6. SUMMARY

The present study describes isolation, characterization and analysis of antibacterial activity of a serum lectin from the grub of rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae).

CHAPTER -1:

In the study, for the first time, haemagglutinin was detected in the serum of *Oryctes rhinoceros*.

- The crude serum of the grub *O. rhinoceros* agglutinated 10 out of 11 vertebrate RBC types such as sheep, rabbit, buffalo, human-A, B, O, ox, goat, cow and mouse with varying haemagglutination (HA) titers. Among them, the crude serum of the grub *O. rhinoceros* recorded highest value of HA titer as 64 for sheep and rabbit RBC types. A moderate HA titer of 32 was recorded for buffalo RBC. Nevertheless, buffalo and sheep RBC types were chosen as suitable indicator RBC types for all subsequent experiments since they were collected without any difficulty among these three RBC types.

- This haemagglutinin did not require any cations such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ for agglutination of various RBC types and insensitive to EDTA.

- The experiment on dialysis of crude serum of *O. rhinoceros* in a dialysis tubing with molecular weight cutoff of less than 14 kDa revealed that the haemagglutinin in the serum might be above 14 kDa in native form.

- The haemagglutinating activity of serum was stable between pH 6.0 and 8.5 upon dialysis of serum against buffers with various pH range. The
agglutinating activity of serum was stable up to 50 °C upon incubation of serum with various temperatures for 30 minutes.

- The serum agglutinating activity could be precipitated by ammonium sulphate (70 %) and TCA (10 %), suggesting that serum agglutinin might be proteinaceous in nature.

- Incubation of serum with 2-mercaptoethanal reduced the HA activity. It signifying the denaturing ability of 2-mercaptoethanol on the haemagglutinin in the serum of *O. rhinoceros*.

- Thirty carbohydrates and three glycoproteins were used for haemagglutination inhibition assay. None of the monosaccharide inhibited the agglutinating activity of serum against sheep RBC. However, galactose and lactose inhibited the HA activity against buffalo RBC with minimum inhibitory concentration of 100 and 50 mM respectively. Out of three glycoproteins tested, only the bovine sub-maxillary mucin (BSM) inhibited the agglutinating activity of serum against sheep and buffalo RBC types at a concentration of 3.9 µg and 7.81 µg respectively.

Over all, this preliminary study revealed the presence of haemagglutinin in the grub serum of *O. rhinoceros* with maximum HA activity for sheep and rabbit RBC and moderate HA activity for buffalo RBC. It is heat-labile, cation independent and insensitive to EDTA. The detected haemagglutinin in the grub serum had galactose, lactose and mucin binding specificity.
CHAPTER -2:

Based on the preliminary analysis of haemagglutinin in the grub serum of *O. rhinoceros*, an attempt was made on the isolation of detected agglutinin by affinity column chromatography on acid-treated Sepharose 6B and its characterization.

- The procedure adopted for the isolation of serum agglutinin using acid-treated Sepharose 6B resulted in about 37.78 fold increase in specific activity against buffalo RBC. Further 60 % activity could be recovered from the initial activity of serum.

- The isolated lectin agglutinated sheep, rabbit, buffalo, human-A, B, O, ox, goat, cow and mouse RBC types and did not agglutinate hen RBC.

- The isolated lectin upon gel electrophoresis revealed a single protein band in 7 % acrylamide gel stained with coomassie brilliant blue as well as silver nitrate. On the other hand, SDS-PAGE analysis of isolated haemagglutinin showed three distinct bands on 10 % acrylamide gel with approximate molecular weight of 90 kDa, 78 kDa and 45 kDa.

- The MADTI-TOF/MS and mascot analyses of trypsin digested 45 kDa polypeptide fraction showed maximum matches with amino acid sequences of two lectin molecules of Coelopteran beetles namely, *Tribolium castaneum* and *Tenebrio molitor*. It confirmed that the isolated molecule from the grub serum of *O. rhinoceros* was a lectin.

- Thirty carbohydrates and three glycoproteins were used for haemagglutination inhibition assay. The HA activity of isolated lectin against sheep RBC could be inhibited by dulcitol with the minimum inhibitory concentration of 100 mM.
while dulcitol, galactose and lactose inhibited the HA activity of isolated lectin against buffalo RBC with the minimum inhibitory concentration of 100 mM, 50 mM and 25 mM respectively.

- Out of three glycoproteins tested, BSM inhibited the HA activity of isolated lectin against sheep RBC with minimum inhibitory concentration of 1.95 µg and buffalo RBC with minimum inhibitory concentration of 3.9 µg. The HA activity of isolated lectin was inhibited by thyroglobulin at a minimum concentration of 250 µg against both sheep and buffalo RBC types.

- Isolated lectin required no cations such as Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) for agglutination of various RBC types tested. It was insensitive to EDTA.

- The HA activity of isolated lectin was constant between pH 6 and 8.5.

- The HA activity of isolated lectin was stable up to 50 °C upon incubation of isolated molecule with various temperatures for 30 minutes.

Overall, the analysis of isolated haemagglutinin from the grub serum of *O. rhinoceros* showed a single protein band in native-PAGE. It was stable between pH 6 and 8.5, heat-labile, cation independent and insensitive to EDTA. The isolated lectin had binding specificity to dulcitol, galactose, lactose, mucin and thyroglobulin. The SDS-PAGE analysis of the isolated lectin revealed three distinct polypeptide fractions with approximate molecular weight of 90 kDa, 78 kDa and 45 kDa. The MALDI-TOF/MS analysis and mascot search of mass spectrum of trypsin-digested 45 kDa polypeptide fraction indicated peptide sequences with distinct homology to lectin molecules known for other coleopteran species such as *Tribolium castaneum* and *Tenebrio molitor*.
CHAPTER -3:

Analysis of antibacterial as well as bacterial agglutination properties of both crude serum and isolated lectin was carried out with ten laboratory cultures of bacteria that include 5 Gram-negative, 5 Gram-positive and 7 soil bacteria (isolates I to VII) obtained from native habitat of grub of rhinoceros beetle *O. rhinoceros*.

- Seven different bacterial colonies were isolated from the dung bits i.e., the natural habitat of grub of *O. rhinoceros* based on the growth and morphology of bacterial colonies on agar plate. These bacteria were named as isolate I, II, III, IV, V, VI and VII.

- Analysis of antibacterial activity of crude serum as well as isolated lectin was performed using the zone of inhibition (ZI) assay method.

- Study revealed the presence of antibacterial activity only in the soil bacterial isolates II and IV out of seventeen bacterial species tested. The crude serum with 675 µg of protein in 50 µl inhibited the growth of bacterial isolates II and IV with the zone of inhibition of 11 ± 2.0 mm and 13 ± 3.0 mm respectively. Similarly, the isolated lectin with a concentration of 20 µg in 50 µl of TBS inhibited the growth of soil bacterial isolates II and IV with the zone of inhibition of 6.0 ± 1.0 mm and 8.0 ± 1.0 mm respectively.

- Bacterial agglutination was observed for both crude serum and isolated lectin only for the bacterial isolates II and IV while in other bacteria agglutination was not observed.
These two soil bacterial isolates II and IV were subjected to morphological and biochemical characterizations including molecular analysis. They were identified as *Bacillus subtilis* and *Bacillus pumilus*.

On the whole, the significant findings of this study reveal prospective antibacterial and bacterial agglutination activities of the lectin isolated from the crude serum of the grub of rhinoceros beetle *Oryctes rhinoceros* for two Gram-positive native soil bacteria *viz.*, *Bacillus subtilis* and *Bacillus pumilus*. 