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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 of the *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 HA titre 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<sup>1</sup>. 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<sup>2</sup>. Hence, they play an important role in identification of different blood groups<sup>3</sup>.

Lectins are diversified group of proteins based on their size, aminoacid composition and structure with many biological properties<sup>4</sup>. Lectins were isolated from plants and their various tissues<sup>5</sup>. 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<sup>6</sup>. 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<sup>7</sup>. 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<sup>8</sup>.

*Ipomoea pes-caprae* belongs to Convolvulaceae family which have been recorded with plants containing various medicinal properties. *Ipomoea nil* possesses hepato protective<sup>9</sup>, the leaves of *Ipomoea batatas*– anticancer<sup>10</sup>, anti-inflammatory and anti hyperlycomia<sup>11</sup> properties. *Ipomoea pes-caprae* is widely used in traditional medicine. The plant is mucilaginous and is considered astringent, tonic, alterative, diuretic and purgative<sup>12</sup>, to cure tumours and rheumatic pains<sup>13</sup>, digestive disorders, kidney ailments, internal pains, insulinogenic, and possess hypoglycaemic activity<sup>14</sup>. Many of the medicinal herbs are proved to possess agglutinin<sup>15</sup>. It is reported that lectins have been isolated from *Convolvulus arvensis*, *Ipomoea batatas* and *Calystegia sepium*<sup>8</sup>. 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 Whatman filter paper and centrifuged at 3000 rpm for 10 minutes. The crude extract was analyzed for the presence of hemagglutinin either immediately or stored at -20°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 Alsevier'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<sub>2</sub> 10 mM) and suspended in the same as 1.5% suspension<sup>16</sup>.

### ***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±2°C) 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<sup>16</sup>.

### ***Biochemical Assay***

Water<sup>17</sup>, calcium<sup>18</sup>, lipid and protein<sup>18, 19</sup> 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 (0°C to 100°C) 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<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>) and

chelators (EDTA and trisodium citrate) and was incubated at room temperature ( $30\pm 2^{\circ}\text{C}$ ) for an hour prior to the addition of rabbit erythrocytes and the hemagglutination titre was determined.

### ***Effect of enzyme treated rabbit erythrocytes on hemagglutination of crude flower extract of *Ipomoea pes-caprae****

Protease treated erythrocytes were prepared following the method of<sup>20</sup> and, asialo-erythrocytes were prepared following the method of<sup>21</sup>.

### ***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  $10^{\circ}\text{C}$  overnight (18 hours) with gentle occasional shaking. After centrifugation, the supernatant was tested against the selected erythrocytes for hemagglutination assay.

### ***Hem agglutination Inhibition assay***

To a known concentration of serially diluted inhibitor (sugars/glycoproteins) solution (25  $\mu\text{l}$ ), 25  $\mu\text{l}$  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  $\mu\text{l}$  of 1.5% rabbit erythrocytes suspension was added and incubated for 1 hour at room temperature ( $30\pm 2^{\circ}\text{C}$ ). The minimum concentration of the inhibitors required to completely block the agglutination after 1 hour of incubation at room temperature ( $30\pm 2^{\circ}\text{C}$ ) 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 HA titre 2. The agglutinins failed to agglutinate Human A, B and AB erythrocytes.

**Table 1: Hem agglutination titre 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°C to 50°C and it decreased gradually above 60°C and completely loss above 100°C (Table 3).

**Table 3: Hemagglutination titre of 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)<br>(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

Haemagglutination 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 focuses on the preliminary characterization of lectin distributed in different plant parts of *Ipomoea pes-caprae*. The presence of naturally occurring agglutinin in flower, stem and leaves showed variations in hemagglutination titre with different mammalian erythrocytes. Among various erythrocytes tested, the agglutinin of *Ipomoea pes-caprae* showed maximum 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<sup>22</sup>. The quantitative distribution of lectin differs in different parts of plants<sup>23</sup>. 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 components like protein, calcium, lipid and water content were analyzed<sup>24</sup> 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*<sup>25</sup> leaf lectins are stable at pH 7.5. Lectins are thermo sensitive and temperature may change their activity<sup>26</sup>. The flower agglutinin was stable from 0°C to 50°C and decreased above 60°C. Hem agglutination activity of the extract of the flower *Sansevieria roxburghiana* was stable from 0°C to 45°C and gradually reduced above 55°C<sup>27</sup>. Temperature sensitivity of plant lectin can be attributed to their role in plant defense against environmental stress<sup>28</sup>.

The HA activity of flower agglutinin increased on addition of Ca<sup>2+</sup> but no changes were observed with Mg<sup>2+</sup> 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 showed a notable increase in HA activity whereas protease treated erythrocytes remained same and the neuraminidase enzyme treated cells slightly reduced the HA activity. Addition of protease enzyme removes the cell surface proteins that mask the agglutinin binding sites while trypsin cleaves the residues in protein chain<sup>29</sup> 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<sup>30</sup>. The specificity of lectin to sugars revealed that D-mannose, N-acetyl mannosamine and sucrose has high inhibition and D-glucosamine, L-fucose and  $\alpha$ -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<sup>31</sup>. Mannose – binding lectin (MBL) is a group of III type lectin<sup>32</sup> that can recognize carbohydrates like mannose, glucose, fucose and N-acetyl mannosamine<sup>33</sup>. 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<sup>34</sup>. D-mannose specific lectin was isolated from *Aloe aristata haw.flower*<sup>35</sup>. Crude protein extract of seed from *Centrolobiummicrochaete* showed agglutination against rabbit erythrocytes and was strongly inhibited by D-mannose<sup>36</sup>.

The cross adsorption assay of flower agglutinin revealed the presence of a single lectin as it completely removed the HA activity with 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<sup>37</sup>.

Mannose specific lectins are described as innate host defense molecule and these agglutinins are also used as passive immunotherapy to prevent infection<sup>38</sup>. Since they have high specificity it has become an important tool for potent antiviral<sup>39</sup> 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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