



## Purification, Characterization and Antimicrobial Properties of Hemolymph Lectin from the Larva of Red Palm Weevil, *Rhynchophorus ferrugineus*

Arokya Glory PT<sup>1</sup>, Basil Rose MR<sup>2</sup>, Josephine Priyatharshini C<sup>3</sup>, Anitha C<sup>4</sup>, Venci Candida X<sup>5</sup>

<sup>1,2,3,4,5</sup>Department of Zoology, Holy Cross College (Autonomous), Nagercoil, Affiliated to Manonmaniam Sundaranar University, Tirunelveli – 627 012, Tamil Nadu, India.

\*Corresponding author's E-mail: [arokyaglory@gmail.com](mailto:arokyaglory@gmail.com)

Article History	Abstract
Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 02 Nov 2023	<p>Lectins are renowned hemagglutinins and multivalent proteins with a well-known quality for sugar-binding specificity that participate significantly in invertebrate defense functions. Studies on biological activity of lectin from coleopteran insect are very scarce. A lectin with specific affinity for N-acetyl neuraminic acid was purified from the hemolymph of the larva of the red palm weevil, <i>Rhynchophorus ferrugineus</i> by biospecific adsorption using formalinized rabbit erythrocytes and affinity chromatography using PSM-linked cyanogen bromide activated Sepharose 4B. The specific activity of the lectin purified by affinity chromatography was much higher than the lectin purified by biospecific adsorption. The binding specificity of the weevil lectin distinguishes it from other known insect lectins. Like the crude agglutinin, the lectin purified by affinity chromatography also showed the same pattern of specificity towards erythrocytes. However, 4-to-8-fold decrease in HA titer was observed when tested with the purified lectin. In the same way, reduction is also observed in the HAI titer of the purified lectin with most of the inhibitors except PSM where the HAI titer was identical both in the crude agglutinin and purified lectin. Sugars N-acetyl neuraminic acid, N-acetyl mannosamine and N-acetyl-D-galactosamine inhibited the HA titer of the purified lectin with greater efficacy than the crude agglutinin. The sialic acid specificity of the lectin was confirmed by 16-fold reduction in HA titer with asialo rabbit erythrocytes and 32-fold reduction in HAI titer with desialylated PSM. The purified lectin is homogenous on sodium dodecyl sulphate-polyacrylamide electrophorogram with a molecular weight of about 60 kDa. The lectin showed antimicrobial activity against pathogenic bacteria <i>Streptococcus mutans</i>, <i>Escherichia coli</i>, <i>Klebsiella pneumoniae</i> and <i>Pseudomonas aeruginosa</i> and fungi <i>Candida albicans</i> and <i>Aspergillus niger</i>.</p>
CC License CC-BY-NC-SA 4.0	<b>Keywords:</b> Antimicrobial, hemolymph, lectins, SDS-PAGE

### 1. Introduction

Agglutinins/lectins are one of the several types of biological resources endowed with biomedical values which could be exploited for their therapeutic and diagnostic potential. They have many biologically significant activities such as, the ability to agglutinate red blood cells, lymphocytes, fibroblasts, spermatozoa, fungal, bacterial and plant cells (Lis and Sharon, 1986) and have found application in cell identification and separation (Kobayashi et al., 1989), detection, isolation and structural studies of glycoproteins (Alroy et al., 1984), investigation of carbohydrates on cells and subcellular organelles (Sharon and Lis, 1989), glycoprotein biosynthesis (Wang et al., 2006) and in diverse fields of clinical interest such as typing of blood cells, carriers of chemotherapeutic agents and as epidemiological and taxonomic markers of specific microorganisms (Slifkin and Doyle, 1990), mapping of neuronal pathways (Yoshihara et al., 1999), mitogenic stimulation of lymphocytes (Gunther et al., 1974), purging of bone marrow for transplantation (Rhodes, 1998) and to study the cell surface receptors of various bacteria, protozoa and fungi and to determine bacterial cell wall components and bacteriophage receptors (Sharon and Lis, 1989).

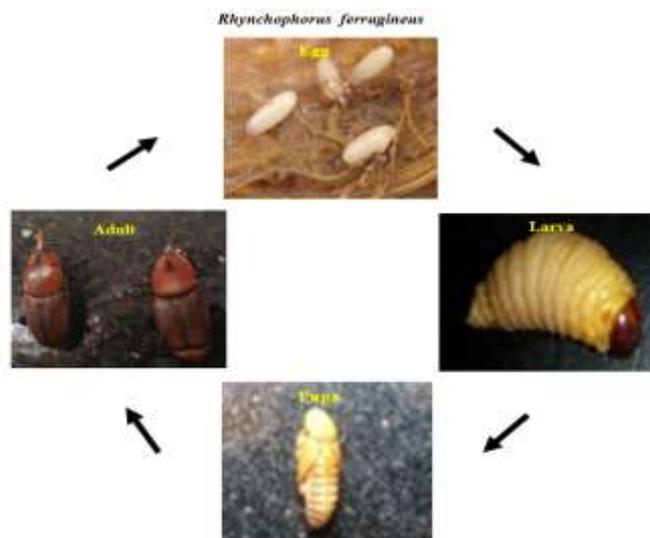
In the past few years, several hundreds of lectins have been purified and characterized in detail taking into consideration their biochemical properties, sugar-binding specificities and several biological activities (Tian et al., 2009). The methods used involve salt induced crystallization, ethanol precipitation, ion exchange chromatography, gel filtration (Sharon and Lis, 2004) ammonium sulfate precipitation followed by ionic and affinity chromatographic steps and a final protein-polishing step (Spelzini et al., 2005). The introduction of affinity chromatography for the purification of lectin was a turning point in the field of lectinology and immensely increased the pace of purification of lectins from various sources (Goldstein et al., 1980).

Affinity chromatography depends on the specific interaction between the lectin and a carbohydrate structure attached to an inert matrix (Agrawal and Goldstein, 1967) which depends essentially on the interaction between the molecule to be purified and a solid phase where mono, di- and polysaccharides as well as glycoprotein ligands are immobilized which will allow the separation of contaminants. Usually, glycans contain a variety of carbohydrates and therefore can be used for the isolation of lectins with various specificities (Lis and Sharon, 1981). Since affinity purification results in maximum recovery and great purity of the lectin, efforts are taken to purify the larval hemolymph lectin of the red palm weevil, *Rhynchophorus ferrugineus* by affinity chromatography and investigate its antimicrobial properties.

## 2. Materials And Methods

### Animal collection and maintenance

The larvae of *Rhynchophorus ferrugineus* were collected from dead and decaying coconut trees found in the coconut groves of Azhagappapuram, Kanniyakumari District, Tamil Nadu, India. The collected larvae were maintained in plastic containers containing cut petiole or stem tissues from coconut tree as recommended by Rananavare et al. (1975).



**Fig. 1.** Life cycle of red palm weevil, *Rhynchophorus ferrugineus*

**Table 1.** Systematic position of the red palm weevil, *Rhynchophorus ferrugineus*

<b>Phylum</b>	Arthropoda
<b>Sub phylum</b>	Mandibulata
<b>Class</b>	Insecta
<b>Order</b>	Coleoptera
<b>Family</b>	Curculionidae
<b>Genus</b>	<i>Rhynchophorus</i>
<b>Species</b>	<i>ferrugineus</i>
<b>Common name</b>	Red palm weevil

### Purification of lectin

#### Materials

Polypropylene econo columns were purchased from Bio-Rad; Enzymes, Cyanogen bromide activated Sepharose 4B, sugars, glycoproteins and *Clostridium perfringens* Neuraminidase (Type X) were purchased from Sigma, Bengaluru, Karnataka, India.

## Buffers

Buffers used in this study are identified in the text by the following abbreviations: TBS (Tris Buffered Saline): 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM CaCl<sub>2</sub>; TBS-BSA: (Tris Buffered Saline with Bovine Serum Albumin): TBS, pH 7.5, with 0.05% BSA; HSB (High Salt Buffer): 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM CaCl<sub>2</sub>; LSB (Low Salt Buffer): 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 10 mM CaCl<sub>2</sub>; EB: (Elution Buffer): 50 mM Tris-HCl, pH 8.2, 0.3 M NaCl, 10 mM disodium EDTA; Acetate buffer: 0.1 M sodium acetate, 0.5 M NaCl, pH 4; Coupling buffer: 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.3; PBS-BSA: (Phosphate-Buffered Saline): 75 mM NaCl, 75 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0 and PBS-BSA (Phosphate-Buffered Saline with Bovine Serum Albumin): PBS, pH 7.0, with 0.05% BSA.

## Preparation of larval hemolymph

The larvae were exposed to chloroform vapours for 10 minutes to avoid clotting or clumping of blood cells. The rare end of the larva was cleaned with cotton swab containing 70% ethanol. The posterior tip of the larva was cut with sterile scissors and the hemolymph was collected in a sterile centrifuge tube containing phenylthiourea placed on ice. The cellular elements were removed by centrifugation for 10 minutes at 2000 rpm at 4°C and the serum was stored at -20°C. Prior to the purification of the lectin, the major portion of the high molecular weight proteins were made to sediment by centrifuging the hemolymph at 3 x 10<sup>4</sup> g for 5 hours at 4°C (Beckman x L90 Rotor SW 28).

## Affinity chromatography

As PSM inhibited the hemagglutination activity of the hemolymph with rabbit erythrocytes, the hemolymph was purified using PSM-Sepharose 4B affinity column.

## Preparation of PSM agarose affinity gel

The affinity gel was prepared using the commercially available Cyanogen bromide activated Sepharose 4B.

## Swelling and washing the gel

One gram of freeze-dried cyanogen bromide activated Sepharose 4B powder swollen for 15 minutes in 1 mM HCl was washed in a sintered glass funnel (porosity G3) with the same solution. A total of 200 ml of 1 mM HCl per gram dry gel powder was added in several aliquots. One gram freeze dried powder gave a gel volume of approximately 3.5 ml. The HCl is used to preserve the activity of the reactive groups which hydrolyze at high pH.

## Coupling of glycoproteins

After washing the gel with 5 ml of coupling buffer, it was immediately transferred to a solution of porcine stomach mucin (5 mg/ml). As the reactive groups on the gel may get hydrolyzed at the coupling pH, the above stage was finished fast with no delay. A gel buffer ratio of 1:2 provides a suitable suspension for coupling. The mixture of porcine stomach mucin (PSM) and swollen gel was rotated end over end for 2 hours at room temperature (30°C ± 2°C).

The degree of coupling was monitored by the concentration of PSM (protein) in the coupling medium. Finally, to quench the excess of activated group (if present), 5 ml of 1 M ethanolamine-HCl, pH 8.3 was added and gently mixed for an additional hour. Approximately 70-80% of the PSM got coupled. The PSM-Sepharose 4B was then packed and stored in polypropylene econo column containing cold TBS (pH 7.5) with 0.02% sodium azide.

## Purification of lectin from the hemolymph of the larva of red palm weevil, *Rhynchophorus ferrugineus* using affinity chromatography

Clarified serum (50 ml) was applied to 3.5 ml of PSM Sepharose 4B in an econo column (Bio-Rad) previously equilibrated with TBS at 4°C. The eluant was collected at a rate of 0.6 ml/minute. The column was washed with HSB until A<sub>280</sub> of the effluent was <0.002 and the fractions collected were tested for HA activity. The column was further washed with LSB at 4°C until the A<sub>280</sub> of the effluent was < 0.002 and then it was washed with warm LSB (30 ± 2°C) until the A<sub>280</sub> of the effluent was <0.002. This step further eluted additional inert proteins and was necessary for obtaining homogenous lectin. All the buffers used so far contained the calcium required for lectin binding to PSM-Sepharose 4B. The elution of lectin was done with elution buffer that contained 10 mM disodium EDTA and collected as 1 ml fractions in polypropylene tubes containing 10 µl of 10 mM calcium chloride placed on ice at the rate of 0.3 ml/minute. The fractions were vortexed immediately after collection and kept on ice.

Fractions containing lectin were pooled on the same day and dialyzed against 10 mM CaCl<sub>2</sub> at 4°C for 5 minutes and the dialysate was then aliquoted, lyophilized and stored at -20°C. The protein concentration of the lectin was estimated by Folin Ciocalteu method (Lowry et al., 1951). The specific lectin activity was obtained by dividing the lectin titer with the protein concentration of the sample (Wang et al., 2000).

### **Purification of lectin from the larval hemolymph of the red palm weevil, *Rhynchophorus ferrugineus* using formalinized rabbit erythrocytes as the affinity adsorbent**

#### **Preparation of formalinized rabbit erythrocytes**

Following the method of Nowak and Barondes (1975), formalinized rabbit erythrocytes were prepared. Fresh rabbit erythrocytes were washed three times in 20 volumes of PBS, pH 7.2 (75 mM NaCl, 75 mM Na<sub>2</sub>HPO<sub>4</sub>) per packed cell volume by centrifugation at 1000 x g for 5 minutes. The cells were suspended at a concentration by volume of 8% in PBS, pH 7.2 and an equal volume of formalin (3% solution in PBS with pH adjusted to 7.2 with 0.1 M NaOH) was added. This mixture was incubated at room temperature for 16 hours with moderate shaking. The cells were then washed four times in five volumes of PBS, pH 7.2 per packed cell volume and stored at 4°C as 10% suspension in the same buffer.

#### **Adsorption of agglutinin to formalinized rabbit erythrocytes**

The formalinized cells were washed six times in 10 volumes of TBS, pH 7.2 (50 mM Tris-HCl; 100 mM NaCl). The packed cells were then incubated with 20 volumes of clarified hemolymph in plastic tubes for 2 hours with moderate shaking at 4°C and then washed 3 times with 20 volumes of TBS, pH 7.2, containing 0.01 M CaCl<sub>2</sub>.

#### **Elution of adsorbed agglutinin**

Elution of adsorbed hemagglutinin was accomplished by incubation of the cells with 10 volumes of 10 mM disodium EDTA in TBS pH 8.2 for 10 minutes at 4°C and the elution mixture was then centrifuged for 10 minutes at 28000 x g to remove any residual particulate material and the resultant supernatant was dialyzed and tested for hemagglutination with 1.5% rabbit erythrocytes in TBS, pH 7.5. The protein concentration of the lectin was estimated by Folin Ciocalteu method (Lowry et al., 1951).

#### **Hemagglutination assay**

Hemagglutination assays were performed in a microtiter plate at room temperature (30 ± 2°C). The purified lectin (25 µl) was serially diluted with TBS-BSA (25 µl) and mixed with 25 µl of 1.5% rabbit erythrocyte suspensions and incubated for 1 hour at room temperature (30 ± 2°C). HA titers were reported as the reciprocal of the highest dilution of the lectin giving complete agglutination after 1 hour.

#### **Hemagglutination inhibition assay**

Inhibitors (25 µl) of known concentration (sugar: 100 mM; glycoprotein: 5 mg/ml) were serially diluted with 25 µl of TBS-BSA in microtiter plates. Then to each well, 25 µl of purified lectin diluted to subagglutination concentration (to give a HA titer of 2) was added and incubated for 1 hour at room temperature (30 ± 2°C). After incubation, 25 µl of 1.5% rabbit erythrocyte suspension was added, mixed and incubated. Hemagglutination inhibition titer was reported as the reciprocal of the highest dilution of inhibitor giving complete inhibition of agglutination after 1 hour.

#### **Sialidase treatment of rabbit erythrocytes**

A reaction mixture (total 1.0 ml) containing 10% washed rabbit erythrocytes in PBS-BSA (pH 7.0) and 140 milliunits of neuraminidase of *Clostridium perfringens* (Type X; Sigma) was incubated at 37°C for 4 hours. Neuraminidase treated and untreated erythrocytes were washed with PBS-BSA three times and pelleted by low-speed centrifugation and finally washed in TBS-BSA (pH 7.0). HA assays were performed against the native and desialylated rabbit erythrocytes using purified lectin.

#### **Sialidase treatment of sialoglycoprotein**

Asialo PSM was prepared by incubating 2 mg of glycoprotein (PSM) with 0.1 unit of *Clostridium perfringens* sialidase (Type X; Sigma) in 400 µl of 5 mM acetate buffer, pH 5.5 for 2 hours at 37°C. As a control, PSM was treated similarly without sialidase. HAI assay was performed with purified lectin for sialidase treated and untreated PSM against 1.5% rabbit erythrocyte suspension.

#### **Polyacrylamide gel electrophoresis**

Sodium dodecyl sulphate polyacrylamide 11% slab gel electrophoresis was performed according to Laemmli (1970). Samples were heated for three minutes at 100°C in sample buffer (25% 1 M Tris-HCl,

pH 6.8; 4% SDS; 2%  $\beta$ -mercaptoethanol and 5% glycerol). Gels were fixed and stained with a solution containing 0.25% Coomassie blue R-250, 7% acetic acid and 50% isopropyl alcohol and destained with a solution containing 50% ethanol and 7% acetic acid at room temperature ( $30 \pm 2^\circ\text{C}$ ).

### Screening for antibacterial and antifungal activity

The antibacterial and antifungal activity were carried out by employing 24 hours cultures of *Escherichia coli*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. Activity of crude hemolymph and purified hemolymph lectin of *Rhynchophorus ferrugineus* larva was tested using agar well diffusion method (Mbata et al., 2006).

Petriplates containing about 20 ml of sterilized agar medium were seeded with respective strains of bacteria and fungi. The plates were left at room temperature for solidification. A well of 6 to 8 mm diameter was made using sterile cork borer. The standard drug and samples were added into the wells and the plates were incubated at  $37 \pm 2^\circ\text{C}$  for 24 hours. Streptomycin and Clotrimazole were used as positive control for antibacterial and antifungal activity respectively. The diameter of the zone of inhibition was measured.

## 3. Results and Discussion

### Purification of lectin

The lectin purified by biospecific adsorption resulted in 10 fold increase in specific activity of about 6468 (Table 2).

**Table 2.** Purification of hemolymph lectin from the larva of red palm weevil, *Rhynchophorus ferrugineus*

Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)	Purification fold
Crude hemolymph	50	3200	$2.048 \times 10^6$	640	1
Clarified hemolymph	35	1085	$1.43 \times 10^6$	1,321	2
Lectin purified using formalinized rabbit erythrocytes	8	25.33	$1.64 \times 10^5$	6,468	10
Lectin purified using PSM - Sepharose 4B affinity column	22	3.3	$2.25 \times 10^5$	68,266	107

The specific activity of the lectin purified by affinity chromatography revealed 107-fold increase in specific activity (68,266 HA units/mg of protein) which was comparatively higher than the agglutinability of the lectin purified by biospecific adsorption (Table 2). The column profile showing the purification of hemolymph lectin from the larva of the red palm weevil, *Rhynchophorus ferrugineus* on PSM-Sepharose 4B is shown in figure 2.

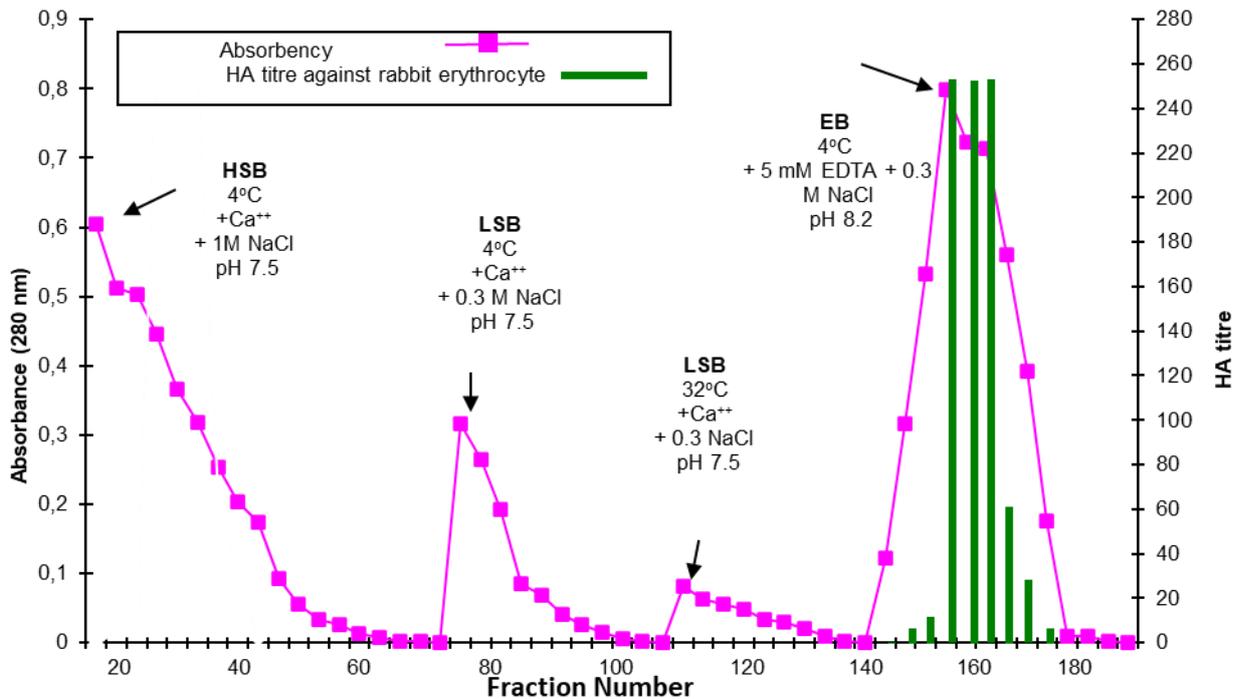


Fig. 2. Porcine stomach mucin - affinity elution profile

### Hemagglutination assay

Like the crude hemolymph agglutinin, the purified lectin from the larva of the red palm weevil, *Rhynchophorus ferrugineus* agglutinated all the mammalian erythrocytes tested in the same pattern. But the HA titer was found to be less with all the tested erythrocytes than the crude agglutinin (Table 3). However, 107-fold increase in specific activity was observed with regard to protein concentration (Table 2).

Table 3. HA and HAI titer of the crude agglutinin and purified lectin of the larva of the red palm weevil, *Rhynchophorus ferrugineus*

Erythrocytes	HA titer		Inhibitors	HAI titer	
	Crude (n = 5)	Purified (n = 5)		Crude (n = 5)	Purified (n = 5)
Rabbit	1024	256	PSM	512	512
Guinea pig	256	64	Fetuin	512	16
Horse	256	64	Bovine Thyroglobulin	256	4
Human A	16	4	Transferrin	32	8
Human B	128	32	Apotransferrin	32	4
Human O	64	16	BSM	8	4
Dog	256	32	ManNAc	<b>128</b>	<b>256</b>
Buffalo	64	8	Melibiose	128	16
Pig	32	8	Lactose	128	8
Goat	32	4	N-acetyl-D - galactosamine	<b>32</b>	<b>64</b>
Rat	16	4	D-galactosamine	64	16
Mice	16	2	N-acetyl neuraminic acid	<b>32</b>	<b>256</b>
Cow	16	2	N-glycolyl neuraminic acid	0	0

### Hemagglutination inhibition assay (HAI)

The agglutinating activity of the purified hemolymph lectin against rabbit erythrocytes was inhibited by glycoprotein PSM followed by fetuin and to a lesser extent by transferrin, bovine thyroglobulin, apotransferrin and BSM (Table 4) and sugars NeuAc, ManNAc followed by GalNAc, melibiose, D-galactosamine and  $\alpha$ -lactose (Table 5). Decrease in HAI titer was observed with all glycoproteins except PSM and all sugars except N-acetyl neuraminic acid, N-acetyl mannosamine and N-acetyl-D-galactosamine where an increase in HAI titer was observed.

**Table 4.** Hemagglutination inhibition (HAI) of the hemolymph lectin in the larva of the red palm weevil, *Rhynchophorus ferrugineus* by different glycoproteins

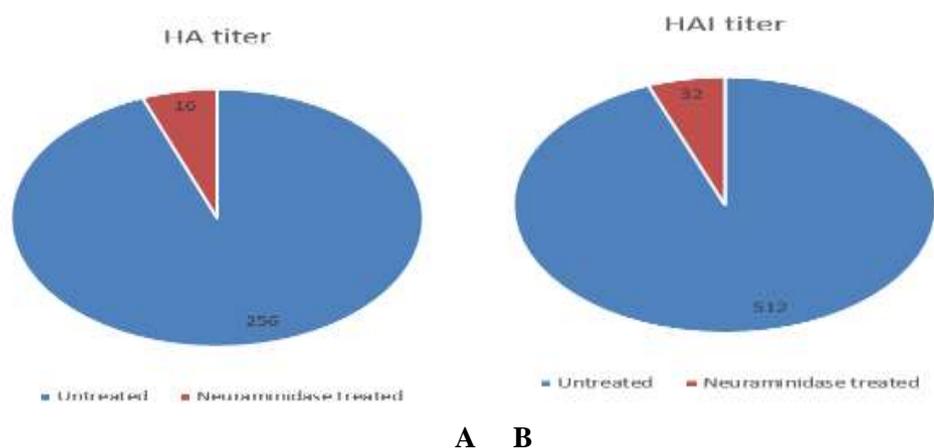
Glycoproteins (n = 5)	HAI titer	Minimum concentration for inhibition ( $\mu\text{g}$ )	Relative inhibitory potency (%)
Porcine stomach mucin	512	9.76	100
Fetuin	16	312.5	50
Transferin	8	625	25
Bovine thyroglobulin	4	1250	12.5
Apotransferin	4	1250	12.5
Bovine submaxillary mucin	4	1250	12.5

**Table 5.** Hemagglutination inhibition (HAI) titer of the hemolymph lectin in the larva of the red palm weevil, *Rhynchophorus ferrugineus* by different sugars

Sugars (n = 5)	HAI Titer	Minimum concentration for inhibition (mM)	Relative inhibitory potency (%)
N-acetyl neuraminic acid	256	19.53	100
N-acetyl mannosamine	256	19.53	100
N-acetyl-D-galactosamine	64	78.12	50
Melibiose	16	312.5	25
D-galactosamine	16	312.5	25
$\alpha$ -Lactose	8	625	12.5

### Sialidase treatment of rabbit erythrocytes and glycoprotein, PSM

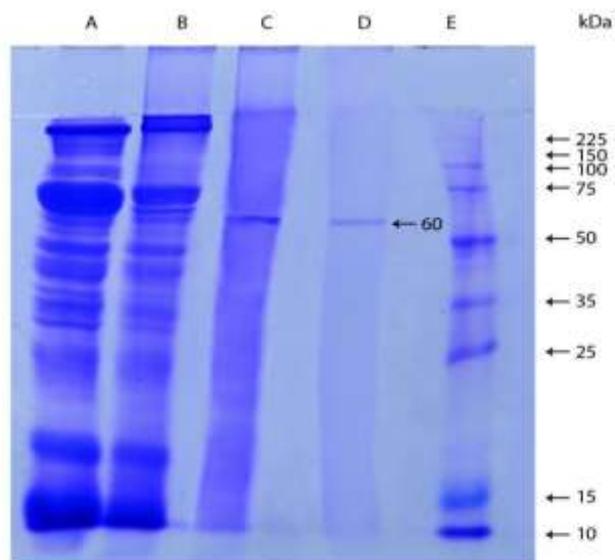
Purified lectin showed 16 fold reduction in HA titer when tested with sialidase treated rabbit erythrocytes (Figure 3A) and 32 fold reduction in HAI titer when analysed with asialo PSM (Figure 3B).



**Fig. 3.** A. Hemagglutination titer of the hemolymph lectin in the larva of the red weevil, *Rhynchophorus ferrugineus* with native and asialo rabbit erythrocytes; B. Effect of neuraminidase treatment of glycoprotein on the HAI of the hemolymph lectin in the larva of red palm weevil, *Rhynchophorus ferrugineus*

### Electrophoretic analysis of the purified lectin

The molecular weight of the purified lectin was found to be around 60 kDa on SDS PAGE (Figure 4).

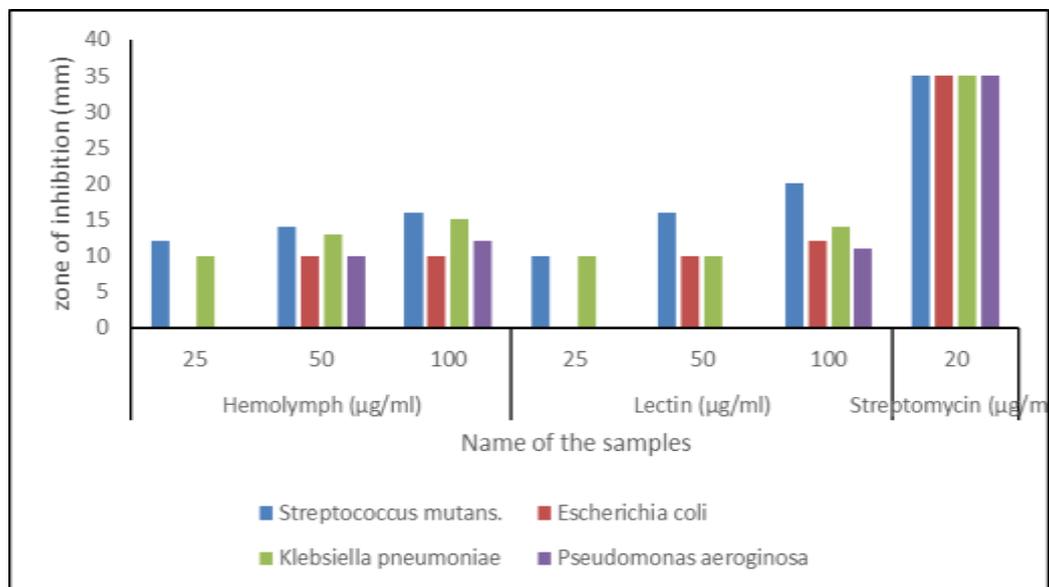


Lane A-Crude hemolymph; Lane B-Clarified hemolymph; Lane C-Lectin purified by affinity adsorption using formalinized rabbit erythrocytes; Lane D-Lectin purified by affinity chromatography; Lane E-Protein molecular weight markers.

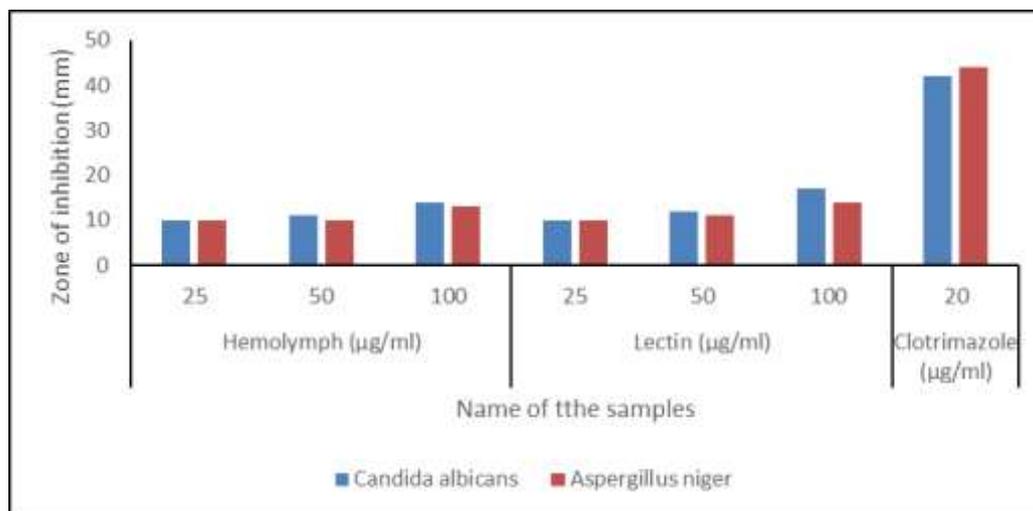
**Fig. 4.** SDS-PAGE of purified lectin from the larval hemolymph of the red palm weevil, *Rhynchophorus ferrugineus*

#### Antimicrobial activity of the lectin

The hemolymph lectin of the larva of *Rhynchophorus ferrugineus* inhibited the growth of Gram-positive bacteria *Streptococcus mutans*, Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Figure 5) and fungi *Candida albicans* and *Aspergillus niger* (Figure 6).



**Fig. 5** Antibacterial activity of crude hemolymph and larval hemolymph lectin of *Rhynchophorus ferrugineus*



**Fig. 6.** Antifungal activity of crude hemolymph and larval hemolymph lectin of *Rhynchophorus ferrugineus*

In the present investigation, a sialic acid specific lectin with a molecular mass of 60 kDa was purified by both affinity chromatography and biospecific adsorption from the larval hemolymph of the red palm weevil, *Rhynchophorus ferrugineus*. Among the two methods of purification employed, affinity chromatography is more preferable because both the rate of recovery and the specific activity of the affinity purified lectin was found to be higher than the lectin purified by biospecific adsorption. Lectins from insect species like *Manduca sexta* (Yu and Kanost, 2000), *Oryctes rhinoceros* (Jayalakshmi, 2005), *Schistocerca gregaria* (Dorrah et al., 2009), *Musca domestica* (Cao et al., 2009), *Bradinyopyga geminata* (Learnal-Sudhakar, 2014), *Odoiporus longicollis* (Tamilarasan et al. 2021), *Dermestes frischii* (Arokiyaraj et al., 2022) have been also purified. The purified lectin was homogenous on SDS-PAGE with a single band at 60 kDa.

When the hemagglutinability of the purified lectin was compared to that of the unfractionated hemolymph, 4-8 fold decrease in HA titer was observed invariably in all the mammalian erythrocytes tested. This uniform decrease in HA activity of the purified lectin with all the erythrocytes may be due to the removal of certain stabilizing hemolymph components during the process of purification which may be necessary for erythrocyte binding leading to hemagglutination, as reported in the hemolymph lectin of the insect *Teleogryllus commodus* (Hapner and Jermyn, 1981).

Just like the whole hemolymph, the purified lectin also agglutinated rabbit erythrocytes with great avidity than the erythrocytes of guinea pig, horse, human, dog, buffalo, pig, goat, rat, mice and cow. This differential agglutinability of the lectin towards the diverse species of mammalian erythrocytes could be due to the presence of a particular sugar on the erythrocyte membranes of all the tested animals with quantitative difference. Presence of sialic acid on erythrocyte membrane that were highly agglutinated by the hemolymph lectin of *Rhynchophorus ferrugineus* larva was reported in rabbit and guinea pig (Klein and Roussel, 1988), horse (Gaza et al., 1983), human (Reuter et al., 1980), dog (Hamanaka et al., 1979), buffalo (Chien et al., 1978) and pig (Suzuki et al., 1985). So, it could be stated that the lectin may bind to sialic acid expressed on the erythrocyte membrane.

Just like the HA titer, the HAI titer of the purified lectin also showed significant quantitative difference with the tested inhibitors. When all the inhibitors expressed 2-to-32-fold decrease in HAI titer, the HAI titer of the purified lectin remained unaltered with PSM, showed a 2-fold increase with N-acetyl mannosamine and an 8-fold enhancement with N-acetyl neuraminic acid, revealing its unique affinity to N-acetyl neuraminic acid. In PSM, the potent inhibitor of hemagglutinability, the carbohydrate accounts for about 80% of the whole mass, and the protein backbone is formed of 20% of the weight. These mainly O-linked glycans such as N-acetyl galactosamine, N-acetyl glucosamine, fucose, galactose and sialic acid (N-acetyl neuraminic acid) are attached to the hydroxyl side chains of the threonine and serine (Libao-Mercado and de Lange, 2007). However, the ability of N-acetyl mannosamine, the precursor of N-acetyl neuraminic acid (Roseman et al., 1961) to inhibit agglutinability and N-acetyl neuraminic acid to inhibit the same with even greater potency and the inability of the N-glycolyl neuraminic acid to inhibit agglutinability of the lectin further accounts for the affinity of the lectin to N-acetyl neuraminic acid.

Pig gastric mucin glycans, although they have in common the Gal  $\beta$  1,3 GalNAc core unit show heterogeneity and they may carry one or more N-acetyl lactosamine branches and the latter may be

terminated either by Fucose in  $\alpha$  1, 2 linkage or by GlcNAc  $\alpha$  1, 4 linkages to Gal (Van Halbeek et al., 1982). Gastric and duodenal mucins generally contain the core-1 (Gal  $\beta$  1-3 GalNAc  $\alpha$  1- Ser/Thr) and the core-2 (Gal  $\beta$  1-3 (GlcNAc  $\beta$  1-6) GalNAc  $\alpha$  1-Ser/Thr) structure (Larsson et al., 2009). The predominant glycolipid present in the erythrocyte of rabbit is Gal  $\alpha$ , 1-3 Gal  $\beta$ 1-3/ 4GlcNAc  $\beta$ 1-3 Gal  $\beta$  1-4 Glc $\beta$ 1- ceramid. So it could be suggested that the sialic acid specific lectin isolated from the hemolymph of the larva of the red palm weevil, *Rhynchophorus ferrugineus* may have binding affinity towards Gal  $\beta$  1-3 linkage, which is present in Porcine stomach mucin the potent inhibitor and in the surface of the high agglutinating rabbit erythrocytes (Yamakawa, 2005).

The sialic acid specificity of the purified lectin was further revealed by reduction in HA titer with asialo rabbit erythrocytes and HAI titer with desialylated PSM. The reduction in HA titer with desialylated rabbit erythrocytes and HAI titer with asialo PSM affirm that the purified larval lectin from the red palm weevil, *Rhynchophorus ferrugineus* is a sialic acid specific lectin. Sialic acid specific lectins were also purified from insects, *Teleogryllus commodus* (Hapner and Jermyn, 1981), *Bombyx mori* (Kitagaki et al., 1986), and *Bradyinopyga geminata* larva (Learnal-Sudhakar, 2014).

Lectins that recognize the linkages or modifications of sialic acids are indispensable in biochemical research and diagnostic analysis. Hence sialic acid binding lectins can be used as valuable diagnostic tools for identifying the surface components of pathogenic bacteria and tumor-associated sialylated antigens (Ravindranath and Cooper, 1984; Ravindranath et al., 1988; Chatterjee et al., 2008; Elaya Bharathi et al., 2020; Vargila et al., 2023).

To know the therapeutic potential of the lectin, the purified hemolymph lectin from *Rhynchophorus ferrugineus* larva was tested for its antimicrobial potential. The results revealed antibacterial activity against Gram-positive *Streptococcus mutans* and Gram negative *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. These pathogenic microbes may possess the specific sugars on their cell surface and the lectin may bind to them and inhibit their growth. These findings were in accordance with the findings of Naganuma et al. (2006) where a galactoside binding pearl shell lectin purified from marine bivalve, *Pteria penguin* effectively inhibited the growth against both gram-positive and gram-negative bacteria. Tateno et al. (2002) have also reported a rhamnose-binding lectin from the egg of steelhead trout (*Oncorhynchus mykiss*) which inhibited the growth of gram positive and gram-negative bacteria by recognizing lipopolysaccharide or lipoteichoic acid present on their surface. Recently, it was reported that snake venom lectins are able to inhibit growth of phytopathogenic bacteria (Barbosa et al., 2010).

The purified lectin also showed antifungal activity against *Candida albicans* and *Aspergillus niger*. Iijima et al. (1995) have also suggested the presence of antifungal activity in lectin isolated from sea hare. The inhibition of fungal growth can occur through lectin binding to hyphae resulting in poor absorption of nutrients as well as by interference on spore germination process (Lis and Sharon, 1981). The polysaccharide chitin is constituent of fungi cell wall and chitin-binding lectins showed antifungal activity; impairment of synthesis and/or deposition of chitin in cell wall may be the reasons of antifungal action of the lectin (Selitrennikoff, 2001; Vargila et al., 2023).

#### 4. Conclusion

A sialic acid specific lectin with antibacterial and antifungal properties was purified and characterized from the larval hemolymph of red palm weevil, *Rhynchophorus ferrugineus* for the first time. The identified lectin was proven for its novel and remarkable potential with respect to biological applications such as antibacterial and antifungal properties.

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