	0.098	100
	ManNAc	512	0.195	50
	Sucrose	512	0.195	50
	D-Glucosamine	128	0.781	12.5
	L-Fucose	16	6.25	1.562
	α-Lactose	4	25	0.391
Glycoproteins	Lactoferrin	128	39.06	100
	Apotransferrin	64	78.12	50
	Fetuin	32	156.25	25
	Transferrin	8	625	6.25

Effect of enzymes on HA

The crude flower agglutinin of *Ipomoea pes-caprae* showed a tremendous increase in HA titre with trypsin treated rabbit erythrocytes and slightly decreases in neuraminidase treated erythrocytes. The HA activity was unaffected with protease treated erythrocytes (Table 6).

Table 6: Effect of enzyme treated rabbit erythrocytes on heamagglutination of flower agglutinin of *Ipomoea pes-caprae*

Enzymes(n=5)	**HA Titre**
None	1024
Trypsin (1 mg/ml)	8192
Neutral protease (0.25 mg/ml)	1024
Neuraminidase	512

Cross Absorption assay

The cross adsorption profile showed that the *Ipomoea pes-caprae* flower extract was adsorbed to particular erythrocytes species and it failed to agglutinate the erythrocytes of other species. It revealed the presence of single lectin (Table 7).

Table 7: Hemagglutination of flower agglutinin of *Ipomea pes-capre* after adsorption with different erythrocytes

Erythrocyte adsorbed (n=3)	HA Titre						
	Rabbit	**Rat**	**Human A**	**Human B**	**Human O**	**Dog**	**Mice**
None	1024	512	2	2	64	128	16
Rabbit	0	0	0	0	0	0	0
Rat	32(0)	0	0	0	0	0	0
Human A	16 (0)	0	0	0	0	0	0
Human B	16 (0)	0	0	0	0	0	0
Human O	4 (0)	0	0	0	0	0	0
Dog	16 (0)	0	0	0	0	0	0
Mice	4(0)	0	0	0	0	0	0

DISCUSSION

This study focuseson the preliminary characterization of lectin distributed in different plant parts of *Ipomoea pes-caprae*. The presence of naturally occurring agglutinin inflower, stem and leaves showed variations in hemamagglutinationtitre with different mammalian erythrocytes.Among various erythrocytes tested, the agglutinin of *Ipomoea pes-caprae*showedmaximum hemagglutination with rabbit, rat and dog erythrocytes, moderate affinity towards human O and mice and very poor affinity to human A and B, buffalo, goat, cow and human AB erythrocytes.Agglutinating molecules contain one or more combining sites that enables them to agglutinate more than one erythrocyte or a cell at a time[22]. The quantitative distribution of lectin differs in different parts of plants[23].Evaluation of various plant parts like stem, leaf and flower of *Ipomoea pes-caprae*revealed the flower to possess high agglutinin activity, hence the further analysis was carried out with flower agglutinin.

Biochemical compontents like protein, calcium, lipid and water content were analyzed[24] and maximum protein was observed correlating to the high HA titre of the flower extract. The physico-chemical characterization of *Ipomoea pes-caprae* agglutinin revealed that the agglutinin was sensitive to pH and temperature. The HA titre was stable between pH 6.5 and 9.5 and decreased above pH 10 and below 6.5.The pH stability of plant lectins varies. It is reported that *Ipomoea asarifolia*[25] leaf lectins are stable at pH 7.5. Lectins are thermo sensitive and temperature may change their activity[26]. The flower agglutinin was stable from 0ºC to 50ºC and decreased above 60ºC. Hem agglutination activity of the extract of the flower *Sansevieriaroxburghiana* was stable from 0ºC to 45ºC and gradually reduced above 55ºC[27]. Temperature sensitivity of plantlectin can be attributed to their role in plant defense against environmental stress[28].

The HA activity of flower agglutinin increased on addition of Ca^{2} but no changes were observed with Mg^{2+} and decreased activity was observed with increased concentration of cations indicating that the agglutinin is cation dependent. The decrease in HA activity observed with the treatment of EDTA and trisodium citrate confirms the flower agglutinin to be calcium dependent. The trypsin treated cells showeda notable increase in HA activity whereas protease treated erythrocytes remained same and the neuraminidase enzyme treated cells slightly reduceed the HA activity.Addition of protenase enzyme removes the cell surface proteins that mask the agglutinin binding sites whiletrypsin cleaves the residues in protein chain[29] and it increases the erythrocyte agglutination.

Binding specificity of *Ipomoea pes-caprae* flower agglutinin was analyzed by hapten inhibition assay using glyco protein and sugars. It revealed lactoferrin as a potent inhibitor when compared to apotransferrin, fetuin and transferrin. Lactoferrin consists of a single chain and has two

glycan attached to N-glycosidic linkage. It contains mannose, galactose, fucose, N-acetyl neuraminic acid and N-acetyl galactosamine[30]. The specificity of lectin to sugars revealed that D-mannose, N-acetyl mannosamine and sucrose has high inhibition and D-glucosamine, L-fucose and α-lactose weakly inhibits the flower lectin. This inhibitory effect of sugar can be attributed to their ability to compete for binding sites on the lectin molecule, which can interfere with the attachment of the lectin to sugar units on the surface of the erythrocytes[31]. Mannose – binding lectin (MBL) is a group of III type lectin[32] that can recognize carbohydrates like mannose, glucose, fucose and N-acetyl mannosamine[33]. Based on the hapten inhibition studies, it can be inferred that the lectin may be a mannose specific lectin which also may possess sialic acid specificity. *Galanthusnivalis* agglutinin readily agglutinates rabbit erythrocytes and exhibits exclusive specificity towards mannose[34]. D-mannose specific lectin was isolated from *Aloe aristata haw.*flower[35]. Crude protein extract of seed from *Centrolobiummicrochaete* showed agglutination against rabbit erythrocytes and was strongly inhibited by D-mannose[36].

The cross adsorption assay of flower agglutinin revealed the presence of a single lectin as it completely removed the HA activitywith the tested erythrocytes.Serological studies have shown that agglutinability of the agglutinin to one type of erythrocyte can be adsorbed by that type of erythrocyte, leaving residual agglutinating activity to other type of erythrocyte[37].

Mannose specific lectins are described as innate host defense molecule and these agglutinins are also used as passive immunotherapy to prevent infection[38].Since they have high specificity it has become an important tool for potent antiviral[39] and anti-insect properties.

CONCLUSION

This study provides all preliminary data for further purification of D-Mannose specific lectin from *Ipomoea pes-caprae* flower using affinity column chromatography and the agglutinin of *Ipomoea pes-caprae* flower can be studied for its pharmacological importance.

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