Material and Methods
3. MATERIAL AND METHODS

3.1. Screening of wild pulses for stored-pulse insect resistance:

3.1.1. Collection of seeds of wild pulses:

The Indian wild pulse varieties (Fig. 1) such as *Vigna umbellata* (rice bean - red), *Vigna umbellata* (rice bean - yellow) and *Vigna aconitifolia* (mat bean) were collected from National Pulses Research Centre (NPRC), Vamban, Pudukottai. *Lablab purpureus* (field bean - brown) was collected from Palani Hills, Kodaikanal. *Canavalia virosa* (jack bean) and *Mucuna pruriens* (velvet bean) were collected in and around Chennai. *Phaseolus* sp. (kidney bean) was collected from Dharmapuri, Tamil Nadu. Collected seeds of pulse varieties were brought to the laboratory, shade dried, cleaned and stored at room temperature (26 ± 2 °C). These pulses were then used for further experiments.

3.1.2. Stock culture of stored product insect pest, *Callosobruchus maculatus*:

The culture of bruchid beetle, *Callosobruchus maculatus* were raised from infested seeds collected at warehouses and maintained throughout the study period for insect bioassay experiments. The adult bruchids of both the sexes were reared on a most susceptible cultivated seeds of cowpea, *Vigna unguiculata* (approximately 150 g of pulses) obtained from local market in glass jars (dimension - 6.5 x 17 cm). These jars were covered with muslin cloth tightly and maintained under controlled laboratory conditions (26 ± 2 °C and 60 ± 5% RH) with natural photoperiod. The adult emergence from the stock cultures was monitored daily and freshly emerged adult insects were used for the bioassay experiments.
3.1.3. Insect feeding bioassay:

The collected seeds of various wild pulses were subjected to insect feeding bioassay (insect infestation study) to assess the nature of resistance or susceptibility of these wild pulses. The period of one complete life cycle from oviposition to adult emergence for the stored product insect pest, *Callosobruchus maculatus* was estimated approximately as 27 to 30 days. Five pairs of freshly emerged adult insects of *C. maculatus* (both sexes) were introduced into plastic containers with 100 g of each pulse variety. Five replicates were maintained for each pulse variety. After 5 days, the numbers of eggs laid on the seeds of each variety were counted. Such seeds were observed for a maximum duration of 60 days and the numbers of emerging adults were counted for a duration of 8 days with adults being isolated to prevent further breeding. Developmental period, percentage adult emergence and percentage loss in weight [(initial - final)/initial seed weight x 100] were calculated to evaluate developmental performance (infestation potential) of *C. maculatus* on various wild seeds of pulses. A stock culture of *C. maculatus* was reared on the seeds of cowpea *Vigna unguiculata* an established susceptible variety in glass jars covered with muslin cloth that served as control.

3.2. Detection and preliminary characterization of arcelin-like lectin activity in the seed extracts of *Canavalia virosa*:

3.2.1. Fine chemicals and reagents:

Fine chemicals and reagents used in this study were obtained from diverse commercial sources as listed below:
**Simple sugars:** Pentoses: L-arabinose, D (+) arabinose, xylose; Hexoses: D-glucose, D-galactose, D-mannose; D-fructose, L (-) arabinose; Deoxyhexoses: L-fucose, D-fucose, L-rhamnose; **Alcohol sugars:** Sorbitol, mannitol, adonitol, dulcitol, inositol, sialicin; **Disaccharides:** Maltose, cellobiose, lactose, trehalose, sucrose, melibiose; **Oligosaccharides:** Raffinose; **Polysaccharides:** Inulin, laminarin; **Amino sugar:** D-glucosamine, D-galactosamine, D-mannosamine and **Acetylated amino sugars:** N-acetyl-glucosamine, N-acetyl-galactosamine and N-acetyl-mannosamine.

**Glycoproteins:** Mucin from bovine submaxillary glands, fetuin from fetal calf serum and thyroglobulin from porcine thyroid gland.

**Column chromatographic matrices:** mannose-Sepharose 6B, BSM-Sepharose 4B, maltose-Sepharose 6B and CM-Cellulose.

The carbohydrates were purchased from BDH, Serva, Fluka, Merk, Hi-Media or Sigma. The chemicals used in hemagglutination inhibition assays were products of SDFCL, BDH, CDH, Qualigens and Sigma. The glycoproteins were purchased from sigma and polysaccharides from Hi-media and column matrices were purchased from Sigma/GE Healthcare/Pharmacia. All other chemicals and reagents were of the highest analytical grade and were supplied by local agencies. Sigma: St, Louis, Missouri, USA; Fluka: Basal, Switzerland; Merck: Darmstadt, Germany; GE Healthcare (Bioscience division)/Pharmacia: Uppsala, Sweden; Serva: Heidelberg, Germany; SRL: Mumbai, India; BDH: Mumbai, India; s.d. Fine Chemicals: Mumbai, India; CDH: Mumbai, India; Hi-Media: Mumbai, India; Qualigens: Mumbai, India.
3.2.2. Preparation of solutions and buffers:

Physiological saline (0.9 % NaCl)

9 g of sodium chloride was dissolved in 1 liter of double distilled water.

Alsever's solution

Alsever’s solution was prepared according to the method of Garvey et al. (1979). Briefly, 10.25 g dextrose, 4 g tri-sodium citrate, 0.28 g citric acid and 2.10 g sodium chloride were successively dissolved in 500 ml of double distilled water. The solution was autoclaved at 15 psi for 15 min, cooled to room temperature and then 50 mg of streptomycin was dissolved in this solution and stored at 10 ºC until use.

HCl (2 N)

18 ml of hydrochloric acid were made up to 100 ml with double distilled water to get a final concentration of 2 N HCl.

NaOH solution (0.5 N)

2 g of NaOH were dissolved in 50 ml of double distilled water to get a final concentration of 0.5 N NaOH.

Tris-HCl buffer (50 mM, pH 7.4)

3.028 g of tris (hydroxymethyl) aminomethane were dissolved in 500 ml of double distilled water. The pH of this solution was adjusted to 7.4 using 2 N HCl.

Tris-buffered saline (50 mM TBS, pH 7.4, 300 mOsm)

3.360 g of sodium chloride were dissolved in 500 ml of tris-HCl buffer to give a final concentration of 115 mM NaCl.
**Sodium acetate buffer (0.2 M, pH 3 - 6)**

16.406 g sodium acetate was dissolved in 1000 ml double distilled water and 1.21 ml of acetic acid was made up to 1000 ml in double distilled water to give a concentration of 0.2 M sodium acetate and 0.2 M acetic acid, respectively. The pH of acetate solution was adjusted to 3 - 6 using 0.2 M acetic acid.

**Tris – HCl buffer (0.2 M, pH 7 - 9)**

12.14 g tris was dissolved in 1000 ml double distilled water and 17.391 ml HCl was made up to 1000 ml in distilled water to give a concentration of 0.2 M tris and 0.2 N HCl, respectively. The pH of tris solution was adjusted to 7 - 9 using 0.2 N HCl.

**Glycine – NaOH buffer (0.1 M, pH 10, 11 & 12)**

4 g NaOH and 7.507 g glycine were dissolved separately in 1000 ml each double distilled water, to give a concentration of 0.1 M NaOH and 0.1 M glycine, respectively. The pH of the solution was adjusted with the other to give a pH of 10, 11 and 12.

**Tris-buffered saline + CaCl$_2$ (50 mM, pH 7.4, 300 mOsm)**

605 mg of tris was dissolved in 90 ml of double distilled water and pH was adjusted to 7.4 using 2N HCl, finally the volume was made up to 100 ml. To that 584 mg of NaCl and 294 mg of CaCl$_2$ were added to get the concentration of 0.1 mM and 0.01 M respectively.
**Tris-buffered saline + MgCl₂ (50 mM, pH 7.4, 300 mOsm)**

605 mg of tris was dissolved in 90 ml of double distilled water and pH was adjusted to 7.4 using 2N HCl, finally the volume was made up to 100 ml. To that 584 mg of NaCl and 203 mg MgCl₂ were added to get the concentration of 0.1 M and 0.01 M respectively.

**Tris-buffered saline + MnCl₂ (50 mM, pH 7.4, 300 mOsm)**

605 mg of tris was dissolved in 90 ml of double distilled water and pH was adjusted to 7.4 using 2N HCl, finally the volume was made up to 100 ml. To that 648 mg of NaCl and 19.97 MnCl₂ were added separately to get the concentration of 0.111 M and 0.001 M respectively.

**Tris-buffered saline + NiCl₂ (50 mM, pH 7.4, 300 mOsm)**

605 mg of tris was dissolved in 90 ml of double distilled water and pH was adjusted to 7.4 using 2N HCl, finally the volume was made up to 100 ml. To that 584 mg of NaCl and 237 mg of NiCl₂ were added to get the concentration of 0.1 M and 0.01 M respectively.

**Tris-buffered saline + EDTA (10 mM, pH 7.4, 300 mOsm)**

3.068 g of NaCl and 1.86 g of ethylene diamine tetra acetic acid-disodium salt (EDTA) were dissolved successively in 500 ml of 50 mM Tris-HCl buffer (pH 7.4) to give final concentrations of 105 mM NaCl and 10 mM EDTA respectively. The pH of the final solution was adjusted to 7.4 using 1 N NaOH solution.

**Note:** The osmolality of various buffers used in this study was determined using 50 μl of buffer solutions from the molecular formulae of the constituent salts and adjusted to desired osmolarity using crystals of sodium chloride (NaCl) or double distilled water in a cryoscopic osmometer (Model: Osmomat 030, Gonotec, Germany).
Reagents for protein estimation:

**1 N NaOH**: 4 g of NaOH was dissolved in 100 ml of double distilled water.

**0.1 N NaOH**: 10 ml of 1 N NaOH solution was made up to 100 ml with double distilled water.

**1% sodium potassium tartrate**: 1 g of sodium potassium tartrate was dissolved in 100 ml of double distilled water.

**Reagent A**: 2 g of sodium carbonate was dissolved in 100 ml of 0.1 N NaOH.

**Reagent B**: 500 mg of cupric sulphate (CuSO₄·5H₂O) was dissolved in 100 ml of 1% sodium potassium tartrate.

**Reagent C**: 2 ml of Reagent B was mixed with 100 ml of Reagent A.

**1N Folin-phenol reagent**: 2 N Folin-phenol reagent was diluted with an equal volume of double distilled water.

**Protein standard**: 5 mg of bovine serum albumin (BSA) was dissolved in 1 ml of 1 N NaOH.

**Note**: All reagents were prepared fresh prior to protein determinations.

### 3.2.3. Preparation of extracts using wild seeds of *Canavalia virosa*:

The seed coats of dried jack bean, *C. virosa* was manually removed and the kernels (cotyledons) alone were finely powdered using a mortar and pestle. The seed powder (10 g) was added with small quantity of TBS buffer (50mM Tris; 115mM NaCl, pH 7.4, 300 mOsm), mixed well and made up to 100 ml with the same buffer. This suspension (10% dry weight/volume) was stirred well on a magnetic stirrer kept overnight at 10 °C. Finally, the kernel extract was centrifuged
(8400 x g, 30 min, 4 °C), and the resultant supernatant was filtered using Whatmann No. 1 filter paper and clear filtrate was collected (10% seed kernel extract). This extract was stored at 10 °C and used for further analyses.

### 3.2.4. Determination of protein concentration:

Protein concentrations of the samples were estimated following the method of Lowry et al. (1951). The seed kernel extract was prepared and mixed with 20 volumes of 80% ethanol to enable complete precipitation of proteins (Subhashini and Ravindranath, 1980). The mixture was centrifuged at 400 x g, 5 min at 28 °C and the resulting supernatant was discarded. The precipitate was completely dissolved in 1 ml of 1 N NaOH and then mixed with 5 ml of Reagent C. A standard BSA solution (40 µl containing 200 µg BSA) was also dissolved separately with 1 ml of 1 N NaOH and 5 ml of Reagent C. After 10 min, 0.5 ml of 1 N Folin-phenol reagent was added to each tube, mixed thoroughly and left for 30 min. A reagent blank containing 1 ml of 1 N NaOH, 5 ml of Reagent C and 0.5 ml of 1 N Folin-phenol reagent was concurrently maintained as mentioned above. After incubation, the optical density (O.D.) of the blue colour developed in the sample and standard was measured against the reagent blank at 600 nm in Shimadzu (UV-160A) spectrophotometer. The protein concentration of the sample was calculated as follows:

\[
\text{Protein concentration in the sample (mg/ml)} = \frac{\text{OD of sample}}{\text{OD of standard}} \times \text{Concentration of standard (40µg)}
\]
3.2.5. Collection of vertebrate blood samples and preparation of erythrocytes suspension:

Human blood samples (A, B and O blood groups) were obtained from blood bank, Stanley Medical College, Chennai. Sheep, goat, ox, cow and buffalo blood samples were collected from the Corporation Slaughter House, Perambur, Chennai. Rabbit (Reg. No. 360/01/a CPCSEA dt. Jan 2001), rat and mice (JAEC: No. 02/016/2011/Sep11) blood samples were received as gift from control animals maintained in our laboratory by venous or cardiac puncture. The hen blood cells were collected from chicken shop, Kotturpuram, Chennai. All blood samples were collected in Alsever’s solution, stored at 10 °C and used within five days.

The collected blood samples were washed thrice with 0.9% physiological saline and once with TBS by centrifugation (400 x g, 5 min, 20 °C). Unless otherwise specified, the washed erythrocytes pellet was finally re-suspended in TBS to make up to 1.5% (v/v) erythrocytes suspension.

3.2.6. Hemagglutination (HA) assays:

The presence of arcelin-like lectin in the jack bean (10%) TBS extract was detected by the hemagglutination (HA) assays which were routinely performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial dilution of a 25 µl sample with an equal volume of appropriate buffer (TBS). Under certain experimental conditions, TBS without any divalent cations and TBS with specific divalent cations (CaCl₂, MgCl₂, MnCl₂ and NiCl₂) were used for HA. After dilution, 25 µl of RBC suspension (1.5 % v/v) was added to each well and incubated for
45 min at 25 °C. The hemagglutination titer was recorded as reciprocal of the highest dilution of the sample causing complete agglutination of RBCs (Garvey et al., 1979). Controls consisted of the substitution of the sample by buffer. Each experiment was performed in duplicate for three to five times using samples from different preparations, and the agglutinating activity of the test sample was analyzed based on the median agglutination titer values obtained for each RBC type.

3.2.7. Preparation of trypsin and pronase treated erythrocytes:

RBC in the blood samples were washed thrice with 0.9% physiological saline by centrifugation (400 x g, 5 min, 20 °C). Two hundred microliter of vertebrate RBC pellets was re-suspended with 5 ml of trypsin (5 mg/ml) or pronase (0.5 mg/ml) prepared in TBS (pH 7.4). The suspension was incubated for 1hr at 37 °C in water bath with occasional shaking. After incubation, the trypsinated RBC cells were washed once with 0.9% physiological saline by centrifugation (400 x g, 5 min, 20 °C) and finally the enzyme treated RBC cells (200 µl) were suspended at a concentration of 1.5% (v/v) in TBS.

3.2.8. HA assay with trypsin and pronase treated erythrocytes:

Trypsin and pronase treated erythrocyte suspensions (25 µl in 1.5% v/v 50 mM TBS) were mixed with 10% crude seed extract of C. virosa sample (25 µl). After incubation for 45 min at 37 °C, the assayed microtitre plate was observed for hemagglutination activity.
3.2.9. Cross-adsorption tests:

The crude *C. virosa* seed extract-10% (each 400 µl) was mixed with an equal volume of washed and packed human A, B, O, rabbit, sheep, buffalo, rat, mouse, rabbit, and hen RBC and incubated for 1 h at 28 °C with occasional shaking. The suspension was centrifuged at 400 x g, 5 min, 20 °C, the supernatant was collected and adsorbed for a second and third time with fresh RBC types under the same conditions. The sample adsorbed thrice was subsequently tested for hemagglutinating activity using all the above mentioned RBC types.

3.2.10. pH and thermal stability:

The stability of 10% crude seed extract of *C. virosa* hemagglutinating activity in different pH was tested by dialyzing 500 µl of seed extract of *C. virosa* against the following buffers at pH ranging from 3 to 12: acetate buffer (pH 3 to 6), tris-HCl buffer (pH 7 to 9) and glycine-NaOH buffer (pH 10 to 12). All the samples were finally re-equilibrated by dialysis in TBS at 4 °C and centrifuged (400 x g, 5 min, 20 °C). The resultant supernatant was tested for hemagglutinating activity using only the indicator RBC types (RBC types with highest HA titer - sheep and buffalo RBCs).

The thermal stability of 10% crude extract of *C. virosa* was assessed by holding 200 µl aliquots at temperatures ranging from 10 to 100 °C for 30 min. The samples were centrifuged (400 x g, 5 min, 20 °C) to remove debris if any, and the hemagglutinating activity in the supernatant was assayed against indicator RBC types (sheep and buffalo RBCs).
3.2.11. Precipitation by ammonium sulfate:

Protein was serially precipitated (Jakoby, 1971) using 30 ml of crude seed extract of C. virosa by stepwise addition of solid ammonium sulfate (10% - 90%) with constant stirring. The mixture was incubated at 10 °C for 12 hr with frequent shaking. Then the mixture was subjected to centrifugation at 8400 x g for 20 min at 4 °C. The pellet obtained after centrifugation (for each percentage of the ammonium sulfate) was re-suspended in 4 ml of TBS (50 mM Tris-HCl, pH 7.5 and 115 mM NaCl) and dialysed against same buffer for 12 hr at 4 °C. Finally, the dialysate was centrifuged at 8400 x g for 20 min at 4 °C and the HA activity in the supernatant was determined against the indicator RBC types.

3.2.12. Precipitation by trichloroacetic acid:

250 µl of 10% crude seed extract was mixed with an equal volume of ice-cold 20% trichloroacetic acid (TCA), to give a final TCA concentration of 10%. The mixture was incubated for 45 min at 10 °C with frequent shaking and centrifuged (400 x g, 5 min, 20 °C). The supernatant was dialysed extensively against TBS (Link and LaBaer, 2009). The dialysate was centrifuged (400 x g, 5 min, 20 °C) and the hemagglutinating activity in the supernatant was determined against indicator RBC types (sheep and buffalo RBCs).

3.2.13. Divalent cation requirement for HA activity in the crude seed extract of C. virosa:

In an attempt to ascertain the divalent cation requirement for HA activity in the crude seed extract of C. virosa, assays were performed using 50 mM TBS alone.
and TBS containing different divalent cations at varying concentrations. The arcelin-like lectin activity of the extract was tested against sheep and buffalo RBC types suspended in the respective buffers. In these assays, the extract (25 µl) was serially diluted with either cation-free TBS or TBS containing various cations (CaCl₂, MgCl₂, MnCl₂ and NiCl₂). After dilution, 25 µl of RBC suspension (1.5% v/v) in respective buffer was added to each well and incubated for 45 min at 25 °C, and then HA titer was recorded.

3.2.14. Dialysis experiments:

The dry dialysis tubing with MW exclusion limit of 12-14 kDa were purchased from Himedia, Mumbai and this membrane was pre-treated before use. This involved soaking the membrane in double distilled water, heating the membrane to 50 °C for 10 min and rinsing in double distilled water extensively to remove preservatives. The pretreated membrane was used for dialysis of samples.

Dialysis experiments were carried out to gain insight into approximate molecular size of arcelin-like lectin molecules present in the extract. Two aliquots of C. virosa crude seed extract (each 500 µl) were separately loaded into dialysis membrane with MW exclusion limit of 12-14 kDa and they were extensively dialysed at 10 °C against cation-free TBS (pH 7.4). The resulting dialysates were centrifuged (4000 x g, 10 min, 4 °C) and the residual lectin activity of the supernatant was determined by hemagglutination assays performed using indicator RBC types (sheep and buffalo RBC). Undialysed extract kept at the identical condition served as control.
3.2.15. EDTA sensitivity for HA activity in the crude seed extracts of *C. virosa*:

Samples of *C. virosa* seed kernel extract (each 250 µl) were dialysed (MW exclusion limit of 12-14 kDa) extensively against TBS containing 10 mM EDTA, pH 7.4 and subsequently re-equilibrated by dialysis in cation-free TBS (pH 7.4) to remove EDTA from the extract. The resulting dialysates were centrifuged (8400 x g, 5 min 4 ºC) and the agglutinating activity of the supernatant was determined using indicator RBC types (sheep and buffalo RBC) in the presence of cation-free TBS or TBS containing various cations (CaCl₂, MgCl₂, MnCl₂ and NiCl₂).

3.2.16. Hemagglutination-inhibition assays:

The tested carbohydrates (stock solutions at 200 mM) were prepared in TBS (50 mM tris, 55 mM NaCl, pH 7.4, 135 mOsm) and glycoproteins (stock solutions at 10 mg / ml in TBS - 50 mM tris, 115 mM NaCl, pH 7.4, 300 mOsm) were tested for their ability to inhibit the HA activity in *C. virosa* seed extract against the indicator RBC types. The pH of these test solutions were adjusted to pH 7.4 using NaOH pellets.

The carbohydrate binding specificity of the lectin activity detected in jack bean seed kernel extract was assessed by competitive inhibition of its agglutinating activity against sheep and buffalo RBC types using various carbohydrates and glycoproteins. The seed extract was first diluted with TBS (pH 7.4) to give a hemagglutination titer of 4 against sheep and buffalo RBC types. The diluted seed
extract (25 µl) was added to each well of the microtiter plate, then 25 µl of each test carbohydrate or glycoprotein solution was added to the first well and serially diluted up to 8 wells in microtiter plates and incubated for 1 h at 25 ºC. After incubation, 25 µl of 1.5% indicator RBC suspension was added to each well, incubated for 45 min at 25 ºC, and occurrence of hemagglutinating activity in each well was carefully observed. The minimal concentration of the test substance that completely inhibited the hemagglutinating activity was recorded.

3.3. Isolation and characterization of arcelin-like lectin molecules from the seed extracts of Canavalia virosa:

3.3.1. Preparation of solutions and buffers:

Tris buffers, saline and carbohydrate solutions were prepared as mentioned in the section of 3.2.2.

3.3.2. Batch assay:

The binding ability of arcelin-like lectin molecules in the seed kernel extracts of Canavalia virosa was analyzed with various types of column matrices using batch assays to find a suitable matrix for isolation of these molecules. The column matrices used were BSM-Sepharose 4B (Navens et al., 1992), mannose-Sepharose, maltose-Sepharose (Vretblad, 1976; Ostermann, 1986) and CM-Cellulose (Sigma Product Information-C4146, 1997). Each synthetic polymer (200 µl) was extensively washed with 50 mM TBS buffer (pH 7.4), except CM-Cellulose, which was washed extensively using 25 mM sodium acetate buffer (pH 4.6). Then equal volume of C. virosa kernel extract (HA titer = 64 for sheep and 128 for buffalo RBC types) was applied and it was incubated at room
temperature for about an hour. The effluent was monitored for HA activity against indicator RBC types (sheep and buffalo RBC) to determine lectin binding capacity of the column matrices used.

3.3.3. Isolation of arcelin-like lectin molecule from the seed kernal extracts of *C. virosa* using affinity column of mannose-Sepharose 6B.

**3.3.3.1. Coupling of mannose to epoxy activated Sepharose 6B:**

2 g of the gel matrix was suspended in 25 ml of double distilled water containing 0.02 % sodium azide (NaN₃) in a conical flask and allowed to swell overnight at room temperature with gentle shaking.

1. Swollen gel matrix was washed (approximately 6 ml) with 50 ml of double distilled water for 5 times followed by 20 ml of 100 mM NaOH for three times. The washing was performed by allowing the gel matrix with passive settlement.

2. 6 ml of the washed gel matrix was mixed rapidly and gently with equal volume of 100 mM NaOH containing 300 mg mannose and incubated at room temperature for 16 hrs with continuous gentle shaking.

3. The supernatant was removed and the gel matrix was washed sequentially as given below.

   a. It was washed with 20 ml of double distilled water containing 0.02 % NaN₃ for five times.

   b. Blocking the excess reactive group in matrix was done by re-suspending the matrix in 20 ml of 1M ethanolamine in double distilled water overnight at 45 °C in a shaker.
c. The matrix was washed with 100 mM carbonate buffer containing 500 mM NaCl and 0.02 % NaN₃ (pH 8.0) and 100 mM acetate buffer containing 100 mM NaCl and 0.02 % NaN₃ (pH 4.0).

4. Finally the gel matrix was resuspended in 0.9% saline containing 0.02% NaN₃ and stored at 8-15 °C for further use.

3.3.3.2. Isolation of arcelin-like lectin molecule:

Briefly, 1 ml of mannose-Sepharose 6B was packed in a glass econo-column (10 × 0.5 cm diameter; Bio-Rad, India) and equilibrated with TBS (Tris-buffer saline: 50 mM Tris-HCl buffer + 300 mM NaCl, pH 7.4) at a flow rate of 2 ml / hr. A total of 2 ml of filtered supernatant of 10% seed kernel extracts of *C. virosa* (=titer 8192 and 2048 for buffalo and sheep RBC respectively) was applied to this affinity column at a flow rate of 2 ml / hr. The column was washed with 10 ml of TBS to completely remove unbound or free proteins at a flow rate of 12 ml / hr. Bound protein on the column matrix was eluted with 6 ml of TBS containing 0.2 M mannose. Fractions of 1 ml were collected at a flow rate of 20 ml / hr and absorption at 280 nm was measured for each fraction. The fractions were also analyzed using both native (Maurer, 1971) and denatured (Laemmli, 1970) gel electrophoresis.

3.3.4. Determination of protein concentration of isolated molecule:

Protein concentration of isolated lectin was determined following the procedure as described in the section 3.2.4.
3.3.5. Analysis of isolated arcelin-like lectin molecules:

3.3.5.1. Non-Denaturing Polyacrylamide Gel Electrophoresis (Native-PAGE):

Preparation of solutions

Resolving gel buffer (pH 8.9)

9.15 g of tris was dissolved in 10 ml of glass distilled water. The pH was adjusted to 8.9 using 2 N HCl. To this 0.058 ml of TEMED (N, N, N’, N’-tetramethylethylenediamine) was added and was made up to 25 ml.

Acrylamide for resolving gel

7 g of acrylamide and 0.184 g of methylene bisacrylamide were dissolved and made up to 25 ml with distilled water.

Ammonium per sulphate (1.4%)

140 mg of ammonium per sulphate was dissolved in 10 ml of distilled water.

Stacking gel buffer (pH 6.7)

1.495 g of tris was dissolved in 10 ml of glass distilled water. The pH was adjusted to 6.7 using 2 N HCl. To this 0.115 ml of TEMED was added and made up to 25 ml.

Acrylamide for stacking gel

2.5 g of acrylamide and 0.625 g of methylene bisacrylamide were dissolved and made up to 25 ml with distilled water.

Sucrose (40%)

4 g of sucrose was dissolved in 10 ml of distilled water.
**Ammonium per sulphate (0.14%)**

14 mg of ammonium per sulphate was dissolved in 10 ml of distilled water.

**SDS- sample buffer**

- Bromophenol blue : a pinch (or 0.05%)
- Distilled water : 7.75 ml
- Stacking gel buffer : 1.25 ml
- Glycerol : 1.00 ml

**Tank buffer (pH 8.3)**

3 g of tris and 14.4 g of glycine was added and made up to 500 ml with distilled water. The pH was adjusted to 8.3.

**Staining solution (100 ml)**

- Coomassie brilliant blue (R-250) : 200 mg
- Ethanol or methanol : 50 ml
- Acetic acid : 7 ml
- Distilled water : 43 ml

**Destaining solution (100 ml)**

- Ethanol or methanol : 30 ml
- Acetic acid : 7 ml
- Distilled water : 63 ml

**Gel storing Solution**

7 ml of acetic acid was made up to 100 ml using distilled water.
The gel plates were assembled according to the manufacturer’s instruction and the volume of the gel mould was determined (slab gel: 170 x 150 x 1.5 mm). In a beaker, the resolving (separating) gel (7%) was prepared by mixing resolving gel buffer, acrylamide for resolving gel, distilled water and ammonium per sulphate in the ratio of 1:2:1:4. The components were mixed thoroughly. Without delay, the mixture was poured into the glass mould and overlaid with distilled water. After polymerization (approximately 20 minutes), the overlay was removed. The stacking gel (3%) was prepared by mixing the stacking gel buffer, acrylamide for stacking gel, ammonium per sulphate and sucrose in the ratio of 1:2:1:4. These components were mixed and poured over the resolving gel. Immediately a comb was inserted and the gel was allowed to polymerize. After polymerization the comb was removed from the mould and the wells were washed with distilled water. The gel was mounted in the electrophoretic apparatus. In order to load the sample, 30 or 15 µg of each sample were mixed thoroughly with sample buffer. The samples were loaded in the wells. The native PAGE tank buffer of pH 8.3 was added to the upper and the lower tanks of the electrophoretic apparatus. The apparatus was attached to power supply unit and 60V for stacking gel and 120V for resolving gel was applied. Electrophoresis was carried out at 10 ºC. The power supply was turned off, once the bromophenol blue reached the bottom of the resolving gel. The gel mould was removed from the apparatus and the plates were removed apart using a spatula. The orientation of the gel was marked. The gel was immersed in 5 volumes of staining solution and stained for 4 hrs at room temperature. The gel was destained with the destaining solution. It was stored in 7% acetic acid. The bands were visualized using an illuminator and then photographed.
3.3.5.2. Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Preparation of Solutions

Acrylamide (30% stock)

Acrylamide : 30 g
Bisacrylamide : 0.8 g

They were dissolved in 70 ml of distilled water and made up to 100 ml. The stock solution was stored in amber colour bottles at 4 °C (3 months).

Resolving gel buffer, 1.5 M Tris-HCl, pH 8.8

18.17 g tris base were dissolved in 70 ml distilled water. The pH was adjusted to 8.8 with 2 N HCl and made up to 100 ml. This buffer was stored at 4 °C.

Stacking gel buffer, 0.5 M Tris-HCl, pH 6.8

6.05 g tris base were dissolved in 70 ml of distilled water. The pH was adjusted to 6.8 with 2N HCl and made up to 100 ml. This buffer was stored at 4 °C.

Electrophoresis (tank) buffer, pH 8.3

3 g tris base, 14.4 g glycine and 1 g SDS were dissolved in 1000 ml of distilled water. The pH without adjustment was 8.3.

Ammonium per sulphate (10%) - initiator

Ammonium per sulphate : 0.1 g

This was dissolved in 1 ml of distilled water. It was prepared freshly.

TEMED (N, N, N’, N’-tetramethylethylenediamine)

It was used as a catalyst.
**SDS (10%)**

2.5 g SDS was dissolved in 25 ml of double distilled water.

**SDS-sample buffer**

- Bromophenol blue : a pinch (or 0.05%)  
- 2-mercaptoethanol : 0.50 ml  
- SDS : 0.15 ml  
- Glycerol : 1.00 ml  
- Stacking gel buffer : 1.25 ml  
- Distilled water : 7.10 ml

The gel plates were assembled according to the manufacturer’s instruction and the volume of the gel mould was determined (slab gel: 170 x 150 x 1.5 mm). In a beaker, the acrylamide mixture for 12 % resolving gel (4.00 ml of 30% acrylamide stock + 2.50 ml of resolving gel buffer, pH 8.8 + 3. 30 ml of distilled water + 100 µl of 10% SDS + 100 µl of 10% ammonium per sulphate + 4 µl of TEMED) was prepared, mixed thoroughly and without delay the acrylamide mixture was poured into the glass mould and overlaid with distilled water. After polymerization (approximately 20 minutes), the overlay was removed. A stacking gel (5%) was prepared by mixing 0.5 ml of 30% acrylamide stock, 0.38 ml of stacking gel buffer, pH 6.8, 2.10 ml of distilled water, 20 µl of 10% SDS, 20 µl of 10% ammonium persulphate and 2 µl of TEMED. These components were mixed and poured over the resolving gel. Immediately a comb was inserted and the gel was allowed to polymerize.
In order to load the sample, all the sample containing 10 or 6 µg of protein were mixed separately with equal volume of SDS-sample loading buffer. The samples were heated in a boiling waterbath for 1 minute to denature the proteins. They were kept on ice to retain the denatured stage. The comb was removed from the mould and the wells were washed with distilled water. The gel was mounted in the electrophoretic apparatus. The samples were loaded on the wells.

The SDS-tank buffer, pH 8.3 was added to the upper and the lower tank of the electrophoretic apparatus. The apparatus was attached to power supply unit and the voltage of 60 for stacking gel and 120 for resolving gel was applied. The power supply was turned off, once the bromophenol blue reached the bottom of the resolving gel. The gel mould was removed from the apparatus using a spatula. The orientation of the gel was marked.

The separated proteins in the gel were stained with Coomassie brilliant blue R-250 for 4 hrs at room temperature. The gel was then destained with the destaining solution. It was stored in 7% acetic acid. The gels were visualized using an illuminator and then photographed.

**3.3.5.3. Silver staining:**

The silver staining of isolated arcelin-like lectin molecules in both native and denatured gel was followed according to the procedure of Merril et al. (1981).

**Fixative solution**

50% ethanol, 12% acetic acid and 0.1 ml formaldehyde were dissolved in 200 ml of distilled water.
Silver nitrate solution

0.4 g of silver nitrate and 160 µl of formaldehyde solution were mixed with 200 ml of distilled water.

Sodium thiosulphate solution

0.04 g of sodium thiosulphate was dissolved in 200 ml of distilled water.

Developer solution

6 g of sodium carbonate, 50 µl of formaldehyde, a pinch of sodium thiosulphate were mixed together with 200 ml of distilled water.

Initially the gel was kept in the fixative solution for about 2 hrs to overnight and washed with 50% ethanol three times (each 20 minutes). The gel was then treated in sodium thiosulphate solution for one minute and then washed with distilled water three times (each 20 sec). Finally, the gel was treated with silver nitrate solution added with 160 µl formaldehyde for twenty minutes and then was washed with distilled water three times (each 20 sec). At the end of the staining procedure a brown precipitate may appear upon contact of protein fractions in the gel with the developer. Finally, the gel was rinsed with distilled water for 10 sec and it was preserved in 12% acetic acid.

3.3.5.4. Hemagglutinating (HA) assays:

The fractions collected in affinity chromatography were dialyzed against TBS at 4 °C for 12 hr and HA activity was measured using indicator RBC types (sheep and buffalo RBC) as described in section 3.2.6.
3.3.5.5. Cation dependency, EDTA sensitivity, pH and thermal stability:

Cation dependency, EDTA sensitivity, pH and thermal stability of isolated molecule were analyzed following the procedures as described in the sections 3.2.13; 3.2.15 and 3.2.10.

3.3.5.6. Carbohydrate binding specificity:

The carbohydrate binding specificity of the isolated arcelin-like lectin molecule was examined by hemagglutination-inhibition assays using various sugars and glycoproteins, and the assays were performed as described in section 3.2.16. The inhibitory potency of the test substance was expressed at its minimal concentration that completely inhibited the HA activity of the isolated molecule against indicator RBC types (sheep and buffalo RBC).

3.3.5.7. Matrix-assisted laser desorption / ionization - time of flight-mass spectrometry (MALDI-TOF-MS) analysis:

Separation and detection of isolated molecule was performed on SDS-PAGE. The gel stained with coomassie brilliant blue revealed five polypeptide fractions. One major subunit that was electrophoresed at 34 kDa was subjected to peptide mass fingerprinting and peptide sequencing using the standardized protocol for MALDI-TOF-MS spectrometry (Wilm et al., 1996).

The excised gel piece containing 34 kDa polypeptide subunit was destained by dispensing in 50% (v/v) acetonitrile and 50 mM NH₄HCO₃ solution and incubated at room temperature for 15 min with gentle agitation. The solution was replaced several times until gel pieces were clear and dried at room temperature for
10-20 min in a centrifugal evaporator (Genevac, NY, USA). A volume of 10 mM dithiothreitol (Sigma, USA) in 100 mM NH$_4$HCO$_3$ sufficient enough to cover the gel pieces were kept at 56 °C for 30 min. After cooling at room temperature, 10 µl of alkylation solution (50 mM iodoactamide in 100 mM NH$_4$HCO$_3$) was added and incubated for 30 min in the dark at room temperature with occasional vortex. The gel pieces were washed with 100 µl of 100 mM NH$_4$HCO$_3$ solution for 10 min, dehydrated by the addition of acetonitrile, swelled by rehydration in 100 mM NH$_4$HCO$_3$ and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed and the gel pieces were completely dried in a centrifugal evaporator (Genevac, NY, USA). The gel pieces were allowed to swell in a digestion buffer containing 50 mM NH$_4$HCO$_3$ and 20 µg/ml trypsin (Promega, Madison, WI, USA) in an ice-cold bath. After 45 min the supernatant was removed and replaced with 10 µl of the same buffer and incubated at 37 °C for 16 h. Peptides were extracted by centrifugation at 12,500 x g for 30 sec. The supernatant was transferred to a sterile tube. To this, 50 µl of extraction solution was added [60% (v/v) acetonitrile, 1% trifluoroacetic acid (TFA)] and sonicated in ultrasonic water bath (Citizen Scale, Mumbai, India) for 10 min.

The separated polypeptide subunit fractions were dried by centrifugal evaporation and 5 µl of suspension solution [50% (v/v) acetonitrile, 0.1% TFA] was added, gently agitated on a vortex at lowest setting. Mass spectra were obtained under a Daltonic Ultraflex III MALDI-TOF/TOF (Bruker, Germany). MALDI peptide spectra were calibrated using matrix ion peaks as per international standard.
The data obtained under mass spectrum were subjected to database (NCBI nonredundant/Swiss-Prot) searching using the programme MASCOT (http://matrixscience.com) analysis. The calibrated peptide masses were searched with 100-500 ppm mass accuracy.

3.3.5.8. Multiple sequence alignment using ClustalW:

The general purpose multiple sequence alignment program namely, ClustalW (Thompson et al., 1994) was used for multiple sequence alignments of predicted peptide sequences from MASCOT search for the major polypeptide subunit of the isolated molecule subjected to MALDI-TOF/TOF.

3.4. Insect bioassay:

To examine the effects of arcelin-like lectin molecule isolated from *C. virosa* on the development of *C. maculatus* was performed using an artificial seed system previously developed by Shade et al. (1986). Artificial seeds (250 mg each seed) containing the isolated molecule at different concentrations (0.2%, 0.4% 0.8%, 1.0%, 1.5% and 2.0% w/w) were obtained by thoroughly mixing the isolated arcelin-like lectin molecules (lyophilized) with seed flour of the most susceptible variety of cowpea seeds (*Vigna unguiculata*). The susceptible cowpea seeds were milled into a powder. The resulting flour was mixed with TBS buffer, pH 7.4 in 2:1 ratio and was made until a smooth paste was formed. The paste was then transferred to an acrylic mold. These plates were frozen at -20 °C for 12 h and lyophilized for 12 h. After lyophilization, the solid artificial seeds were removed from the wells of the acrylic mold by gentle pressure. The seeds were then placed in plastic petri
plates and maintained for hydration at a constant temperature (25 °C) and relative
humidity (60 ± 5%) for 48 h. During hydration, the plates were closed with fine
mesh to prevent accidental infestation. The seeds were then coated with 8% gelatin
to mimic the seed coat texture. The artificial seeds (10 numbers of seeds/treatment)
were placed in glass jars for *C. maculatus* infestation. Each treatment had ten
artificial seeds and was replicated three times for each of the above concentrations.
Newly emerged adults (five pairs of both the sexes) were introduced into the plastic
container for oviposition. This set up was maintained in the insect growth chamber
with constant temperature and humidity as stated earlier. Effect of various doses of
isolated arcelin-like lectin molecule on oviposition, adult emergence and percentage
of infestation/seed damage was studied. Control artificial seeds were made with
susceptible variety of cowpea seed (*V. unguiculata*) flour with appropriate buffer.

3.5. **Analysis of arcelin-like lectin gene mRNA expression and their relative quantification:**

3.5.1. **Isolation of total RNA:**

All the pulse varieties were germinated under laboratory conditions. After
four days, the tender leaves were used for isolation of total RNA using Mini RNA
extraction kit (Shrimpex Biotech Limited, India) following the manufacturer's
protocol. Briefly, the germinated tender leaves of different wild pulses were surface
sterilized with glass distilled water followed by physiological saline (0.9%) and
DEPC treated water. The leaf samples (100 mg) were homogenized using RNase
free teflon micropestle in 1.5 ml centrifuge tubes containing 650 μl of extraction
buffer / SL buffer and 6.5 μl of β-mercaptoethanol. The homogenized tissue sample was incubated at room temperature for 5 min and centrifuged at 14200 x g for 10 min. The supernatant (350 μl) was collected and loaded with pre filter column placed in 2 ml collection tube. It was again centrifuged at 8400 x g for 30 s and lysate collected was added with an equal volume (350 μl) of 70% ethanol, mixed thoroughly and loaded to SMS column placed in a 2 ml collection tube. This column was centrifuged at 8400 x g for 15 sec and the flow through was discarded. The column was washed with 550 μl of 70% ethanol and centrifuged again at 8400 x g for 15 sec. Finally, the column was transferred to a new 2 ml collection tube, added with 50 μl of SE buffer, incubated for 5 min followed by centrifuging at 14200 x g for 1 min. Later, the column was discarded and the RNA was collected at the tube bottom.

3.5.2. cDNA synthesis:

The prime RT premix (2X) kit purchased from Genetbio Inc. was used for synthesizing single strand cDNA. Briefly, cDNA was synthesized in a volume of 20 μl with reaction mixture containing:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo dT (50 μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Total RNA (10 pg - 5 μg)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>DEPC water</td>
<td>8.5 μl</td>
</tr>
</tbody>
</table>

The mixture was incubated at 65 °C for 5 minutes and then immediately placed on ice for 5 min, added with 10.0 μl of 2X RT premix and mixed gently by
pipetting up and down. This reaction tube was preheated at 37 °C for 2 min. The tube was incubated at 37 °C for 60 minutes. The reaction was stopped by giving a heat shock at 70 °C for 10 minutes. The tube was chilled on ice for approximately 30 minutes. It was then briefly centrifuged to collect the contents and stored at -20 °C.

3.5.3. Spectrophotometric analysis of cDNA:

Freshly synthesized cDNA were subjected to spectrophotometric analysis in the wavelength of 260 and 280 nm to determine enough quantity and quality of cDNA for PCR analysis using NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) at King Institute of Preventive Medicine, Chennai, India.

3.5.4. Agarose Gel Electrophoresis of DNA:

Preparation of solutions:

Stock solutions:

**1 M tris-HCl pH 8.0 (100 ml)**

12.11 g tris was dissolved in 80 ml distilled water. The pH was adjusted to 8.0 using concentrated HCl. It was then made up to 100 ml.

**0.5 M EDTA pH 8.0 (100 ml)**

7.31 g EDTA (disodium salt) was dissolved in 80 ml of distilled water. The pH was adjusted to 8.0 using concentrated NaOH crystals. It was made up to 100 ml.
**TAE (electrophoresis) buffer (50X stock)**

- Tris base : 25 g
- Glacial acetic acid : 50 ml
- 0.5 M EDTA : 4 g

The solution was made up with distilled water to 1000 ml and diluted to 1X for use.

**TAE working buffer (1X concentration)**

- 40 mM tris acetate $p$H 8.0
- 1 mM EDTA $p$H 8.0

**Sample buffer (10 ml)**

- 0.1 M EDTA $p$H 8.0 : 0.40 ml of 0.5 M stock
- 0.01 M Tris HCl $p$H 8.0 : 0.02 ml of 1 M stock
- 0.25 % Bromophenol blue : 5.00 mg
- 50 % Glycerol : 9.00 ml

To this 0.58 ml of distilled water was added.

**Ethidium Bromide Solution**

10 mg of ethidium bromide was dissolved in 1 ml of 1X TAE buffer.

**1% Agarose gel**

1 g of agarose was dissolved in 100 ml 1X TAE buffer.

Appropriate amount of agarose was mixed in 1X agarose gel buffer. The mixture was heated in a boiling waterbath by rotating the flask occasionally until agarose was dissolved. The agarose was cooled to 65-70 °C in a water bath and was
poured on to the gel tray to a thickness of 3-5 mm, the comb was inserted before adding the agarose. The gel was allowed to polymerize for 30 minutes. It was placed in the electrophoresis tank and filled with 1X electrophoresis buffer. The comb was removed carefully from the gel. One volume of DNA sample was mixed with 5 volumes of DNA sample buffer. It was applied to the wells of the gel. The sample was run until the bromophenol blue migrated approximately 2/3 of the way through the gel. The gel was stained after electrophoresis with ethidium bromide and illuminated under UV (Gel Documentation System, BioRad) at 254-366 nm (Sambrook et al., 1989).

3.6. Real-time RT-PCR analysis of arcelin-like lectin gene in various wild pulses:

3.6.1. Primer designing, specificity verification and selection of housekeeping gene:

Arcelin cDNA sequence of *Lablab purpureus* submitted to NCBI from our earlier work was retrieved (GenBank Accession No DQ985699.1) for designing of primers specific for arcelin-like lectin gene for real time RT-qPCR. Primer sequences were designed using Primer Express-3 software provided by Applied Biosystems, USA. A set of primer sequences was designed (Forward: ACTGGGTTCCTCGTTGTTGTA and Reverse: CAATGCCACCGTCTCATTCA), and synthesized (Eurofins MWG Operon, Germany). These primers were used for real time RT-qPCR. To optimize polymerization efficiency with reduced impact of RNA integrity on gene expression in RT-qPCR, primers with 58–62 °C for Tm, 18-24 bp length and 45-55 % GC content were selected. Amplicon lengths were
optimized to 90-155 bp on relative quantification of gene expression. Specificity of the amplified product of primer pair was verified by the presence of a single peak in melting curve analysis which were performed at the end of PCR run, and the presence of a single band of the expected size using agarose gel electrophoresis.

The normalization of real-time RT-PCR housekeeping gene was carried out using various universal housekeeping genes such as, β-actin, GABDH and 18S rRNA and were checked with RT-PCR conditions to select suitable internal control for gene expression study.

3.6.2. Real-time RT-PCR:

The following thermal cycling conditions were used for Real Time PCR:

Initial hold at 95°C for 10 min for the activation of Taq DNA polymerase present in the master mix, an amplification program repeated 40 times (95 °C for 15 sec and 60 °C for 1 min) and a melting-curve program (55 to 95 °C with warming of 0.2 °C per sec). RT and PCR positive controls (cDNA) and negative controls (deionized water) were included in each run. Among various housekeeping genes, 18S rRNA was selected for the normalization by real-time RT-PCR.

3.6.2.1. Melt curve analysis:

Melting curve analysis, a dissociation protocol with a gradient from 55-95 °C was used for each primer pair to verify the specificity of the RT-qPCR reaction and the absence of primer dimer. All the RT-qPCR were subjected to melting curve analysis which were performed at the end of the PCR run over the range 60-95 °C, increasing the temperature stepwise by 0.5 °C every 10 sec. Each
experiment was carried out with a negative control containing SYBR green with primer without template cDNA to detect possible reagent contamination.

3.6.2.2. Relative quantification and data analysis:

The relative expression ratio was calculated for the gene of interest using a mathematical model described by Pfaffl (2001).

3.7. Statistical analyses:

All the data were subjected to Student's t-test and the level of significance was tested at P < 0.05. Tukey’s test was performed using SPSS Software Package.