A mannose-specific lectin from the seeds of Canavalia rosea and its potential insecticidal activity against the stored product pest Callosobruchus maculatus

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Abstract

An attempt is made in the present investigation to test the efficacy of lectin purified from *C. rosea* against the major stored product insect pest, *C. maculatus*. Biochemical analysis carried out as the part of preliminary characterization revealed the presence of a mannose-specific lectin (*Cr*MBL) from the crude seed kernel extract of *C. rosea.* The haemagglutinating (HA) activity of the extract was found to be divalent cation independent and EDTA insensitive. Further, the HA activity remained stable between pH 5 and 8 and unaffected between temperatures 10ºC and 70ºC. The lectin, *Cr*MBL from the crude seed kernel extracts of *C. rosea* was purified through single step affinity chromatography using the mannose-coupled epoxy activated Sepharose 6B. The purified lectin showed highest affinity to the simple carbohydrate, mannose. Various biochemical assays of the *Cr*MBL revealed a similar profile of results as observed in the preliminary characterization. Its molecular characterization using gel electrophoresis revealed five different polypeptide subunits *viz*., 98 kDa, 80 kDa, 50 kDa, 30 kDa, and 15 kDa with a native mass of 273 kDa. Q-TOF-LC/MS of *Cr*MBL and MALDI-TOF-MS analysis of the 30 kDa polypeptide subunit of *Cr*MBL showed molecular similarity to several lectins, especially from Leguminosae family. The feeding bioassay with *Cr*MBL showed significant protection against the infestation of the stored product insect pest, *C. maculatus*. Lectin from *C. virosa* with more resistance to the activity of insect detoxifying enzymes could offer effective way of insect pest control with its suitability for employing this molecule as a component of integrated pest management.

Introduction

Lectins are known to possess several important functions in biological systems. Plants rely heavily on lectins for growth and protection and they release these molecules mainly to defend themselves towards pathogens and insect pests (Meyer et al. 2008; Karimi et al. 2012; Peumans and Van Damme EJ 1995a; Van Damme et al. 1998; Trigueros et al. 2003). Lectins possess an adverse effect on insect biological traits such as larval weight, mortality, feeding inhibition, abnormalities in overall development, adult emergence, and fecundity (Powell et al. 1993; Habibi et al. 1993). In addition, the insecticidal properties of certain lectins against a wide range of significant pests of insects have been thoroughly established, demonstrating their potential as natural insecticides. These molecules from many different sources of plants are proven to be lethal to those belonging to several groups of insects (Gatehouse et al. 1995; Powell 2001; Carlini and Grossi-de-Sá 2002).

Seeds particularly those from leguminous plants are contemplated to be copious source of lectins (Van Damme 2014; Van Damme et al. 1998; Lannoo and Van Damme 2010). Over the past two decades, significant advancement has been made in research on the application of biological effects of plant lectins against pest insects, particularly phytophagous insects (Peumans and Van Damme 1995b; Vasconcelos and Oliveira 2004; Sakthivelkumar et al. 2016). Many research efforts indicate that typical expression of plant-derived lectins in genetically manipulated crop plants, as well as the incorporation of purified lectins in artificial diets, have a negative impact on many pest insects from various orders such as Lepidoptera, Coleoptera, Diptera, and Hemiptera. It is conceivable that following ingestion of plant seeds or tissues by phytophagous insects, plant lectins are released from the broken cellular structures and come in contact with carbohydrate structures found in the midgut of insects (Vandenborre et al. 2011). Such plant-derived lectins have enormous promise as insecticides due to their capability to glycosylate the cells of insects' midguts. Although the actual mechanism of lectins working against insects is unknown, it is believed that lectins attach to certain glycoprotein structures found in insect midgut cells and impede nutrition absorption, resulting in the mortality (Macedo et al. 2015; Reyes-Montaño and Vega-Castro 2018). The quantity of lectins isolated from different bean cultivars varies substantially (Lam and Ng 2011). Lectin concentration among several legume seeds varies with the protein content of seeds that they have such as *Phaseolus vulgaris* (2.4–5.0%), *Glycine max* (0.8%), and *Pisum sativum* (0.6%) (Rüdiger and Gabius 2001; Ye and Ng 2001; Ye et al. 2000). In certain species, lectin concentrations can reach 50%, but in others, it accounts for less than 0.1% of total seed protein (Etzler 1986).

The biological activities of lectins include agglutination, antibacterial, antifungal, antiviral, anticancer and entomotoxic *etc* (Katoch and Tripathi 2021). Janzen and others (Janzen et al. 1976; Gatehouse et al. 1984) were the very first to describe the entomotoxicity with legume lectins, such as *Phaseolus vulgaris* lectin (PHA-E). In an artificial diet feeding with isolated lectin, insects were unable to survive at doses of even 0.10%. Many bean lectins have been studied for entomotoxic action against various insect pests. Melander et al. (2003) assessed the consequences of the agglutinin (PSA) from *Pisum sativum* on the development and longevity of the pollen beetle, *Meligethes aeneus*. Consequently, lectins have the potential to be a safer alternative to several chemical-based insecticides. Many research investigations suggest that plant lectins, particularly legume lectins through plant breeding, are capable of controlling stored product insect pests without depending on chemical insecticides. Based on this research background, the study was aimed at the detection, preliminary characterization and purification of lectin from the seed protein extracts of *Canavalia rosea* and to evaluate its insecticidal role on the stored product insect pest, *C. maculatus*.

Materials and methods

Wild pulse seeds

The seeds of coastal jack bean, *Canavalia rosea* were collected at the Thiruvanmiyur seashore in Chennai district, Tamil Nadu, India. The seeds were taken to the laboratory, cleaned, shade-dried and kept at room temperature or -20°C for long-term storage.

Seed kernel extract

The kernels of seeds were carefully pulverised with a mortar and pestle. 1 g of seed kernel powder was mixed and suspended with 10 ml of TBS buffer (50 mM Tris; 100 mM NaCl, pH 7.5). The resultant 10% dry weight/volume suspension was swirled well on a magnetic stirrer and left overnight at 10°C. The extract was centrifuged at 12000 rpm for 20 min at 4°C and the supernatant was collected and utilised for the work. Protein concentration was calculated using the method of Lowry et al. (1951) with BSA as a standard.

Physico-chemical characterization of crude seed kernel extract and purified lectin from C. rosea

Haemagglutination (HA) assay

To determine the presence of lectin in crude seed kernel extract of *C. rosea* and purified lectin sample, HA assays were conducted in V-bottom microtiter plates (Tarson). A 25 µl of sample was serially diluted with an equal volume of TBS buffer. Immediately, 25 µl of erythrocyte suspension (1.5% v/v) was added to every well and incubated at room temperature for 45 min. The HA titer was calculated as the reciprocal of the sample's maximum dilution resulting in the complete agglutination of erythrocytes (Garvey et al. 1977). Control consisted of replacing the sample with the buffer. Each experiment was repeated approximately three times, and then the agglutinating activity of the test sample was determined using the median agglutination titer values recorded for each RBC type. Human blood samples [A, B, and O (all positive blood types)] were collected from blood bank as gift. Blood samples including sheep, goat, ox, cow, and buffalo were collected at the Corporation slaughterhouse in Chennai, Tamil Nadu, India. Blood samples of rat (IEAC No.09/08/2021), and mice (IEAC No.01/15/2022) were obtained as gift from control animals at the laboratory through a venous puncture. The rabbit and hen blood cells were acquired from a local butcher’s shop in Chennai, India. Blood samples were taken in Alsever’s solution, refrigerated at 10°C, and utilised within five days.

Cross-adsorption assay

The 200 µl of 64 times diluted 10% crude seed extract of *C. rosea* was mixed with an equal volume of washed and packed RBCs (RBCs achieved higher agglutination titer in HA tests *i.e.*, buffalo, sheep, rat and mice erythrocytes as indicator cells) and incubated for 1 h at 4ºC with occasional shaking. The resulting suspension was centrifuged at 5000 rpm for 10 min at 10ºC. The supernatant was taken out and adsorbed again with fresh RBC types under the same conditions. The sample that absorbed three times was evaluated for HA activity.

Dialysis experiments

Dialysis experiments were performed to determine the approximate molecular size of the lectin molecules present in the crude seed extract. Two 500 µl aliquots of *C. rosea* crude seed extract were placed into a pretreated dialysis membrane that had an MW exclusion limit of 12–14 kDa (pre-treated dialysis tubing, Hi-Media, Mumbai). These samples were completely dialyzed at 10ºC against TBS pH 7.5. The dialysates were centrifuged at 12,000 rpm for 10 min at 4ºC. The residual activity of the supernatant was measured using HA analyses with indicator RBC types. Controls consisted of an un-dialyzed extract stored in the same conditions.

Divalent cation dependency and EDTA sensitivity on HA activity

To determine the divalent cation required to achieve HA activity in the crude seed extract of *C. rosea* and purified lectin, experiments were done with 50 mM TBS pH 7.5 alone and TBS containing various divalent cations at varied concentrations. The lectin activity in the samples was assessed using the indicator RBC types suspended in the appropriate buffers. In these tests, an extract of 25 µl was serially diluted using either cation-free or cation-containing TBS pH 7.5 ($CaCl_2$, $MgCl_2$ and $MnCl_2$). Following serial dilution, 25 µl of RBC suspension (1.5% v/v) was added in an appropriate buffer to each well and incubated for 45 min at room temperature. The HA titer was assessed.

The *C. rosea* crude seed kernel extracts and purified lectin (each 250 µl) were dialyzed (with MW exclusion limit of 12–14 kDa) against TBS pH 7.5 containing 10 mM EDTA, and then re-equilibrated by dialysis in cation-free TBS pH 7.5 to remove EDTA. The dialysates were

centrifuged (12000 rpm, 5 min, 10ºC) and the HA activity of the supernatant was checked through indicator RBC types in the presence of cation-free or cation-containing TBS pH 7.5 (10 mM $CaCl_2$, 10 mM $MgCl_2$, and 1mM $MnCl_2$).

pH stability and thermo-tolerance for HA activity

To assess the stability of 10% crude seed extract of *C. rosea* and purified lectin, HA activity over various pH ranges, 500 µl of the extract was dialyzed against the following buffers: acetate buffer (pH 3 to 6), tris-HCl buffer (pH 7 to 9), and glycine-NaOH buffer (pH 10 to 12). All samples were then re-equilibrated by dialysis in TBS (pH 7.5) at 4°C and centrifuged at 12000 rpm, 10 min at 10°C. The supernatant was then evaluated for HA activity using the indicator RBC type. The temperature stability was tested by keeping its 200 µl aliquots at temperatures ranging from 10 to 100°C for an hour. The samples were centrifuged at 12000 rpm for 10 minutes (10°C) to remove any debris, and the HA activity in the supernatant was assessed against the indicator RBC type.

HA inhibition assay

The carbohydrates (stock solutions at 200 mM) and glycoproteins (stock solutions

10 mg/ml) prepared in TBS pH 7.5 were used to examine for their efficacy in inhibiting HA activity using *C. rosea* crude seed extract and purified lectin against the indicator RBC types. The 10% crude seed kernel extract was diluted initially in TBS pH 7.5 to attain the HA titer of 4 towards indicator RBC types. The 25 µl of diluted crude seed extract was added to every well in the microtiter plate, followed by 25 µl of each test carbohydrate or glycoprotein solution, serially diluted up to 8 wells, and incubated for 1 h at room temperature. Immediately following incubation, 25 µl of 1.5% indicator RBC suspension was added to every well, incubated for 45 min at room temperature, and the HA activity was observed. The lowest possible concentration of the tested substances that completely inhibited HA activity was determined.

Purification of lectin molecule from the crude seed extract of C. rosea

Briefly, 1 ml of the affinity matrix *i.e.*, mannose-coupled epoxy activated Sepharose 6B was packed into a column (4.8 cm x 1.0 cm) and then equilibrated using

five-column volume of TBS (Tris-buffer saline: 50 mM Tris-HCl buffer + 110 mM NaCl, pH 7.5) at a flow rate of 5 ml/h. A total of 10 ml (0.5 ml filtered supernatant of 10% *C. rosea* seed kernel extract diluted with 9.5 ml TBS buffer, yielding a HA titer value of 4096) was passed on to the affinity column at a flow rate of 5 ml/h. The column was washed with 5 ml of TBS at a flow rate of 5 ml/h to remove unbound or free proteins. The bound proteins on the column matrix were eluted with 10 ml of TBS containing 0.2 M mannose. Each fraction of 1 ml was collected at a flow rate of 20 ml/h, and its absorbance at 280 nm was recorded. The purified lectin was analysed using denatured (Laemmli 1970) gel electrophoresis to determine the polypeptide fractions of purified lectin molecule.

Molecular characterization of purified lectin

The Agilent Q-TOF LC/MS, a liquid chromatograph Q-TOF mass spectrometer equipped with a quadrupole, a hexapole (collision cell), and a time-of-flight unit was used to generate MS/MS spectra with purified native lectin and the MS spectra was used to identify proteins. Clustal W was used to compare predicted peptide sequences from mass spectrometry (Thompson et al. 1994). MALDI-TOF-MS analysis was performed on the purified native lectin to determine molecular ion mass using absolute masses or m/z ratios. The purified lectin was initially run through SDS-PAGE. The major polypeptide band with mw of 30 kDa from PAGE was carefully exercised subjected to tryptic digestion of in-gel proteins (Huynh et al. 2009) for mass spectrometry using MALDI-TOF Brucker Autoflex max LRF. The mass spectra produced were then analysed to get the molecular ion mass.

Insect feeding bioassay to analyse the insecticidal properties of purified native lectin (CrMBL)

To assess the insecticidal effect of lectin isolated from the seeds of *C. rosea* on the development of *Callosobruchus maculatus*, an artificial seed system was used as described by Shade et al. (1986). Artificial seeds (~ 380 mg each seed) using native purified *C. rosea* mannose-binding lectin (named as *Cr*MBL) at the concentrations of 0.1%, 0.5%, and 1% were prepared by mixing them completely with the seed flour prepared from the most susceptible cowpea seeds *i.e.*, *Vigna unguiculata* to bruchid beetle infestation. The prepared flour was mixed with appropriate amount of autoclaved double distilled water to obtain a paste for the preparation of artificial seeds. The paste made was then applied to an acrylic seed mould. These acrylic seed moulds fixed with the seed paste were then air-dried at room

temperature for approximately 12 h and then the solid artificial seeds were gently taken out from the acrylic moulds. Finally, it was lyophilised for 6 h. After lyophilisation, these artificial seeds were used for insect feeding bioassay. Artificial seeds (12–14 seeds per treatment) were placed in plastic containers for infestation of *C. maculatus*. Newly emerged adults (5–6 pairs of both sexes) were released in to the plastic containers for mating and oviposition at ambient temperature. The control artificial seeds consisted of flour prepared only from susceptible cowpea seeds of *V. unguiculata* without *Cr*MBL. The infestation potential or seed damage was evaluated after 22 days in both control and experimental artificial seed bioassays to analyse the insecticidal effect native lectin.

RNA isolation, cDNA synthesis and spectrophotometric analysis

After the artificial insect bioassay, matured fourth instar larvae of the bruchid beetle, *C. maculatus* were collected from each treatment and utilised for total RNA isolation using TRIzol method. cDNA synthesis from the isolated RNA was carried out with RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific Company, USA). It was spectrophotometrically evaluated at 260 and 280 nm using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

Primer designing

Primers for the genes of insect detoxification enzymes such as glutathione S-transferase, cytochrome p450 and esterase were designed using IDT software (Coralville, IA, USA). Primer compatibility, including melting temperature, percentage of guanine (G) and cytosine (C), length, molecular weight, self and 3' complementarity were evaluated using the same software. To optimise polymerization efficiency and limit the influence of RNA integrity on gene expression in RT-qPCR, primers with a Tm of 58–61°C, length of 20–24 bp, and GC content of 45–60% were used. The primers are commercially synthesised by Eurofins Genomics India Private Ltd., India. 18S rRNA was chosen as an appropriate internal control for real-time RT-qPCR gene expression studies.

RT-qPCR

Real-time PCR amplification for all the detoxification enzymes from the larvae of *C. maculatus* for the various artificial seed bioassay experiments were done using an 8-well optically active strips (Applied Biosystems) under sterile conditions. The reaction conditions were: initial hold – 95ºC for 10 min; initial cycling stage – 95ºC for 15 sec; annealing – 58ºC (glutathione S-transferase), 59ºC (esterase) and 60ºC (cytochrome p450) for 1 min; initial melting curve stage – 95ºC for 15 sec; second melting curve stage – 60ºC for 1 min; final holding stage – 4ºC for 10 min. Reaction was carried out for 40 cycles. The melt curve programme was included with a warming rate of + 0.3ºC per sec. Each run included negative controls, such as nuclease-free water and internal control *i.e.*, 18S rRNA. Four replications were performed for each sample. Pfaffl's (2001) mathematical model was utilised to compute the relative expression ratios for each gene of interest.

Results

Detection and preliminary characterization of lectin in the crude seed kernel extract of C. rosea

A protein concentration of 14.87 µg/µl was observed in the 10% (w/v) crude seed kernel extract of *C. rosea*. HA profiles of the extract with TBS pH 7.5 free from cations demonstrated highest titre of 131072 against buffalo erythrocytes among various kinds of erythrocytes examined. The erythrocytes from sheep and rabbit demonstrated an equal titer value of 4096. This was followed by rat erythrocytes with a titer value of 512. Interestingly, no activity was recorded against goat, ox, cow, hen, human A, B, and O erythrocyte types. The 10% seed kernel extract diluted to 64 times with TBS buffer was adsorbed three times with erythrocytes *viz*., buffalo, sheep, mouse and rat which showed titer values in the HA assay were failed to agglutinate other RBC types. The EDTA sensitivity experiment demonstrated that the HA titer levels were 131072, as observed without EDTA using buffalo erythrocytes as indicator RBC type. Similarly, no influence of cations on the HA activity was observed when the sample was dialysed against Ca^{2+}, Mg^{2+} and Mn^{2+} containing 10 mM EDTA. Divalent cation dependency experiment demonstrated with the indicator RBC revealed similar HA titer value of 131072 for $MnCl^{2+}$, $MgCl^{2+}$ and $CaCl^{2+}$. The HA activity of the sample was unaffected between pH 5 and 8. HA activity of the sample was unaffected at 10ºC and 20ºC temperatures. But it was increased between 30ºC and 50ºC and unaltered at 70ºC. Out of 32 carbohydrates examined, 15 carbohydrates inhibited the HA activity of *C. rosea* crude seed kernel extract against indicator RBCs at concentrations ranging from 1.562 to 100 mM. Among the monosaccharides examined, 3.125 mM mannose was known to cause effective HA inhibition of the crude seed kernel extract against buffalo erythrocytes. Likewise, trehalose was also displayed as the most potent inhibitor of all disaccharides tested. The glycoproteins BSM, fetuin, and thyroglobulin were inhibited the HA activity with minimum inhibitory concentrations of 0.976 µg/µl, 3.906 µg/µl and 0.061 µg/µl, respectively.

Purification, physicochemical and molecular characterisations of lectin (Cr MBL) from the crude seed kernel extracts of C. rosea

Batch assay

The monosaccharide D-mannose, which showed a minimum inhibitory concentration of 3.125 mM showed through HA assay, was coupled to epoxy-activated Sepharose 6B for lectin purification from 10% crude seed kernel extract. Batch adsorption test was carried out to examine the binding ability of the lectin present in the crude extract of *C. rosea* with the coupled matrix. D-mannose coupled epoxy-activated Sepharose 6B (200 µl) was incubated with an equal volume (200 µl of 1:16 diluted 10% crude seed kernel extract) of sample after equilibrating with TBS buffer (110 mM NaCl, pH 7.5). The unadsorbed effluent was analysed for HA activity using the indicator RBC type, which showed a zero titer indicated the complete adsorption of the HA activity of the sample to the coupled matrix. Based on the positive results of the batch adsorption assay, which validated the efficacy of the D-mannose coupled epoxy-activated Sepharose 6B, this matrix was chosen for further large-scale purification of lectin using single-step affinity chromatography from crude seed kernel extract of *C. rosea*.

Purification of lectin from the seed kernel extracts of *C. rosea*

The extract (0.5 ml of 10% crude seed kernel extract of *C. rosea* diluted with 9.5 ml of TBS) with an initial HA titer of 4096 against indicator erythrocytes and a total protein concentration of 6.9 mg was passed through the column packed with 1 ml of D-mannose coupled epoxy-activated Sepharose 6B matrix at a flow rate of 5 ml/h. The matrix was then washed with 5 ml of 50 mM TBS buffer (110 mM NaCl, pH 7.5) at a flow rate of 5 ml/h to eliminate any unbound or free proteins. No loss of activity was observed in the unbound as well as in the washed fractions. After absorbance of the fraction (wash) nearly reached zero at 280 nm, the lectin molecules bound to the matrix were eluted using 5 ml of 200 mM D-mannose in 50 mM TBS buffer at a flow rate of 20 ml/h by competitive elution strategy. An elution profile for the purification of lectin from the crude seed kernel extract of *C. rosea* by affinity chromatography was depicted in Fig. 1. The eluted lectin fraction (E1) was dialysed against TBS that revealed the highest activity of 8192 against indicator erythrocytes. The purified lectin was designated as *Cr*MBL (*Canavalia rosea* mannose binding lectin) in the subsequent sections. Further, Table 1 summarizes the purification summary of *Cr*MBL that included the total activity, specific activity, purification fold and recovery. A purification fold of 10.50 and a recovery of 91.30% of HA activity was obtained.

Table 1
Purification summary of *Cr*MBL by affinity chromatography using D-mannose coupled epoxy activated Sepharose 6B

Sample	Volume (ml)	HA titer	Total protein concentration (mg)	Total activity (Units)[a]	Specific activity (Units/mg of total protein)	Purification fold[b]	Recovery (%)[c]
Crude	10	4096	6.9	1642857.14	238095.237	1	-
Eluate	4	8192	0.6	1500000	2500000	10.50	91.30

Data represent mean values of three separate experiments

[a] One unit of activity is defined as the minimum amount of protein required to give one well of haemagglutination

[b] Purification fold = Specific activity of total isolated protein / Specific activity of the crude protein

[c] Recovery of activity (%) = (Total activity of isolated protein / Total activity of crude protein) x 100

Minimum protein required for one well of HA activity	
Sample	Protein concentration (µg)
Crude	0.0042
Eluate	0.0004

HA and HA inhibition assay for the *Cr*MBL

HA profile for the *Cr*MBL from the seed kernel extract of *C. rosea* with TBS (pH 7.5) free from cations revealed the highest activity of 8192 for indicator erythrocytes. Out of 30 carbohydrates examined, 13 carbohydrates inhibited the HA activity of *Cr*MBL against indicator

erythrocytes *i.e.*, buffalo RBCs at minimal concentrations ranging from 3.125 to 100 mM. Among the carbohydrates used in the HA inhibition experiment, the most effective inhibitors of HA activity of *Cr*MBL of *C. rosea* were mannose (among monosaccharides), maltose and trehalose (among disaccharides) at its 3.125 mM concentration (Table 2). The glycoproteins namely, BSM and fetuin inhibited HA activity with minimum inhibitory concentrations of 3.906 µg/µl (Table 3).

Table 2
HA inhibition activity (HA Titer = 4) of *Cr*MBL from *C. rosea* with various carbohydrates

Carbohydrates tested	Concentration (mM*) of carbohydrates that inhibited the HA activity of purified lectin against buffalo erythrocytes
1. MONOSACCHARIDES	
Pentoses	
L-Arabinose	-
D-Xylose	-
Hexoses	
Dextrose	25
D-Galactose	50
D-Mannose	3.125
D-Fructose	6.25
L-Sarbose	50
Alcohol sugar	
Sorbitol	-
Mannitol	-
Adonitol	-
Inositol	-
Dulcitol	-
Salicin	25
Deoxy hexoses	
L-Fucose	-
L-Rhamnose	-
Hexosamines	
D (+) Glucosamines	100
D (+) Galactosamines	-
D (+) Mannosamines	-
N-Acetyl amino sugars	
N-Acetyl D-glucosamine	25
N-Acetyl D-galactosamine	-
N-Acetyl neuramic acid	-
2. DISACCHARIDES	
Maltose	3.125
Cellobiose	6.25
Lactose	-
Trehalose	3.125

*All the carbohydrates were examined at a concentration of 200 mM. HA inhibition assay was carried out three times; (-) No inhibition observed

Carbohydrates tested	Concentration (mM*) of carbohydrates that inhibited the HA activity of purified lectin against buffalo erythrocytes
Sucrose	50
Mellibiose	-
3. OLIGOSACCHARIDES	
Raffinose	25
4. POLYSACCHARIDES	
Inulin	-
Laminarin	-
*All the carbohydrates were examined at a concentration of 200 mM. HA inhibition assay was carried out three times; (-) No inhibition observed	

Table 3
HA inhibition activity (HA Titer = 4) of *Cr*MBL from *C. rosea* using glycoproteins

Glycoprotein tested (10 mg/ml)	Concentration (µg/µl*) of glycoprotein that inhibited the HA activity of purified lectin against buffalo erythrocytes
BSM	3.906 µg/µl
Fetuin	3.906 µg/µl
*HA inhibition assay was carried out three times	

Divalent cation dependency and EDTA sensitivity of the *Cr*MBL

The *Cr*MBL from *C. rosea* was assessed for its HA activity using various divalent cations for their dependency. This experiment demonstrated similar HA titer values with indicator erythrocytes that showed its insensitive nature to the tested cations such as Ca^{2+}, Mg^{2+} and Mn^{2+} (Table 4). The *Cr*MBL of *C. rosea* was assessed through HA activity for its EDTA sensitivity also demonstrated similar HA titer values as against titer value observed without EDTA. Likewise, adding cations such as Ca^{2+}, Mg^{2+} and Mn^{2+} resulted a same HA activity (Table 5).

Table 4
The divalent cation dependency on the HA activity of *Cr*MBL from *C. rosea*

*Cr*MBL treatment	Cations tested	HA titer* (Buffalo RBCs)
Untreated sample (control)	None	8192
Purified molecule dialysed against TBS	None	8192
	10 mM $CaCl_2$	8192
	10 mM $MgCl_2$	8192
	1 mM $MnCl_2$	8192
*Data based on the median value of three distinct determinations		

Table 5
The sensitivity of EDTA on the HA activity of *Cr*MBL from *C. rosea*

***Cr*MBL treatment**	**Cations tested**	**HA titer* (buffalo erythrocytes)**
Untreated sample (control)	None	8192
Purified lectin dialysed against TBS containing 10 mM EDTA and re-equilibrated with TBS	None	8192
	10 mM $CaCl_2$	8192
	10 mM $MgCl_2$	8192
	1 mM $MnCl_2$	8192
*Data based on the median value of three distinct determinations		

The pH stability and thermal tolerance for the *Cr*MBL

The HA activity of the purified lectin molecule, *Cr*MBL was unaffected at pH 7 and 8 and the activity was significantly reduced at pH above and below this range. The HA activity of *Cr*MBL was unaffected between temperatures 30ºC and 50ºC but decreased at temperatures < 30ºC and > 50ºC.

Analysis of *Cr*MBL using gel electrophoresis

The protein profile of *C. rosea* crude seed kernel extract and purified lectin *Cr*MBL fractions were analysed under denatured conditions using 12% SDS-PAGE in sample buffers with and without β-mercaptoethanol. Five different polypeptide subunits were observed in both the eluted lectin samples treated with and without β-mercaptoethanol showed no intermolecular disulfide bonds. The molecular weight of each subunit was calculated approximately as 98 kDa, 80 kDa, 50 kDa, 30 kDa, and 15 kDa, respectively (Fig. 2) with a total native molecular weight of 273 kDa.

Lane I	**:**	**10% Crude seed kernel extract (75 µg protein)**
Lane II	:	Purified *Cr*MBL from elution-1 with β-mercaptoethanol (35 µg protein)
Lane III	:	Purified *Cr*MBL from elution-1 without β-mercaptoethanol (35 µg protein)
Lane IV	:	Purified *Cr*MBL from elution-2 with β-mercaptoethanol (25 µg protein)
Lane V	:	Purified *Cr*MBL from elution-2 without β-mercaptoethanol (25 µg protein)

Mass spectrometry of purified CrMBL using Q-TOF LC/MS

Q-TOF-LC/MS analysis performed using *Cr*MBL revealed a deconvoluted chromatogram with four major peaks and other related ESI scan with various peptide mass values represented homology to several of the lectin molecules including Leguminosae

(Fig. 3).

The purified native *Cr*MBL analysed using Q-TOF LC/MS revealed a partial peptide sequence of m/z value 14312.950 was IHPSGHLELTNTSMRQIGQAFHGFPIPFLNPNS SNLVSFPTSFVFAITPGPGAPGHGLAFVISPSLDFSGALPSNYLGLFNTSNNGNSLNCILAVEFDTVQAVELNDIDDNHVGIDLNGVISIESTSAEYFDDR. This sequence revealed a peptide matching with protein sequence coverage of 21% to putative inactive L-type lectin-domain containing receptor kinase III.2 from OS = *Arabidopsis thaliana* OX = 3702 PE = 3 SV = 1. Another partial peptide sequence with m/z value 1170.5354 was VLPKVMAHMK. This sequence demonstrated peptide matching with protein sequence coverage of 55% to Melectin from OS = *Melecta albifrons* OX = 582867 PE = 1 SV = 1. The partial peptide sequence of m/z value 1170.5354 was AFDDGAFTGIR and that demonstrated peptide matching with protein sequence coverage of 40% to agglutinin alpha chain from OS = *Artocarpus tonkinensis* OX = 3492 PE = 1 SV = 1. The other partial peptide sequence of m/z value 1287.479210 with ANQTYFNFQR confirmed peptide matching with protein sequence coverage of 66% to 31 kDa lectin subunit from OS = *Vigna unguiculata* subsp. Sesquipedalis OX = 138955 PE = 1 SV = 1, a leguminosae plant lectin.

MALDI-TOF-MS analysis of a major 30 kDa polypeptide from purified *Cr*MBL

The MALDI-TOF-MS analysis of the 30 kDa polypeptide from purified native *Cr*MBL showed several m/z values with homologies against several lectin molecules from various sources were represented below (Fig. 4A & B).

The partial peptide sequence of m/z value 654.814 was FYETN. This sequence demonstrated peptide matching with protein sequence coverage of 33% to 29 kDa subunit lectin (fragment) from OS = *Vigna unguiculata* subsp. sesquipedalis OX = 138955 PE = 1 SV = 1. Another partial peptide sequence of m/z value 861.965 was EITKFPK and that confirmed peptide matching with protein sequence coverage of 43% to lectin from OS = *Delonix regia* OX = 72433 PE = 1 SV = 1. The partial peptide sequence of m/z value 3282.020 with IGFSATTGAEFAAHEVLSWSFHSELGGTSASK established peptide matching with protein sequence coverage of 59% to lectin alpha-1 chain from OS = *Lathyrus hirsutus* OX = 3857 PE = 1 SV = 1. One more partial peptide sequence with a m/z value 3282.020 was IGFSATTGAEFAAHEVLSWSFHSELGGTSASK. This sequence showed peptide matching with protein sequence coverage of 58% to mannose/glucose-specific lectin alpha chain from OS = *Lathyrus sativus* OX = 3860 PE = 1 SV = 1.

Insect feeding bioassay to analyse the insecticidal properties of purified CrMBL

Artificial insect feeding bioassay

The results of the artificial bioassay (Fig. 5) confirmed the anti-metabolic or insecticidal properties of the purified *Cr*MBL from the seeds of *C. rosea*. In control artificial seeds, 80% adult emergence of the stored product pest, *C. maculatus* was observed. Further, artificial seed damage was reduced from 90% recorded in control to 12%, 2.5% and 7% in various *Cr*MBL treated insects. The normal developmental time of the bruchid pest, *C. maculatus* was observed to be 22 days as against 17 days in the case of various *Cr*MBL treatment groups (Table 6).

Table 6
Effect of purified *Cr*MBL from *C. rosea* analysed through artificial feeding bioassay on the development of *Callosobruchus maculatus*

Purified lectin	Concentration of lectin (w/w) in artificial seeds (%)	Developmental periods (days)	Adult emergence (%)	Seed damage (%)
Control	0.0	22	80	90.00
*Cr*MBL	0.1	Nil	Nil	12.00
	0.5	Nil	Nil	2.50
	1.0	Nil	Nil	7.00

*Cr*MBL – *Canavalia rosea* mannose binding lectin

Isolation of total RNA, cDNA synthesis and quantification

It was observed that the presence of RNA in sufficient quantity for the synthesis of first-strand cDNA for real-time PCR experiments from the total RNA was isolated from control and purified *Cr*MBL treated fourth instar larvae of *C. maculatus*. The absorbance ratios of cDNA at 260 and 280 nm were almost 1 to 1.2 for control and treated samples, respectively, confirming the appropriate quality and amount of cDNA for real-time RT-qPCR experiments.

Retrieval of detoxifying enzyme gene sequences and primer design for Real Time RT-qPCR

Three detoxifying insect digestive enzymes were selected for the present study. The cytochrome p450 (accession number - FK669166) enzyme gene sequence was accessed. The glutathione-s-transferase (accession number - FK669177.1) and esterase (accession number - FK669192.1) were retrieved from GenBank, NCBI. All identified genes were the digestive enzymes from the genus *Callosobruchus*. Primers intended to amplify the chosen detoxifying digestive enzymes in control and treated fourth instar larvae of *C. maculatus* is presented in Supplementary Table 1. Furthermore, the housekeeping gene 18S rRNA as endogenous control was chosen, with a gene product predicted to be around 151 bp.

RT-qPCR analysis on cytochrome p450 gene for CrMBL treated larvae of *C. maculatus*

The mean threshold cycle (CT) values and the relative quantification (RQ) of target detoxification enzyme cytochrome p450 and endogenous control are summarised in Supplementary Table 2. The threshold fluorescence for cDNA of various doses of *Cr*MBL treated larvae demonstrated a higher level of expression of the cytochrome p450 gene in the *Cr*MBL treated larvae when compared to control except 1% *Cr*MBL treatment. An increase in amplification fold of cytochrome p450 gene at 0.1% *Cr*MBL treatment was 9.88 and 0.5% *Cr*MBL treatment was 9.78 over control larvae. But at 1% *Cr*MBL treatment, the amplification fold was 0.53 times less in expression of the cytochrome p450 gene in the fourth instar larvae of *C. maculatus* when compared to the control (Fig. 6).

RT-qPCR analysis on glutathione S-transferase gene for *Cr*MBL treated larvae of *C. maculatus*

The mean CT value and the relative quantification of target detoxification enzyme glutathione S-transferase and endogenous control are summarised in Supplementary Table 3. The threshold fluorescence for cDNA of various doses of *Cr*MBL treated sample attained 9.61 cycles for 0.1%, 9.07 cycles for 0.5%, and 11.47 cycles for 1% *Cr*MBL treatments demonstrating a high level of expression of the glutathione S-transferase gene when compared to control larvae. An increase in amplification fold of glutathione S-transferase gene at 0.1% *Cr*MBL treatment was 5.55, 0.5% *Cr*MBL treatment was 8.0 and 1% *Cr*MBL treatment was 41.49 as overexpression of the gene when compared to control larvae (Fig. 7).

RT-qPCR analysis on esterase gene for *Cr*MBL treated larvae of *C. maculatus*

The mean CT value and the relative quantification of target detoxification enzyme esterase and endogenous control are summarised in Supplementary Table 4. The threshold fluorescence for cDNA of various doses of *Cr*MBL treated sample attained 17.70 cycles for 0.1%, 20.94 cycles for 0.5%, and 18.93 cycles for 1% *Cr*MBL treatments demonstrating a high level of expression of the esterase gene when compared to control larvae. An increase in amplification fold of esterase gene at 0.1% *Cr*MBL treatment was 26.86, 0.5% *Cr*MBL treatment was 14.73 and 1% *Cr*MBL treatment was 10.42 by means of overexpression of the gene when compared to control (Fig. 8).

Discussion

Legumes are essential crops in several tropical countries and serve as vital dietary sources of carbohydrates and proteins for mankind (Singh et al. 1985). The bruchid weevil, *C. maculatus* is a major insect pest of legumes that attacks the seeds during storage and possesses detrimental effects on the produce's quality and storability. During severe infestations, postharvest seed losses due to *C. maculatus* can reach up to 100% (Hall et al. 1997). The bruchid beetle, *C. maculatus* is a significant storage pest of cowpea, *Vigna unguiculata* and other legumes throughout many developing countries (Taylor 1981). Owing to their short life cycle and high fertility, even modest infestations at harvest can lead to an entire loss of stored pulses in a short time. Substantial research was carried out to find plant proteins having insecticidal activities against major insect pests (Ferrari et al. 1991; Aronson 2010). Among them, two families of proteins namely, lectins and protease inhibitors, possessed the highest promise for application in insect pest control (Jouanin 1998). The wheat (WGA) and bean (PHA) lectins have been demonstrated to be particularly efficient under *in vitro* experiments towards coleopteran and lepidopteran insects (Janzen et al. 1976; Gatehouse et al. 1984; Czapla and Lang 1990; Murdock et al. 1990; Harper et al. 1995). Hemipteran insects are highly sensitive to mannose and glucose specific lectins (ConA), especially those from *Canavalia ensiformis* (Van Damme et al. 1988). Among the most extensively researched of these in terms of insecticidal characteristics is the snowdrop lectin (*Galanthus nivalis* agglutinin; GNA), with its negative effects *in vitro* on the biology of Aphididae, Delphacidae, and Cicadellidae (Rahbé et al. 1995; Down et al. 1996; Sauvion et al. 1996; Powell et al. 1998). GNA expression in plants, such as potatoes, rice, and wheat, was known to adversely affect sap-sucking insects (Gatehouse et al. 1996; Rao et al. 1998; Stoger et al. 1999; Foissac et al. 2000).

Despite being substantially similar in structural features of lectin molecules, Leguminosae contains lectins with diverse carbohydrate specificities (Sharon and Lis 1990). Their particular carbohydrate-binding ability plays an essential role in their interactions with other organisms (Diaz et al. 1989). It was hypothesised that lectin binding in the peritrophic matrix or contact with glycoproteins on the epithelial cells of insect gut may lead to nonspecific interference with food digestion and absorption (Lannoo and Van Damme 2010; Chrispeels and Raikhel 1991; Eisemann et al. 1994; Zhu-Salzman et al. 1998). Lectins' resistance to proteolysis allows them to resist the activity of multiple insect proteases and reach their target locations in insect guts. Their carbohydrate affinity then directs the lectins towards certain cells or tissues. Biochemical stability and carbohydrate binding are essential to the defensive action of anti-insect legume lectins (Zhu-Salzman and Salzman 2001). Several natural lectins, including *Datura stramonium* lectin, *Solanum tuberosum* lectin, *Griffonia simplicifolia* seed lectin II, *Ulex europaeus* lectin II, and wheat germ agglutinin, are resistant to the activity of insect digestive enzymes (Rahbé et al. 1995; Powell et al. 1998; Zhu-Salzman et al. 1998).

In the present research study, an attempt was made to initially identify lectin from the seed kernel extracts of *C. rosea* followed by its physiochemical characterisations. Haemagglutination tests were carried out using various vertebrate erythrocytes. The crude seed extract of *C. rosea* as well as the purified lectin, *Cr*MBL revealed a highest titre against buffalo RBCs. This was followed by rabbit and sheep erythrocytes with an equal titre value. Santiago et al. (2014) discovered that the crude extract of *Calceolaria oxyphylla* seeds exhibited high agglutination activity against native rabbit erythrocytes. Studies on lectins in many species of seeds from *Canavalia* genus revealed that the activity was dependent of divalent-cations and was found to be EDTA sensitive. In contrast to these reports, experiments on cation dependency and EDTA sensitivity revealed that the HA activity of crude seed extract of *C. rosea* and *Cr*MBL was found to be cation-independent and insensitive to EDTA *i.e.*, the presence or absence of divalent cations and EDTA had no effect on HA activity. After treating the lectin from *C. oxyphylla* with EDTA, its haemagglutination activity remained unaltered. CoxyL, unlike other leguminous lectins, was found to be divalent cations independent perhaps due to their strong binding to the protein (Loris et al. 1998; Cavada et al. 2001). Although this characteristic feature is notably unusual in the case of lectins from the *Diocleinae* subtribe where the haemagglutinating activity associated with other *Diocleinae* lectins, especially those from *Canavalia cathartica*, was not impacted by EDTA (Susheelan et al. 2007). Previous studies have reported that the HA activity of lectin from genus *Canavalia* remained stable up to a temperature of 60ºC. *Cr*MBL remained thermally stable even up to a temperature of 100ºC. Further, studies on the effect of pH on the HA activity revealed that the activity remained stable over wide range of pH. The lectin, CoxyL from *C. oxyphylla* was demonstrated to be thermostable, with complete activity up to 60°C. CoxyL at various pH levels revealed that the lectin displayed maximal activity at pH 7.0, indicating that the lectin was more stable in this condition (Santiago et al. 2014). Interestingly, among 30 different carbohydrates tested, trehalose and mannose were found as a potent inhibitor of HA activity of crude seed kernel extracts of *C. rosea* and purified *Cr*MBL. The lectin (CoxyL) was found to be specific for D-glucose, D-mannose, and α-methyl-D-mannoside. Glycans found in ovalbumin and fetuin could inhibit CoxyL's haemagglutinating action, possibly due to the inclusion of mannose in its composition. CoxyL's specificity profile is comparable to that of other *Canavalia* lectins.

C. rosea lectin purified from the seed cotyledons was found to be D-mannose specific with a MIC of 3.125 against buffalo erythrocytes. Further the activity was found to be independent of divalent cations and EDTA insensitive. A high tolerance to pH and temperatures was noticed over similar lectins isolated and characterized from the seeds of other *Canavalia* species (Santiago et al. 2014; Loris et al. 1998; Cavada et al. 2001). The ultimate purity of isolated *Cr*MBL in our study was validated by gel electrophoresis. It revealed five polypeptide subunits with different molecular weights *viz.*, 15 kDa, 30 kDa, 50 kDa, 80 kDa and 98 kDa with a total molecular mass of 273 kDa. The 30 kDa major subunit analysed using MALDI-TOF-MS showed partial peptide sequence of m/z values that matched with already known plant lectin, especially with legume lectin sequences.

From the last few decades, the insect pest control approach for the stored product pests has been dominated by the use of synthetic pesticides through fumigation, and satisfy increased food demand. However, the extensive and constant application of such chemicals caused environmental damage, produce contamination, and the development of resistant pest populations (Xu et al. 2013; Akami et al. 2017; de Souza et al. 2018). Insect resistance to chemicals may be due to enzymatic reactions such as mutation, repression, or the overexpression of insecticide-targeted genes (Whalon et al. 2008; Ranson et al. 2011; Montella et al. 2012) and that permit insects to overcome and degrade active chemicals into hydrophilic constituents less harmful to the insects (Hammer and Bowers 2015). In the present work, the purified mannose-binding lectin *Cr*MBL from *C. rosea* were examined for their insecticidal action through an artificial diet by an insect feeding bioassay experiments against the bruchid pest, *C. maculatus*. This assay revealed that there was no adult emergence in all the concentrations of *Cr*MBL. Similarly, the percentage of seed damage was significantly less in all the tested concentrations when compared to control.

Subsequently, the larvae of *C. maculatus* subjected to various concentrations of lectin purified from *C. rosea* at its native form were tested for the profiles of its detoxification enzymes. After the bruchid feeding bioassay, the larvae of *C. maculatus* obtained from various artificial seed feeding experiments that contain different doses of *Cr*MBL were subjected to RT-qPCR expression analysis for the detoxification enzymes. The results on the expression revealed that there was an upregulation of various detoxification enzymes in the tested lower concentrations of *Cr*MBL indicating the cope up of the larvae with the toxic compound with metabolic resistance. However, at higher doses of *Cr*MBL there was a decline in the levels of its detoxifying enzymes that proved the toxic nature of *Cr*MBL against the insect resistant mechanisms. Developing insect enzyme-resistant defence proteins most likely represents a mechanism established in some plants by evolution against herbivory (Michaud 1997). For example, the inability to digest and detoxify such defence proteins especially vicilins by *C. maculatus*, a kind of storage proteins from diverse legumes explained its insecticidal effect (Yunes et al. 1998). Therefore, higher doses of *Cr*MBL from *C. virosa* with more resistance to the activity of insect detoxifying enzymes could provide effective means of control with its suitability for employing this molecule as a component of integrated pest management.

Declarations

Conflict of interest

The authors have declared no conflict of interests.

Author Contribution

"M. M. Conceptualization, conduct of experiments, validation, data curation, writing-original draft, visualization; R. N. Formal analysis, investigation, data curation; A. P. M. D. Validation, data curation, visualization; S. J. Resources, conceptualization, editing, supervision, project administration. All authors reviewed the manuscript"

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Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

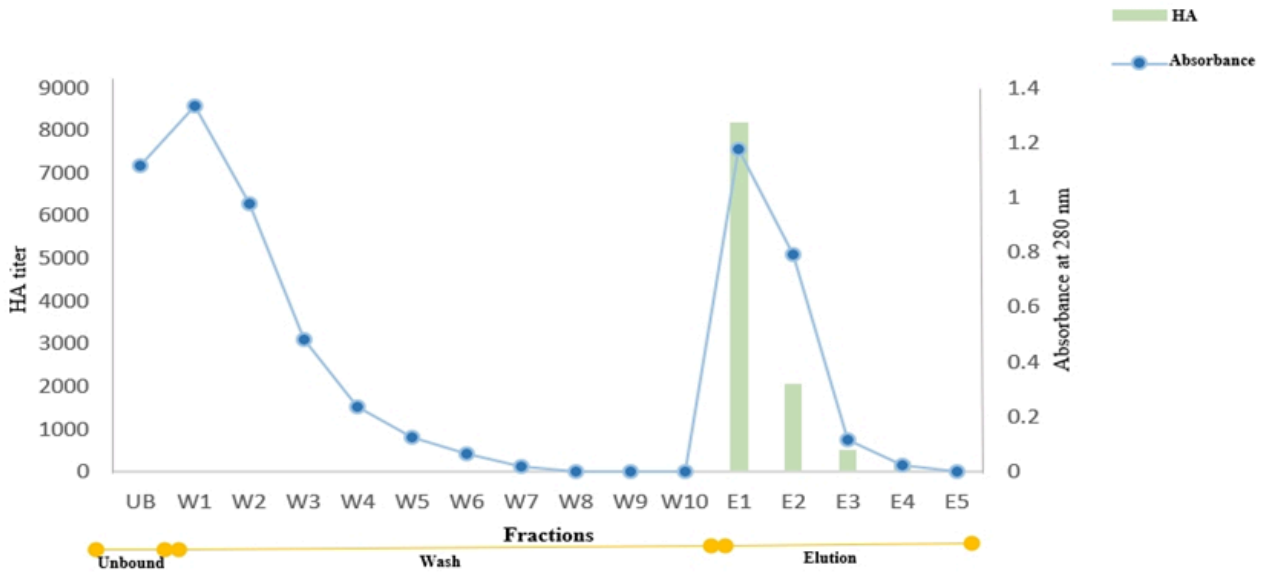

Figure 1

Elution profile of CrMBL with affinity column chromatography on D-mannose coupled epoxy-activated Sepharose 6B

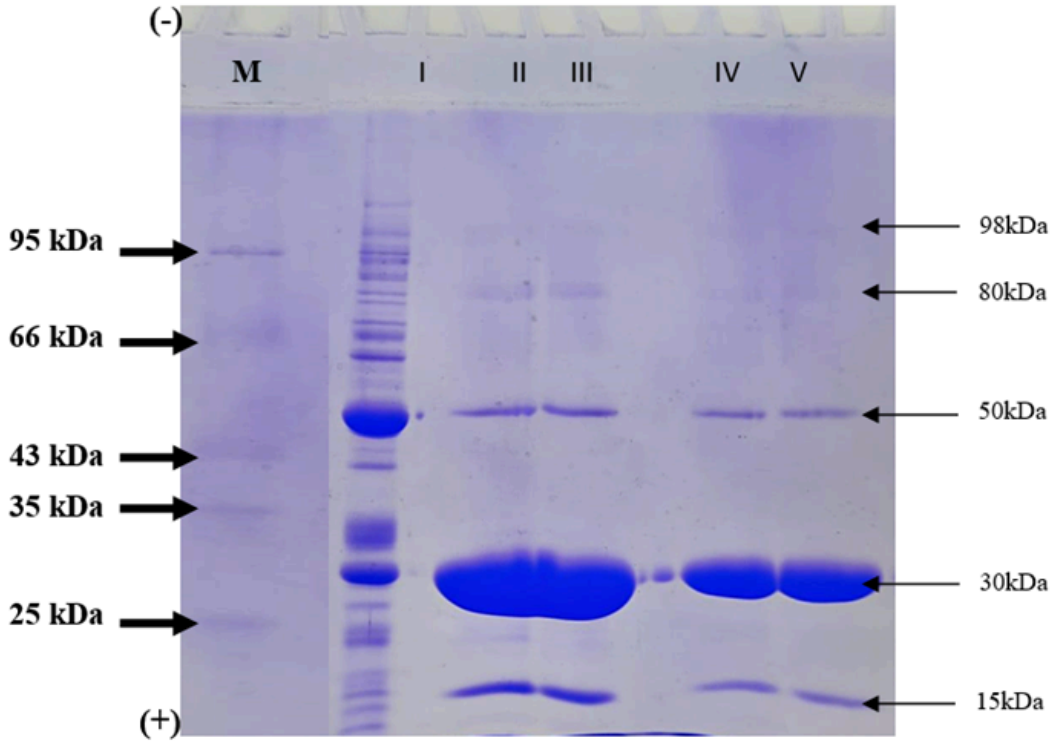

Figure 2

SDS-PAGE (12 %) analysis of total proteins from crude seed kernel extracts of *C. rosea* and purified *Cr*MBL

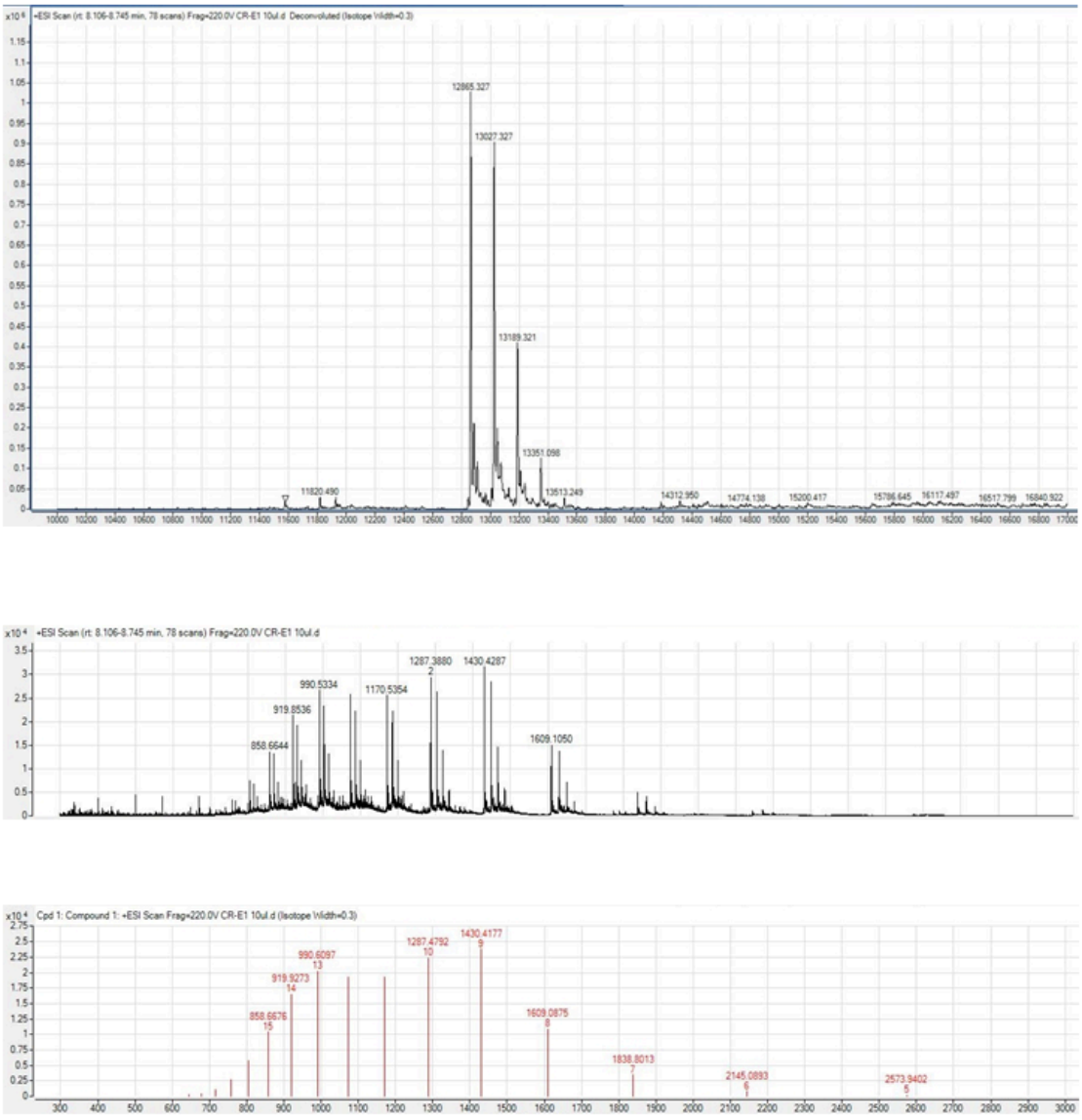

Figure 3

Peptide mass fingerprint of native lectin *Cr*MBL obtained using Q-TOF LC/MS

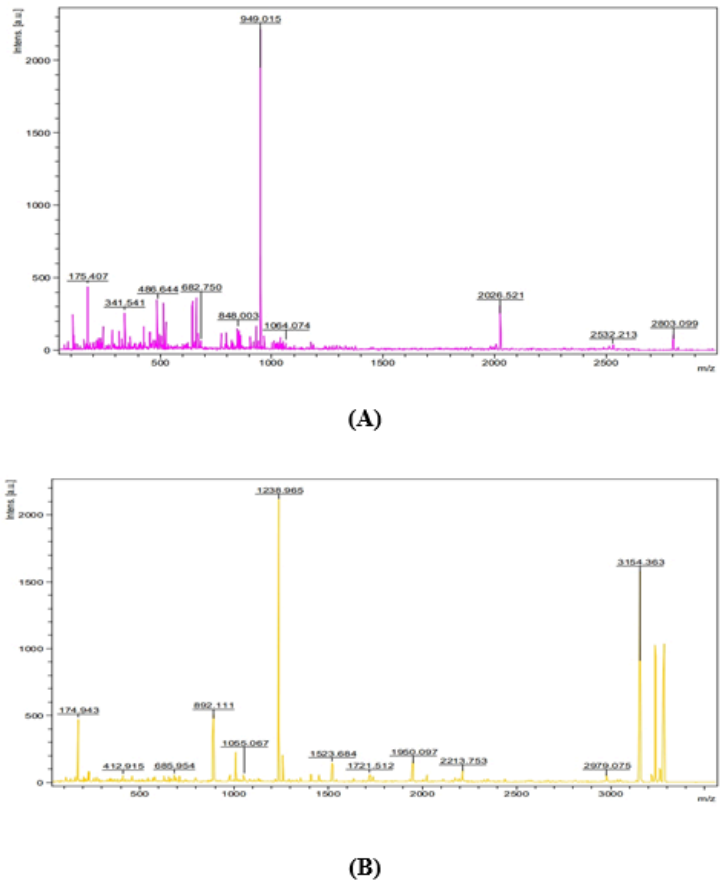

Figure 4

A and B Multiple spectrum report on the peptide mass fingerprint for 30 kDa polypeptide from native *Cr*MBL obtained using MALDI-TOF-MS analysis

Figure 5

Artificial seeds with *Cr*MBL subjected to *C. maculatus* insect feeding bioassay

A - Artificial seeds showing oviposition of eggs by the adult *C. maculatus*

B - Artificial seeds containing developing larvae of *C. maculatus* inside the seeds

C & D - Infestation of artificial seeds showing emergence holes by adults of *C. maculatus*

E & F - Collection of matured larvae from infested artificial seeds

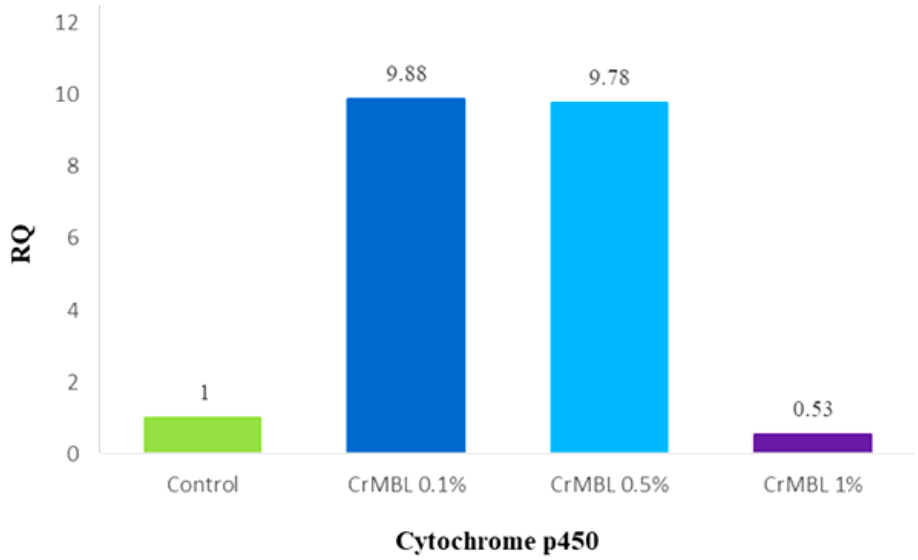

Figure 6

Gene expression plot for *Cr*MBL treated larvae of *C. maculatus*showing RQ for cytochrome p450 gene expression

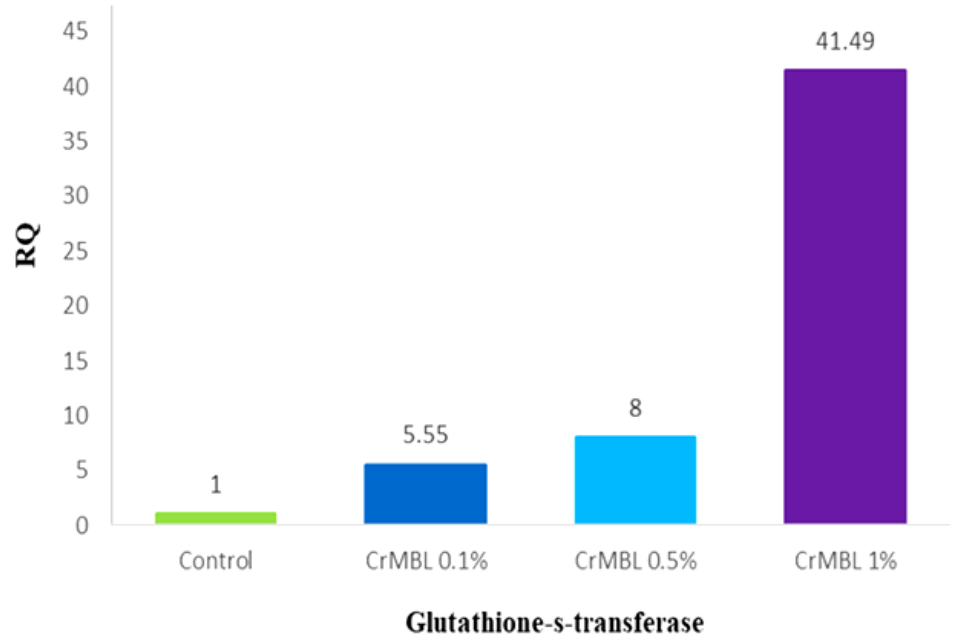

Figure 7

Gene expression plot for *Cr*MBL treated larvae of *C. maculatus*showing RQ for Glutathione S-transferase gene expression

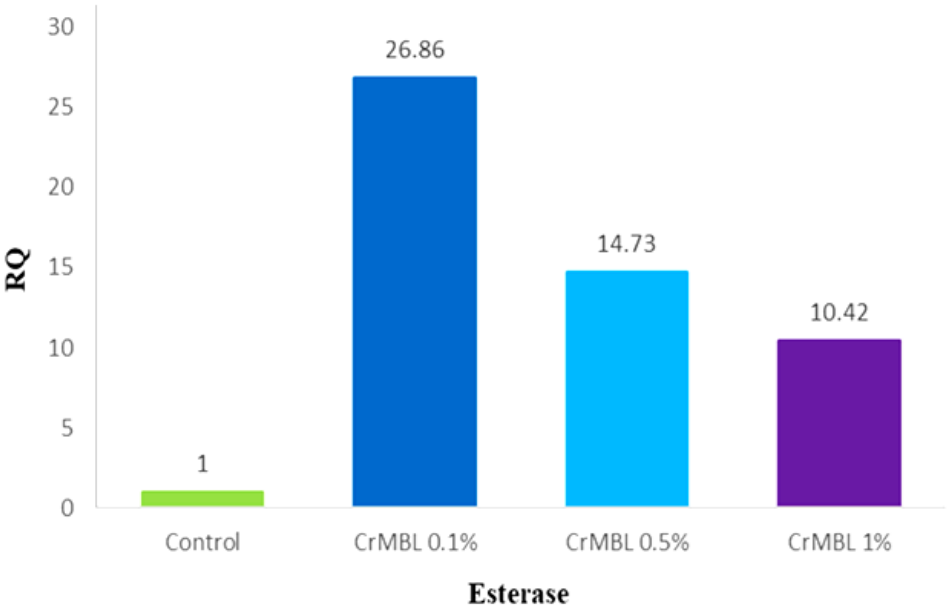

Figure 8

Gene expression plot for *Cr*MBL treated larvae of *C. maculatus* showing RQ for esterase gene expression

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.doc