6. SUMMARY

- Feeding bioassay (= insect infestation) with the stored product bruchid beetle *Callosobruchus maculatus* using seven different wild varieties of pulses was carried out in the present study. The results demonstrated total resistance of five varieties of seeds of pulses such as *Lablab purpureus*, *Canavalia virosa*, *Vigna umbellata* (red), *Phaseolus* sp. and *Mucuna pruriens* against the infestation of *C. maculatus*.

- As a requirement for the isolation of insecticidal arcelin-like lectin molecule, the whole seed kernel extracts of five stored product pest resistant pulse varieties were used for the detection of hemagglutination (HA) activity against 12 vertebrate RBC types. Among them, the seed kernel extracts of *C. virosa* recorded highest value of HA titer as 16384 for native buffalo RBC.

- The seed kernel extract of *C. virosa* also registered a strong HA activity in the case of enzyme treated RBC than native RBC types. Interestingly, this seed extract recorded HA activity as 1073741824 and 67108864 for rabbit RBC treated with 0.05% pronase and 0.5% trypsin respectively. Besides, the native erythrocyte types of cow, ox and goat that were exhibited no HA activity with the extracts of *C. virosa* recorded significant HA titer values due to the treatment of enzymes.

- The nine different RBC types that exhibited HA activity subjected to cross adsorption studies with the crude seed extract of *C. virosa* revealed no cross adsorption within these RBC types. This study provided an evidence that the lectin or arcelin-like lectin molecule in the extract was highly specific to buffalo, sheep, rat and rabbit erythrocytes surface carbohydrates that share some common glycoconjugates on their membrane surface with varying
densities of surface receptors. Based on the erythrocytes agglutination activity, sheep and buffalo RBC were chosen as suitable indicator cells for all subsequent experiments.

- The lectin or arcelin-like lectin activity from extracts was precipitated using 10% TCA. The precipitation of all the HA activity against indicator erythrocytes in the pellet established protein or glycoprotein nature of agglutinin in the extracts. There was no HA activity in the supernatant obtained after TCA precipitation with 10% crude seed kernel extracts of *C. virosa*.

- Dialysis experiments performed using dialysis tubing with 12-14 kDa molecular weight cut-off revealed that the dialysates of the seed kernel extract recovered from tubing only retained all HA activity. It suggested that molecular weight of the agglutinin molecules responsible for HA activity detected in the extract could be above 14 kDa.

- The tests performed with four different divalent cations such as Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) and Ni\(^{2+}\) to assess the requirement of any of these cations for agglutinating activity showed no requirement of such cations for the lectin or arcelin-like lectin molecules in the crude seed kernel extract of *C. virosa* to express full HA activity. The HA activity of crude seed kernel extract was not affected by treatment with 10 mM EDTA.

- The study also revealed that the HA activity in the crude seed extract of *C. virosa* was not affected when the pH range was between 4 and 9. The activity was low at pH less than 4 and more than 10 against the two indicator RBC types. The activity was completely lost above pH 11. HA activity in the crude seed extracts of *C. virosa* remained stable between 10 and 75 °C against buffalo RBC and 10 to 80 °C against sheep RBC.
An extensive hemagglutination-inhibition assay was performed to determine the carbohydrate binding specificity of the lectin or arcelin-like lectin molecule in the seed extract of *C. virosa* using as many as 32 carbohydrates and three glycoproteins. Among them, 21 carbohydrates were found to inhibit the HA activity of crude seed extract of *C. virosa* against buffalo RBC and 13 carbohydrates against sheep RBC. Among the different monosaccharides tested, mannose (3.125 mM) was known to be a potent inhibitor of the hemagglutination activity of the crude seed extract against sheep, buffalo and rabbit RBC types. Trehalose and maltose (both at 3.125 mM) were also reported to be the potent inhibitors among the disaccharides tested.

Three glycoproteins namely, fetuin, mucin and thyroglobulin were used to assess their inhibitory potential on the HA activity of crude seed extract of *C. virosa*. All these glycoproteins inhibited the HA activity of the crude seed extract against buffalo, sheep and rabbit RBC types (except fetuin against rabbit RBC) with concentrations ranging from 0.019 to 1.25 mg/ml. In addition, thyroglobulin was found to be a strong inhibitor of the HA activity among the glycoproteins tested.

The preliminary studies made on the inhibition of HA activity in the crude seed extracts of *C. virosa*, mannose-Sepharose 6B matrix was used for the isolation of arcelin-like lectin in the seed extract of *C. virosa* by single step affinity column chromatography.

The affinity chromatographic procedure consistently yielded about 0.80 mg of arcelin-like lectin from each chromatographic run with about 16 folds increase in specific activity of this isolated molecule for buffalo and sheep RBC types from one milliliter of the seed extract of *C. virosa* containing 4.258 mg of total protein.
The arcelin-like lectin molecule isolated from *C. virosa* seed kernel extract appeared as a single protein band in native PAGE upon staining with coomassie brilliant blue or silver nitrate, thereby indicating that this molecule was isolated with high purity from the crude seed kernel extract, as established by its electrophoretic homogeneity.

Analysis of native molecular mass of the isolated arcelin-like lectin by gel filtration chromatography in FPLC system revealed a molecular weight estimate of approximately 98 kDa.

The protein profile analysis of isolated arcelin-like lectin from the seeds of *C. virosa* under denatured-PAGE with SDS and β-mercaptoethanol revealed five different polypeptide subunits with molecular weights 34, 24, 18, 16 and 14 kDAs respectively.

The HA activity of the pooled isolated fractions eluted from the affinity matrix was tested against twelve vertebrate RBC types. As observed with crude extract, almost the same pattern of HA activity was observed in all those nine RBC types.

The HA activity of isolated molecule was not affected by treatment with 10 mM EDTA. The addition of divalent cations Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ and Ni$^{2+}$ to the EDTA-treated arcelin-like lectin molecule did not cause any significant improvement in its HA titer.

Out of 32 carbohydrates used to assess their inhibitory potential of HA activity of isolated arcelin-like lectin from the seed kernel extract of *C. virosa*, 22 carbohydrates at the minimal inhibitory concentrations ranging from 0.78125 to 100 mM were found to inhibit the HA activity of isolated molecule against buffalo RBC. Likewise, 12 carbohydrates at the minimal
inhibitory concentration ranging from 6.25 to 100 mM inhibited the HA activity of the isolated molecule against sheep RBC and 5 carbohydrates at the minimal inhibitory concentration ranging from 25 to 100 mM inhibited the HA activity of the isolated lectin against rabbit RBC.

- Among the various carbohydrates used for the HA assay, trehalose (6.25 mM) was observed as the potent inhibitor of HA activity of isolated arcelin-like lectin against sheep RBC. Similarly, maltose and trehalose (both at a concentration of 0.78125 mM) were found to potentially inhibit the HA activity of isolated arcelin-like lectin by buffalo RBC. Mannose (1.5625 mM) and trehalose (25 mM) were also observed as potent inhibitor of HA activity of the isolated molecule against rabbit RBC respectively.

- Three glycoproteins inhibited the HA activity of isolated arcelin-like lectin against the various RBC types tested (except the fetuin against buffalo RBC) at the minimal concentration ranging from 0.004 to 5 mg/ml. Among them, thyroglobulin (the minimal inhibitory concentration was 0.004 mg/ml) was found as potent inhibitor of HA activity of isolated molecule against buffalo RBC. Mucin at the minimal inhibitory concentration of 0.009 and 2.5 mg/ml was reported as potent inhibitor of HA activity of isolated molecule against sheep and rabbit RBC types respectively.

- An important revelation in the present study was the finding that trehalose was a potent inhibitor of *C. virosa* arcelin-like lectin. Trehalose in insects play a major role as an energy store, a cryoprotectant, a protein stabilizer and a component regulating feeding behaviour and nutrient intake. Therefore, feeding insect pests such as *C. maculatus* on the arcelin-like lectin could deplete trehalose in the insect system and serve as antibiosis factor/anti-insect activity.
Mass and sequence analysis of 34 kDa polypeptide subunit of the isolated molecule from crude seed kernel extract of *C. virosa* was similar to those of arcelin, arcelin-1, arcelin-2 and arcelin-6 isoforms of *P. vulgaris* and lectin from *Canavalia cathartica*, *C. lineata* and *C. brasiliensis*. The sequences derived from MALDI-TOF/TOF analysis and peptide mass fingerprinting for the isolated molecule thus could be related to genes encoding arcelin cluster together with lectin in a phylogenetic manner. This purified protein molecule from *Canavalia virosa* therefore contained conserved amino acid sequences for multiple isoforms of arcelins and lectin is evidently demonstrated.

The effect of isolated arcelin-like lectin molecule from *C. virosa* on the development of the bruchid beetle, *C. maculatus* was investigated by incorporating the purified molecule in artificial seeds at varying concentrations ranging from 0.2 to 2.0% (w/w). The percentage of seed damage was reduced significantly while increasing the concentration of isolated molecule in the artificial seeds. There was no infestation in the artificial seeds that contain arcelin-like lectin molecule above 0.80%.

Finally, arcelin gene expression was performed in the seeds of selected seven wild pulse varieties using real time RT-qPCR. Arcelin gene specific real time PCR primers were synthesized from arcelin mRNA sequence of the wild pulse variety, *Lablab purpureus*. The results explained different levels of arcelin gene expression in the tested seed varieties. Fortunately, *Canavalia virosa* registered significantly the highest copy numbers of arcelin gene than other wild pulses.

On the whole, the significant findings of this study reveal the potential use of arcelin-like lectin isolated from the seed kernel of the plant species *Canavalia virosa* for the control of the stored product insect pest,
Callosobruchus maculatus. Furthermore, the isolated molecule was reported with a sugar binding specificity to mannose, maltose and trehalose. The trehalose binding specificity of arcelin-like lectin detected provides a new dimension for the application of such biopolymer especially in the control of stored product insect pests.