CHAPTER 4. INSECTICIDAL AND IMMUNOMODULATORY ACTIVITY AGAINST INSECT PEST

4.1. ABSTRACT

The biological characterization of venomous saliva (VS) of *Rhynocoris fuscipes* was tested against the two lepidopteron pest such as *Spodoptera litura* and *Helicoverpa armigera* by the microinjection and oral toxicity bioassay. The VS activity was also analyzed for the immunosuppressive characters against the two pests. The results showed that the administration of VS causes mortality in *S. litura* and *H. armigera*. The LD_{50} values for the microinjection and oral toxicity bioassay for *S. litura* (861.00 and 846.35 ppm) was less than those of *H. armigera* (913.00 and 928.12 ppm) at 96 h observations. The total haemocyte count of *S. litura* and *H. armigera* was 12.85 x 10^6 cells/ml and 13.97 x 10^6 cells/ml, respectively. In *S. litura*, about 48.77% and in *H. armigera* 20.90% cell death was recorded along with the cell apoptosis during the 60 min of observation. The haemolymph protein profile showed that, the VS has made changes in the protein profile in *S. litura* and *H. armigera*. The spreading and haemocyte inhibitory behavior also showed the impact of VS on the haemocytes of both *S. litura* and *H. armigera*. The VS of *R. fuscipes* had its biological activity on *S. litura* and *H. armigera* in *in vivo* and the VS had its *in vitro* effect on the haemolymph protein profile, total haemocyte count and the spreading and spreading inhibitory behavior of haemocytes and on the haemocyte morphology of *S. litura* and *H. armigera*.

4.2. INTRODUCTION

Arthropod venoms are complex mixtures containing a variety of biologically active substances such as proteic and non-proteic toxins, enzymes, nucleotides, lipids, biogenic amines and other unknown substances (Diniz, 1978; Possani, 1984; Jackson and Parks, 1989; Rash and Hodgson, 2002). According to their specificity, the arthropod toxins have been classified into mammal toxins, insect toxins and crustacean toxins (Zlotkin *et al.,* 1971, 1978; Possani, 1984; Gordon *et al.,* 1998; Corzo *et al.,* 2001; Altuntaş *et al.,* 2010). The possibility of using proteinceous toxins highly against the insect crop pests, as a means of strengthening biological control, was suggested in the early 1990s (Maeda *et al*., 1991; Mc Cutchen *et al*., 1991). Animal toxins have high affinity and specificity to neuronal receptors, transporters and ion-channels. Thus they are important tools in the characterization of mammal and insect

nervous system (Usherwood, 1994). True venom of arthropods like spiders; *Parawixia bistriata* (Fontana *et al.,* 2000); *Segestia florentina* (Fitches *et al.,* 2002, 2004); *Loxosceles gaucho, Proneutria nigrivetor, Nephilengys cruentata* and *Titys serrulator* (Manzoli-Palma *et al.,* 2003). *Haronyche versuta* (Mukherjee *et al.,* 2006); *Atrax robustus* (Vonarx *et al.,* 2006); *Latrodectus hasselti* (Nicholson et al., 2006); *Hippasa partita, Hippasa agelenoides* and *Hippasa lycosina* (Siliwal *et al*., 2005; Nagaraju *et al.,* 2006); *Orancistrocerus dreuseni* and *Eumenes pomiformis* (Baek *et al.,* 2010). Hymenoptera; *Aphidius ervi* (Starý, 1978; Powell, 1982); *Pseudaphycus maculipennis* (Sandanayaka *et al.,* 2009); *Bracon hebetor* (Altuntaş *et al.,* 2010); *Orancistrocerus dreuseni* and *Eumenes pomiformis* (Baek *et al.,* 2010); Hemiptera *Acanthaspis pedestris* (Ambrose and Maran, 1999); *Periates turpis, Agriosphodrus dohrini* and *Isyndus obsevrus* (Corzo *et al.,* 2001); *Podisus nigrispinus* (Zanuncio *et al.,* 2008) possesses insecticidal activity against economically important insect pest. Bioassays with the analysis of paralysis and death are important tools for distinguishing the biological effects of venom in insects (Zlotkin *et al.,* 1971; Zlotkin, 1984; Friedel and Nentwig, 1989; Quistad *et al.,* 1994; Boevé, 1994; Escoubas *et al.,* 1995).

Reduviids predators immobilize their prey by injecting the venomous saliva (VS) into their prey (Haridass and Anathakrishnan, 1981; Ambrose, 1988; Morrison, 1989; Cohen, 2000; Sahayaraj, 2007; Sahayaraj *et al.,* 2010). The VS of reduviids appears to be digestive enzymes but it immobilizes the prey and helps in the external digestion (Blum, 1978; Cohen, 1990; Sahayaraj *et al.,* 2010; Kumar, 2011). Previously, the paralytic potential of the salivary gland extract of *Acanthaspis pedestris* was evaluated against *Mylabris pustulata* and *Dysdercus cingulatus* (Ambrose and Maran, 1999). Then, Corzo *et al*. (2001) have studied the toxicity of VS of *P. turpis, A. dohrini* and *I. obsevrus* toxicity against the crickets and cut worms through microinjection assay.

Insects possess potent immune systems comprising cellular and humoral immune response that they deploy to protect themselves from invading parasites (Ratchffe and Gotz, 1990; Otvos, 2000; Lowenberger, 2001; Beckage and Gelman, 2004; Bulet and Stocklin, 2005), pathogens (Schmidt *et al.,* 2001; Cerenius and Söderhall, 2004; Imler and Bulet, 2005) and biomoleclues (Richards and Edwards, 1999; Gundersen-Rindal and Pedroni, 2006) including true venom (Strand and Pech, 1995; Gillespie *et al.,* 1997; Beckage, 1998; Shelby and Webb, 1999) and venomous saliva (Kumar, 2011; Sahayaraj and Muthukumar, 2011). Cellular defense responses refer to haemocyte-mediated immune-responses like phagocytosis, nodulation and encapsulation (Lavine and Strand, 2002), the formation of

circulating haemocyte aggregates (Lavine and Strand, 2002; Jiravanichpaisal *et al.*, 2006; Kumar, 2011; Sahayaraj and Muthukumar, 2011). The role in disabling host haemocytes or inhibiting host aggregation played by venom from two species of pupa specific Ichneumeoid endoparasitiod *Pimpla turionellae* (Osman, 1978); *P. hypochondriace* (Marris *et al.,* 1999; Richards and Parkinson, 2000; Parkinson *et al.,* 2002b); *Pieris rapae* and *Papilio xuthus* (Zhang *et al.,* 2005); *Ovomermis sinensis* (Li *et al.,* 2009); *R. marginatus* (Kumar, 2011) has been reported. Endoparasitiod parasitize their hosts, especially lepidoteran species qualitative and quantitative changes occur in the profile of host plasma proteins (Rolle and Laurence, 1994; Vinson *et al.,* 2001; Consoli and Vinson, 2002; Rahbe *et al.,* 2002; Kaeslin *et al.,* 2005, 2010; Altuntaş *et al.,* 2010; Harvey *et al.,* 2010; Huang *et al.,* 2010).

The total haemocyte count in parasitized hosts is either being increased or decreased (Stettler *et al*., 1998). The process of cellular encapsulation in insects takes place in two phases: the contact with and lyses of the granular haemocytes and the adhesion of the plasmatocytes to the implant site of the lyses of granular cells (Schmit and Ratcliffe, 1977). Furthermore, the efficiency of these immune responses may be influenced by the number and state of health of the haemocytes at the time of stress, and their ability (possibly aided by humoral factors) to recognize and respond to non-self (Ratcliffe and Rowley, 1987; Ratcliffe, 1993; Gillespie *et al*., 1997). In the initial cellular reaction, the filopodial elongation of the granular cells is an essential factor in the recognition of the foreign substances (Wago, 1982; Parkinson and Weaver, 1999; Ergin *et al*., 2006; Kaeslin *et al.,* 2010). The endoparasitoids inject their venom along with their eggs into the host larva at the time of oviposition which infects the haemocytes and causes much change in the host haemocytes such as inhibition of plasmatocyte spreading (Strand, 2008), apoptosis in granular cells (Strand and Pech, 1995), damage to the cytoskeleton of the haemocytes (Webb and Luckhart, 1996), and blebs on haemocytes (Lavine and Beckage, 1996; Luckhart and Webb, 1996). The biochemically isolated protein has been demonstrated to inhibit haemocyte aggregation and to suppress encapsulation responses (Richards and Dani, 2008).

The tobacco army worm, *Spodoptera litura* is one of the serious pests in Asia and other countries. It is a polyphagous pest attacking several agricultural and horticultural crops (Ranga Rao *et al.,* 1993) and causes a major threat to intensive agriculture (Malarvannan *et al.,* 2008). The infestation of the larvae causes 20% yield loss during the seedling or flowering stage (Kulkarni, 1989). The causes of outbreak of this larvae/adult may be due to the heavy rainfall following a prolonged dry spell (Chelliah, 1985; Thanki *et al.,* 2003),

indiscriminate use of chemical insecticides resulting in destruction of natural enemies and development of insecticide resistance (Ranga Rao *et al.,* 1993).

Helicoverpa armigera is a cosmopolitan, multivoltine and agronomically important pest, infesting more than 300 plant species worldwide (Arora *et al*., 2007; Rajapakse and Walter, 2007; Sarwar *et al*., 2011) and it is a major pest, particularly on cotton and legumes, throughout Africa, Asia, Australia, the Pacific and Europe (Hill, 1975; Fitt, 1989; Sharma *et al.,* 2005). Polyphagy, high mobility, fecundity and facultative diapauses are key physiological and ecological characteristics that facilitate survival of *H. armigera* even in unstable habitats (Fitt, 1989). Conventional chemical-based approaches to control this pest have failed due to development of resistance against many insecticides (Kranthi *et al*., 2002; Reddy and Zehr, 2004).*H. armigera* causes high economic losses to the agriculture (Singh and Yadav, 2006; Sarwar *et al*., 2009). As with many other species of insect pest, *H. armigera* tends to develop resistance to a range of pesticides, with recent studies reporting an increase in resistance to the synthetic pyrethroids (Sawiki and Denholm, 1987; Reddy and Zehr, 2004)

Several works had made an attempt using the reduviid predators for the biological control of insect pest and few other literatures have shown the use of true venom on these insects from spiders, scorpions, wasps etc. And hence no one had studied the impact of VS of the reduviid predator, *R. fuscipes*. Hence I proposed to study the biological activity of VS of *R. fuscipes* against the larval mortality; host enzyme regulation and immune system response have been studied on *S. litura* and *H. armigera.*

4.3. MATERIALS AND METHODS

4.3.1. Insect collection

Life stages of *R. fuscipes, S. litura* and *H. armigera* were collected from the agro-ecosystems of Tirunelveli district, Tamil Nadu. The collected predators and pest were reared in the laboratory conditions presented in the first Chapter (Page no. 03). The reduviid predators were given fed with the larvae of *Corcyra cephalonica* (Stainton). The laboratory emerged adult *R. fuscipes* were used for the studies. The laboratory emerged third stadium of *S. litura* (133.00 ± 11.86mmg; 20.50 ± 0.19 mm; n = 15) and *H. armigera* (173.32 ± 2.62 mg; 22.30 ± 0.07 mm; n = 15) were used for the microinjection and oral toxicity bioassay,

whereas for the immunological assay, fifth stadium *S. litura* (317.24 ± 0.12 mg; 38.12 ± 0.12 mm; n = 10) and *H. armigera* (342.11 ± 0.05 mg; 34.41 ± 0.15 mm; n = 10) were used.

4.3.2. VS preparation

Nearly 75 adult *R. fuscipes* were continuously starved for three days and at the end of the starvation, the animals were milked by the method proposed by Sahayaraj *et al.* (2006a); Sahayaraj and Vinoth Kanna (2009). The collected venomous saliva (VS) was pooled and stored at -4 ºC. The VS concentrations such as 200 (0.02% VS), 400 (0.04% VS), 600 (0.06% VS), 800 (0.08% VS) and 1000 (0.10% VS) ppm were prepared using the phosphate buffer (PB) (0.2M Na_2HPo_4 and 0.2M NaH_2Po_4) (pH 7.2) and stored in the sterile vials at -4º C until use.

4.3.3. Microinjection bioassay

In microinjection bioassay, freshly molted healthy third stadium larvae of *S. litura* (n = 15/concentration) and *H. armigera* (n = 15/concentration) were selected and the various concentrations of VS (200, 400, 600, 800 and 1000 ppm) were injected into the third or fourth thoracic segment ventrally at the rate of 1.0 µl/animal using the Hamilton syringe (Hamilton, Switzerland). Control animals received an equal quantity of PB (pH 7.2) for the control and VS treated categories were released into the clean plastic vials contain the natural host (leaves of castor for *S. litura* and fresh pieces of bhendi for *H. armigera*). The animals were observed for a period of 96 h with an interval of 24 h. The larval mortality was observed up to 96 h (Ergin *et al.,* 2006).

4.3.4. Oral toxicity bioassay

A modified method of Vonarx *et al.* (2006) was used for this study using an artificial diet (Soy bean based diet for *S. litura* – Srinivasamurthy *et al.,* 2006 and Kidney bean based diet for *H. armigera* – Ahmed *et al.,* 1998). To the 100 mg of diet, 1.0 µl/animal of VS concentrations were mixed and blended and this was given fed to the animals. For the control category, the artificial diet was mixed along with PB (pH 7.2). Animals were maintained with the artificial diet. The larval mortality was observed for a period of 96 h with an interval of 24 h.

In both the microinjection and oral toxicity bioassay, the larval mortality was corrected to the control (natural morality) (Abott, 1925) and the corrected mortality was subjected for the LD_{50} calculation using Finney's method (Finney, 1971).

4.3.5. Gut enzyme profile

The live larvae of both *S. litura* and *H. armigera* obtained from the microinjection and oral toxicity bioassay were subjected to gut enzyme profile studies. The animal were dissected in the Insect Ringer's solution (IRS) (Nacl – 1.093%; KCl – 0.16%; $CaCl_2$. $2H_2O$ – 0.08%; $MgCl_2$. $6H_2O$ – 0.08%; Li *et al.*, 2009) and the gut was carefully removed and bifurcated into fore (FG), mid (MG) and hindgut (HG). The parts of gut were weighed and homogenized using a tissue homogenizer (Remi, India). Now the homogenate was centrifuged at 8000 rpm for 15 min. After centrifugation, the supernatant was mixed with 10 ml of PB and stored at 4 ˚C.

Digestive enzymes such as amylase (Ishaaya and Swirski, 1970); protease (Morihara and Tsuzuki, 1977) and lipase (Cherry and Crandall, 1932) and the detoxification enzymes such as acid phosphatase (Beaufay *et al.,* 1954) (described in chapter 3), alanine aminotransferase (ALT) and aspartate aminotransferase (AAT) (Bergmeyer and Bernt, 1965) and lactate dehydrogenase (LDH) (King, 1965) were studied.

4.3.5.1. Asparate (AAT) and Alanine aminotransferase (ALT)

The reaction mixture of 1.5 ml contains 100 µ moles of potassium phosphate buffer (pH 7.4), 2 µmoles of α- Ketoglutarate, 0.1 ml homogenate and 100 µ moles of L- aspartic acid for AAT and 100 µmoles of L-Alanine for ALT (Bergmeyer and Bernt, 1965). After incubation for 30 minutes reaction was arrested by addition of 1 ml of 2, 4- dinitro phenyl hydrazine (0.001 M). Later 10 ml of 0.4 N NaOH was added and the color was read at 546 nm in spectrophotometer against a reagent blank contain distilled water.

4.3.5.2. Lactate dehydrogenase

To standardize volumes, 0.2ml of NAD solution was added to the test and 0.2 ml of water was added to the control test tube, each containing 1 ml of the buffered substrate; 0.01ml of the sample was also added to the 'test'. Test tube samples were incubated for

exactly 15 min at 37 ºC and then arrested by adding 1 ml of color reagent (2, 4- dinitrophenyl hydrazine) to each tube and the incubation was continued for an additional 15 min. After the contents were cooled to room temperature, 10 ml of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline to maximize development of hydrazine. At exactly 60 seconds after the addition of alkali to each tube, the intensity of color was measured at 440 nm. Replicated blanks with standards were run through the same procedure. Inclusion of the calculated amount of reduced co-enzyme in the standard makes allowance for the chromogenicity of $NADH_2$ formed in the test. The enzyme activity is expressed as multi international units (mIU) per milligram protein per minute (King, 1965). A mIU is defined as the amount of enzyme that is required to catalyze the conversion of 1µm lactate to pyruvate or pyruvate to lactate per minute per millilitre of the sample under the prescribed assay conditions.

4.3.6. Immunomodulatory

4.3.6.1. Haemolymph collection

Six h starved healthy fifth instar (n = 10) *S. litura* and *H. armigera* were selected and they were immobilized by immersing in distilled water at 4 ºC and they were dried by blotting on a paper towel. The prolegs of the animals were surface sterilized with 70% ethanol and the cuticle at the base of a proleg was pierced with a sterile needle. The haemolymph was collected into a sterile eppendorf tube containing ice cold anticoagulant solution (98 mM NaOH, 145 mM NaCl, 17 mM EDTA and 4 mM Citric acid, pH 4.6) (R. – Xyu *et al*., 2007) and they were pooled.

4.3.6.1.1. Total haemocyte count (THC)

For the THC studies (Cai *et al.,* 2004), the total haemocyte count was carried out using 50 µl of haemolymph. It was mixed with 5 µl of 200, 400, 600, 800 and 1000 ppm VS concentrations and kept undisturbed for 5, 15, 30 and 60 min at 25 ºC. For the control, VS was replaced with the PB. After the stipulated incubation period, the haemocytes were stained with 0.5 µl of Giemsa stain [1.5% giemsa powder (Nice, India) in 100ml of methanol, heated up to 60 ºC for 30 min and 100 ml of glycerin were added and it was filtered. From the above filtrate, the working solution was prepared which contained 1 ml of the filtrate, 8 ml of distilled water and 1 ml of methanol]; and incubated for five seconds; haemocytes were

counted using the Naubauers haemocytometer under a light microscope. The haemocyte numbers were randomly counted from five squares and the total haemocytes were calculated using the method of Jones (1962).

4.3.6.1.2. Haemolymph protein profiling

In another experiment the haemolymph protein profile was recorded using the different concentrations (200, 400, 600, 800 and 1000 ppm) of VS of *R. fuscipes.* From the collected haemolymph, 50 µl of the sample was added with the various concentrations of VS and it was incubated at 25 ºC for 60 min period. Now the sample was mixed with an equal quantity of gel buffer and 30 µl of the sample was loaded onto a SDS PAGE gel (12% separating gel and 4% stacking gel) and they were run at 90 mv using a Tris -glycine buffer (Laemmli, 1970). And after the running of gel, it was stained using staining solution (0.25% commassive brilliant blue in 40 ml of methanol, 10ml of acetic acid and 100 ml of distilled water) for one over night at room temperature. After the staining, the gel was destained (40% methanol and 10% acetic acid). The gel was imaged using the gel documenter (Biotech, India). The molecular weight of the whole haemolymph protein was calculated using the DGel systems (Biotech, India) which was compared with a high molecular protein marker (Genei, Bangalore).

4.3.6.1.3. Inhibition of haemocytes aggregation behavior

Fifth instar larvae of *S. litura* and *H. armigera* insect (n = 10) were individually swabbed with 70% ethanol (v/v), dried and then the cuticle at the base of a larval proleg was pierced with a minute sterile pin under sterile conditions. After bleeding, haemolymph from individual larvae were collected in sterile eppendorf tubes with a few crystals of 1-phenyl-2-thiourea (PTU). Haemocytes sample was prepared for aggregation bioassays according to the method described by Dani and Richards (2009), with a slight modification that, haemocyte concentration was increased (2×10^5 cells per well). 10 µl of VS concentrations was diluted to 100 fold in phosphate buffer (pH 7.2). 20 µl of the diluted VS was applied to 100 µl of haemocytes in strip ELISA plate. Ampicillin (Himedia, India) and a pinch of phenoloxidase inhibitor phenylthiocarbamide (PTC) (Hi-Media) were added to each well to make the final concentration of 100 µg/ml and 20 µm respectively. After incubation at 20 °C, 65% relative humidity for 30 and 240 min, the plates were observed using low-power (40×) Phase contrast microscopy (Olympus CX41, Japan). Inhibition of haemocyte motility was observed in the

absence of haemocyte aggregates, or a reduction in the aggregation compared to control which incubated with PB. Five replications were made for each study period.

4.3.6.1.4. Inhibition of haemocytes spreading behavior

This study was carried out as described by Yu *et al.* (2007) with slight modification. The concentration of haemocytes in the haemolymph was adjusted to $1x10^5$ cells/ml by addition of PBS (pH 6.3), 0.1 mg streptomycin sulfate and 10,000 units of ampicillin per 100 ml medium. Immediately, an aliquot of 50 µl haemolymph was transferred by pipetting into each well of 12-well strip containing 199 ml PBS with 10% bovine serum albumin per well. Then, a 1.0 µl aliquot of VS concentrations (200, 400, 600, 800 and 1000 ppm) and phosphate buffer saline (PBS) (0.8% NaCl in PB) as the control were added to the respective plates. Haemocytes with or without VS treatment were incubated at 27 ºC. After 30 min and 240 min of incubation, the spreading of haemocyte was observed using phase contrast microscope (Olympus CX 41, Japan). Spreading and non-spreading plasmatocytes (PC) and granular cells (GC) (identification as described by Gupta, 1979) were counted from three randomly chosen fields of view at 40x magnification.

Approximately 80 cells were counted in each field of view and totally 240 cells were counted (Sahayaraj and Muthukumar, 2011). The spreading percentage and spreading inhibitory ratio of PL and GC respectively were calculated as follows:

$$\% \text{ spreading} = \frac{\text{No of spreading PC or GC observed}}{\text{Total no of spreading \& non-spreading PC or GC observed}} \times 100$$

$$\text{Spreading inhibitory ratio} = \frac{\text{\% spreading of PC or GC without venom treatment as the control} - \text{\% spreading of PC or GC with venom treatment}}{\text{\% spreading of PC or GC of the control}} \times 100$$

Five wells were evaluated for each venom concentration in three replicates.

4.3.7. Statistical analysis

The statistical analysis was made in comparison between the microinjection and oral administration bioassay methods. All the data were compared using the One way Analysis of

Variance (ANOVA) and post hoc test (Tukey's and DMRT) with 5% significance with the SPSS statistical package ver 11.5 (SPSS Inc., 2005).

4.4. RESULTS

4.4.1. Insecticidal activity

Microinjection of *R. fuscipes* VS caused significantly ($df = 12, 7$; $F = 9.093$; $p < 0.05$) less corrected mortality than the oral administration (Figure 4.1) in *S. litura*. The larvae mortality was recorded within 24 h in both the categories. 50% mortality was recorded even at 48 h of about 53.33 and 60.00% in the 800 and 1000 ppm (LD_{50} = 890.13 ppm/larva) and prolonged time of exposure also showed an increased mortality up to 96 h having a lesser LD_{50} value of 861.60 ppm. However in the oral toxicity bioassay, a dose dependent manner was not observed at the 72 h period, 50% mortality was recorded and at the 96 h the mortality was 21.43, 35.71, 50.00, 71.43 and 71.43% for 200, 400, 600, 800 and 1000 ppm VS concentrations respectively.

The *H. armigera* larvae has an insignificantly ($df = 14, 5$; $F = 3.789$; $p > 0.05$) higher per cent of mortality in the microinjection bioassay than the oral administration bioassay. Those injected with VS died within 24 h and showed an LD_{50} value of 06.24 ppm. At 96 h, the LD_{50} value was 846.35 ppm/larva with a maximum of 84.62% larval morality. However, in the oral toxicity bioassay method, at 96 h observation, the LD_{50} value was 899.91 ppm/larva (Figure 4.2) with 71.43% corrected mortality.

4.4.2. Gut enzyme profile

4.4.2.1. *Spodoptera litura*

Significantly high level of amylase was observed in the midgut for 600 ppm ($df = 2.9$; $F = 1.467$; $P < 0.05$). In control, protease activity was high in the midgut region ($df = 2.9$; $F = 4.171$; $P < 0.05$). At 1000 ppm of VS, midgut showed a higher level of lipase activity (37.35 mequ/min/g), whereaas the detoxicifcation enzymes, the maximum AP activity was recorded in the midgut in 600 ppm category. AAT activity in the control foregut was siginificantly high. LDH avtivity was recorded in the midgut of 1000 ppm ($df = 2,9$; $F = 1.060$; $p < 0.05$) (Table 4.1).

In the oral toxicity bioassay, amylase, lipase, acid phosphatase, ALT and LDH activities increased on a dose dependent maner. Digestive enzymes; amylase activity was siginificantly high in foregut of 1000 ppm ($df = 2,9$; $F = 1.228$; $p < 0.05$) VS. And the foregut of control category had high level (116.61±0.19 µg/g) of protease activity. Lipase activity was higher at the 1000 ppm (12.69 mequ/min/g) VS in hingut region. The detoxicication enzymes such as acid phosphatase was maximum in the midgut at 1000 ppm ($df = 2,9$; $F = 1.241$; $p < 0.05$). The ALT activity was significantly high in the midgut at 1000 ppm ($df = 2,9$; $F = 1.705$; $p < 0.05$). However enzyme activity was not recorded in the foregut and hindgut of control at the 200 ppm category. AAT and LDH activities were not recorded in the control, 200, 400 and 600 ppm categories. However maximum enzyme activites for AAT ($df = 2,9$; $F = 1.619$; $p < 0.05$) and LDH ($df = 2,9$; $F = 4.763$; $p < 0.05$) were recorded in the foregut at 1000 ppm VS (Table 4.2).

4.4.2.2. *Helicoverpa armigera*

In the microinection bioassay, the enzymes such as protease, lipase, AST, ALT and LDH activities were high based on dose dependent manner. Digestive enzymes such as the amylase activity was significantly higher in the hindgut of 600 ppm ($df = 2,9$; $F = 7.841$; $p < 0.05$). In the protease the midgut at 1000 ppm a significantly maximum enzyme activity was observed ($df = 2,9$; $F = 8.326$; $p < 0.05$), whereas in the lipase, the hindgut at 200 ppm had the maximum enzyme activity (13.34 mequ/min/g). The detoxicification enzyme such as acid phosphatase was maximum in the midgut at 600 ppm ($df = 2, 9$; $F = 2.436$; $p < 0.05$) and the ALT ($df = 2,9$; $F = 2.366$; $p < 0.05$) and LDH ($df = 2, 9$; $F = 4.932$; $p < 0.05$) activities were found to be siginificantly higher at the hindgut of 1000 ppm whereas AAT was significantly higher in 1000 ppm ($df = 2, 9$; $F = 2.436$; $p < 0.05$) VS (Table 4.3).

In the oral toxicity bioassay, amylase, lipase, acid phosphatase, ALT and LDH increased enzyme activity was recorded. Digestive enzymes such as amylase was significantly higher at 1000 ppm of hindgut ($df = 2,9$; $F = 1.550$; $p < 0.05$). In the control at foregut protease activity (98.75±0.18 µg/g) was siginificantly higher. In the 1000 ppm VS the lipase had high level of enzyme activity (11.42 mequ/min/g). Detoxification enzymes, the acid phosphatase activity was significantly maximum at foregut of 1000 ppm ($df = 2,9$; $F = 1.148$; $p < 0.05$). In ALT, foregut of 1000 ppm had a siginificantly higher enzyme activity ($df = 2,9$; $F = 2.920$; $p < 0.05$). AST activity was not recorded in the control, 200 and 400 ppm

categories. However, maximum enzyme activities were recorded in the foregut at 1000 ppm ($df = 2,9$; $F = 1.682$; $p < 0.05$). Similarly, no LDH activity was recorded up to 600 ppm VS incorparated artifical diet fed categories and the maximum activity was recorded in the hindgut at 1000 ppm VS category ($df = 2,9$; $F = 9.747$; $p > 0.05$) (Table 4.4).

4.4.3. Immunomodulatory

4.4.3.1. Total haemocyte count assay

The VS mixed *S. litura* haemolymph was less viscous than the control haemolymph. However, in *H. armigera,* the colloidal nature of the haemolymph becomes watery and a significant haemocyte death was recorded. In control, intact haemocyte population was insignificantly reduced up to 20.30% and 12.41% in *S. litura* ($df = 1, 4$; $F = 14.03$; $p > 0.05$) and *H. armigera* ($df = 1, 4$; $F = 225.63$; $p > 0.05$), respectively at 60 min incubation, as the incubation time increased. But the haemocyte population was gradually increased up to 800 ppm of VS (35.20%) (Figure 4.3).

Haemolysis was observed in VS dose dependent [75.4, 66.8, 66.6, 56.1, 50.1, 51.0 for control, 200 ($df = 1, 4$; $F = 2.41$; $p > 0.05$), 400 ($df = 1, 4$; $F = 6.87$; $p < 0.05$), 600 ($df = 1, 4$; $F = 14.46$; $p < 0.05$), 800 ($df = 1, 4$; $F = 54.55$; $p < 0.05$) and 1000 ($df = 1, 4$; $F = 87.04$; $p < 0.05$) ppm, respectively] and time dependent factor [22, 27, 28 and 30% for 5, 15 ($df = 1, 4$; $F = 0.375$; $p > 0.05$), 30 ($df = 1, 4$; $F = 163.84$; $p < 0.05$) and 60 ($df = 1, 4$; $F = 225.63$; $p < 0.05$) min, respectively based on the time dependent manner] in *S. litura* and *H. armigera* in VS dose dependent [87.6, 86.6, 85.2, 83.0, 81.8, 79.1 for control, 200 ($df = 1, 4$; $F = 0.40$; $p > 0.05$), 400 ($df = 1, 4$; $F = 1.12$; $p > 0.05$), 600 ($df = 1, 4$; $F = 2.37$; $p > 0.05$), 800 ($df = 1, 4$; $F = 4.90$; $p > 0.05$) and 1000 ($df = 1, 4$; $F = 4.95$; $p > 0.05$) ppm, respectively) and time dependent factor (11.83, 12.12, 17.94 and 20.90% for 5, 15 ($df = 1, 4$; $F = 5.07$; $p > 0.05$), 30 ($df = 1, 4$; $F = 112.5$; $p < 0.05$) and 60 ($df = 1, 4$; $F = 14.03$; $p < 0.05$) min, respectively) (Figure 4.4).

4.4.3.2. Haemolymph protein profile

The effect of the VS on the *S. litura* haemolymph protein profile showed that six polypeptides had 151 to 10 kDa molecular weight (Plate 4.1a). It was altered by 200 ppm (139 to 3 kDa), 400 ppm (140 to 3 kDa), 600 ppm (136 to 5 kDa), 800 ppm (140 to 4 kDa)

and 1000 ppm (139 to 5 kDa) VS of *R. fuscipes*. The polypeptide having 60 kDa was lost at 400, 600, 800 and 1000 ppm concentrations of VS treated haemolymph.

In *H. armigera* haemolymph, six polypeptides (184 to 21 kDa) (Plate 4.1b) were observed (Plate 4.2). It was altered in the haemolymph treated with 200 ppm (179 to 10 kDa), 400 ppm (179 to 15 kDa), 600 ppm (171 to 10 kDa), 800 ppm (169 to 3 kDa) and 1000 ppm (176 to 19 kDa) venomous saliva of *R. fuscipes*. Furthermore 160 kDa polypeptide was disappeared in VS treated categories, whereas an additional polypeptide (21 kDa) appeared at 400 ppm of VS of *R. fuscipes* (Plate 4.1b).

4.4.3.3. Inhibition of haemocyte aggregation

About 66.67% of the plasmatocyte (PL) and 71.79% of granulocyte (GC) of *S. litura* aggregated in the control during 30 min. However, addition of VS significantly reduced the PL ($t = 49.44$; $p < 0.05$) and GC ($t = 19.00$; $p < 0.05$) aggregation during the 30 min in the 200 ppm and in the 1000 ppm it was further reduced to 16.41% ($t = 26.00$; $p < 0.05$) and 55.88% ($t = 23.56$; $p < 0.05$) for PL and GC, respectively. After 240 min of incubation, haemocyte aggregations significantly decreased in both PL (45.2%) ($t = 57.48$; $p < 0.05$) and GC (35.7%) ($t = 39.65$; $p < 0.05$) respectively in the 1000 ppm category (Figure 4.5 a).

The aggregation at 30 min in the PL (80.00%) and GC (83.33%) and it was significantly increased at the 1000 ppm ($t = 3.00$; $p < 0.05$) in PL (33.33%) and GC (29.41) ($t = 0.124$; $p < 0.05$). At 240 min, the aggregation was significantly increased in PL (73.07%) ($t = 1.481$; $p < 0.05$) and GC (66.67%) ($t = 4.736$; $p < 0.05$) in the 1000 ppm (Figure 4.5 b).

4.4.3.4. Spreading inhibitory behavior

At 30 min incubation period, about 85.7% of PL (600 ppm) and 22.2% in GC (1000 ppm) spreaded was inhibited. While the incubation period was increased (240 min) the spreading inhibition was increased in GC (57.79%) ($t = 2.916$; $p > 0.05$) and PL (30.73%) ($t = 1.851$; $p > 0.05$) decreased insignificantly (Figure 4.5 c)

The spreading inhibitory percentage of *H. armigera* PL and GC were significantly, having 58.3% and 64.7% in 1000 ppm at 30 min inhibition ($t = 3.986$; $p < 0.05$). While the incubation period was increased up to 240 mins the spreading inhibition percentage showed significantly decreased response (45.3% and 57. 2%) for PL ($t = 2.641$; $p < 0.05$) and GC ($t = 4.178$; $p < 0.05$) respectively (Figure 4.5 d).

4.4.3.5. Haemocyte morphogenesis

In *S. litura,* the haemolymph forms a clump and adhesive to the nearby cells and aggregates (Plate 4.2a), which has been inhibited by the VS (Plate 4.2b). When the exposure of VS reacts with the aggregation of haemocytes which may lead to the disintegration of haemocytes which was observed at the 30 min of incubation (Plate 4.2c) while the period of exposure was increased up to 240 min a clear region of haemocyte spreaded was observed (Plate 4.2d) and the VS reacted with the haemocytes and removed the adhesive nature of haemocytes. When considering the morphology of the circulating haemocytes such as PL and GC, it was observed that they were much more affected by the VS addition. The PL was the major one affected by the VS with a capsule like formation (Plate 4.2e, f) on the outer surface of the PL and it was enlarged, which may lead to the bursting of the cell and causes cell death. Similarly another feature is the elongation of filopodial like structure was observed in the PL (Plate 4.2k, l). Other than the PL, GC was also affected by the VS which may cause a pore formation (Plate 4.2i, j) on the outer cell wall followed by the oozing out of the inner cellular contents and lead to the cell death.

In *H. armigera,* the formation of aggregation is very dense (Plate 4.3a), which has been inhibited by the VS (Plate 4.3b).. This may be due to the sticky nature of the haemolymph. The addition of VS may lead to the disintegration of haemocytes. The spreading of haemocytes was very low when compared to that of the haemocytes of *S. litura*. Plate 4.3c showed that at 30 min of incubation with VS causes a very low spreading and when the incubation period was increased upto 240 min, scattered haemocytes were observed in crowded nature (Plate 4.3d). The morphological features of the circulating haemocytes such as PL and GC were also having their significant changes which were observed. Generally the PL was spindle shaped. The addition of VS had its action on the morphology and disintegration of the PL morphology was observed showing the damaged region over the PL (Plate 4.3f, g, h, i). Along with this the granular cells also showed considerable changes such as nodule like formation (Plate 4.3j, k). Filopodial like structure projected on the outer surface of GC (Plate 4.3l). And pore formation on the cell wall of GC led to the oozing out cytoplasmic contents (Plate 4.3m, n).

4.5. DISCUSSION

The analysis of venomous saliva of *Rhynocoris fuscipes* on *Spodoptera litura* and *Helicoverpa armigera* has shown its biological and immunomodulatory activity in *in vivo* and *in vitro* conditions. The paralytic activity of the venomous saliva has toxic nature towards the animals. In the microinjection bioassay, in both *S. litura* (64.29%) and *H. armigera* (84.62%) larval mortality was high at 96 h leading the evidence to the toxicity of the VS. Though enormous number of works are available about the impact of true venom of predatory insects (Schmidt, 1986; Eitan *et al.,* 1990; Stewart *et al.,* 1991; Zilberberg *et al.,* 1991; Zlotkin *et al.,* 1994; Atkinson *et al.,* 1996; Gordon, 1997; Corzo *et al*., 2001; Regev *et al.,*2003; Hisada *et al*., 2005; Inceoglu *et al.,* 2005; Cohen *et al.,* 2006; Mackessy *et al.,* 2006; Miyashita *et al.,* 2007; Whetstone and Hammock, 2007; Herzig and Hodgson, 2008; Abdel-rahman *et al.*, 2010) and parasitoids (Fontana *et al.,* 2000; Rodrigues *et al.,* 2004; Uçkan *et al*., 2004; Dani *et al.,* 2005; Marques *et al.,* 2005; Ergin *et al.,* 2006; Rivers *et al*., 2006; Stocklin *et al.,* 2010) on insect pests, very few reports were about reduviid predators.

The salivary gland extract of *Acanthaspis pedestris* was found to possess paralytic activity against the *Mylabris pustulata* (Ambrose and Maran, 1999). A hunter reduviid paralyses a prey within 3 to 10 seconds (Edwards, 1961; Ambrose, 1999; Sahayaraj, 2007). The injection of VS causes wriggling and restless movement, rapid mastication action of mandible, falling of lateral side and becoming motionless for 30-40 min and then resuming its routine activities. The crude venom of *Eulophus pennicornis*is is highly active and induces developmental arrest at relatively low levels of injected protein (Marris and Edwards, 1995; Weaver *et al*., 1997; Marris *et al.,* 2001; Bell *et al.,* 2010) with insects ceasing to feed 2-4 d after injection, becoming moribund and failing to molt to the next larval stadium. Quistad *et al.* (1994) identified 6 paralytic toxins in the venom of the ectoparasitoid, *B. hebetor*, with at least 2 of the isolated proteins demonstrating high insecticidal activity toward 6 species of lepidopteran larvae and the crude VS of *R. fuscipes* injection causes a rapid mortality and in future studies the toxic components will be dealt with. The VS blended artificial diet showed a lesser mortality in both the animals when considering the microinjection assay. This was previously reported by Vonarx *et al*. (2006) who showed the spider's toxins are also likely to be inactive on ingestion by the *H. armigera* larvae. The VS blended artificial diet fed animals showed no significant changes during the feeding process and after the feeding. Yet the dead larvae were blackish in color. The blended VS pass through the prey gut, the surface epithelial cells may act upon the VS and it gets disintegrated (Fishman and Zlotkin, 1984),

and the direct injection of VS into the haemoceol causes rapid mortality. In the oral administration bioassay, both the *S. litura* and *H. armigera* require a larger quantity of VS for the paralysis of about 913.00 and 928.12 ppm/larva respectively. The LD_{50} doses were obtained even at 24 h from the microinjection bioassay yet in the oral administration bioassay, it may require 72 h for its mortality.

The uptake of a higher quantity of VS in the oral administration bioassay might be due to the disintegration of VS during the passage in the gut which was previously described by Capp *et al.* (1972) and in their studies they have shown that the passage of protein into the gut has to overcome several difficulties. Initially, it has to resist a wide and rich variety of proteolytic enzymes (House, 1974; Sinha, 1976). The gut of the *S. litura* and *H. armigera* contains maximum quantity of protease enzymes followed by the lipase and detoxification enzymes such as LDH, ALT and AAT. Second it has to overcome the multilayered and relatively thick peritropic membranes (Naponitaya and Misch, 1974) and finally it has to pass through the continuous layer of epithelial cells and the basal membrane (Richards, 1975). The toxicity of orally administered VS has been demonstrated through its effects on the survival of larvae and on the growth and food consumption of third stadium larvae. Study shows that oral administration of the VS had proven ineffective (Fitches *et al.,* 1997) and similarly, the VS blended artificial diet fed animal mortality was observed during the 72 h of the experiment. Recombinant fusion proteins combining snowdrop lectin (GNA) linked either to the insect neuropeptide or to an insect spider venom neurotoxin have demonstrated that GNA can be utilized as a transporter to deliver linked peptides to the haemolymph of lepidopteran *L. oleracea* larvae (Fitches *et al*., 2002, 2004), whereas Mukherjee *et al.* (2006) have demonstrated that the peptides from the venom of *Hadronchya versuta* are lethal to the lone star tick *Amblyomma americanum* either by injection or given fed.

A wide range of digestive enzymes were recorded in the alimentary canal of insects (Chapman, 2000). Digestive enzymes play a major role by concerning complex food materials into micromoleclues necessary to provide energy and metabolites (Wigglesworth, 1972). Swingle (1925) have studied that the digestive enzymes are almost produced in the midgut rather than the foregut, whereas in the hindgut, the enzymes are either reabsorbed or destroyed. The gut enzyme profile of *S. litura* reveals that amylase and LDH are localized in HG and MG containing high level of protease, lipase, AP, AAT and ALT and in *H. armigera* high level of enzyme activity was recorded in MG which posses lipase and HG contains amylase, protease, AP, ALT, AAT and LDH activities larvae treated with VS of *R. fuscipes*

have clearly proved that the VS might alter the enzyme profile and it might also evoked the detoxification enzyme level in the gut against the VS. When comparing both the *S. litura* and *H. armigera* the detoxification activity was highly evoked in the latter animal category. In the microinjection bioassay, the activity of amylase was in a reduced state, and it showed that the amylase are feed upon the starchy items (Pereira *et al.,* 1999) in this bioassay, theses animals fed on their natural hosts, whereas in the oral administration bioassay the fed of artificial diet which may evoke the amylase activity and so increased activity was recorded. There is gradual increase in the lipase activity during larval growth. Most of the insects accumulate large quantities of lipid during larval period. The lipase plays an important role in fat mobilization in the epithelium of the lumen. The occurrence of lipase is an indirect indication of the capacity for utilization of fat for energy source (Thomas and Nation, 1984) and hence in both the bioassay in order to uptake the VS of *R. fuscipes*, both *S. litura* and *H. armigera* secretes more quantity of lipase. Higher activity of lipase appears to hydrolyse the fat into fatty acids and glycerol (Kumar, 2011), whereas the increased protease activity was observed in microinjection bioassay and decreased activity in oral administration bioassay. This may be due to the fact that the passage of VS into the gut has to resist a wide and rich variety of proteolytic enzymes (House, 1974; Sinha, 1976) and so in the oral administration bioassay, these proteolytic enzymes may act upon the VS.

Enzymatic detoxification is a major means that insects use to avoid toxicity (Ahmad *et al.,* 1986). In general, the acid phosphatase activity was found to be increased in both the microinjection and oral administration bioassayed animals. Generally the level of AP activity was higher in both the experiment category animals except in the microinjection assayed *H. armigera*. AP is the hydrolytic enzymes, which hydrolyze phosphomonoesters and they are found in the intestinal epithelium of animals and its primary function is to provide phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes. (Sakharov *et al.,* 1989). AP is located in the midgut, malpighian tubules, muscles, and nerve fibers of the lepidopteran insects (Horie, 1958). The AP helps the animal to act against the VS by providing the phosphate ions whereas, *H. armigera* injected with VS causes 50% mortality within 24 h. Senthilnathan *et al.* (2005) have found out the level of AP activities. It was found to be increased in the S. *litura* exposed to azadirachtin. Any impairment in AP level will affect the physiology of the insect gut. The ingestion of lectins, including lectins mixed neuropeptides; leads to changes in the activities of gut and brush border marker enzymes (Pusztai *et al*., 1996). Fitches and Gatehouse (1998) demonstrated that lectins

increased gut protein levels and brush border membrane aminopeptidase activity and also increased trypsin activity, both in the gut and in the faeces. Increased activity of α-glucosidase, but neither lectin had a significant effect on alkaline phosphatase activity. The treatment with *Dysoxylum* triterpenes have a high biological activity towards the rice leaffolder, *Cnaphalocrocis medinalis* larvae, and it has its effect on the gut enzymes such as acid phosphatases, alkaline phosphatases and adenosine tri phosphatases activities were inhibited (Senthilnathan *et al.,* 2007). While the increased level of ALP was observed in both the bioassays it was previously described by Yerasi and Chitra (2000) that the insect tried to metabolize the toxic compound into a non toxic compound by the induction of high level of AAT and ALT activities. In general the activity of LDH was found to be increased simultaneously in all the bioassays. This might be involved in carbohydrate metabolism and has been used as an indicator of exposure to chemical stress (Diamantino *et al.,* 2001). LDH is an important glycolytic enzyme present virtually in all animal tissues (Kaplan and Pesce, 1996). This probably occurs also in situations of chemical stress. Therefore, this enzyme may be a sensitive criterion in laboratory (Wu and Lam, 1997; Diamantino *et al.,* 2001; Senthil Nathan *et al.,* 2005).

There are relatively a few reports in the literature describing the protein profile of haemolymph from *S. litura* (Kaselin *et al.,* 2005) and *H. armigera* (Subramanian and Gujar, 2000). As SDS PAGE analysis of haemolymph from *S. litura* (151 to 10 kDa) and *H. armigera* (184 to 21 kDa), indicates that they contain relatively a few, mostly low molecular weight proteins. Only a limited number of hunter reduviid VS have been reported to affect the behavior of insect haemocyte. Interestingly, under SDS PAGE conditions *R. fuscipes* VS was shown to alter the *S. litura* and *H. armigera* haemolymph protein profile. For instance, we observed for the first time that a polypeptide with 60 kDa and 160 kDa disappeared, while *S. litura* and *H. armigera* respectively were incubated *in vitro* with different concentrations of VS of *R. fuscipes,* indicating that VS of this reduviid alter the arrangement of the haemolymph protein profile. The 60 kDa present in the *S. litura* might be responsible for the storage protein (Baker and Fabrick, 2000) and also it acts as a lipoprotein (Arrese *et al.,* 2001) while the 160 kDa is responsible for the vitellogenin-like protein is involved in arthropod melanin synthesis (Lee *et al.,* 2000). Additionally a 21 kDa protein appeared in the treated *H. armigera* haemolymph and this was responsible for the junivelle hormone proteins. We hypothesized that these proteins are synthesized by the hosts as an immune reaction in response to hunter reduviid. Along with haemolymph protein profile, the haemocyte numbers

have also been altered in *S. litura* and *H. armigera* and this may be due to the suppressing of host immune system which was previously described by Li *et al.* (2009) in *H. armigera.*

It has been observed that the nature (viscosity) of the haemolymph of both hosts change immediately after the addition of VS of *R. fuscipes* as observed earlier in *Pseudaletia separate* (Teramoto and Tanaka, 2004). Possibly humoral factors such as the haematopoiesis inhibiting factor (HIF) or growth blocking peptide (GBP) (Ohnishi *et al.,* 1995). The mean THC for the 60 min incubated group indicated a difference of 20.31% fold in *S. litura* and 12.38% fold in *H. armigera* respectively, when compared to the mean THC of the control group. The mean variation in the overall THC between the control and VS treated groups strongly indicated the likelihood of hemolytic activity. The dose dependent interaction (hemolytic activity) between THC and VS was also observed in our studies. This suggests that the VS cause the first decrease in haemocytes. Direct hemolytic activities have been observed in venom of different bees, wasps and ants (Bettini, 1978).

In our studies, both the time and dose were dependent factors in response towards the total haemocyte numbers. Strand and Noda (1991) observed that the total haemocyte counts were higher in parasitized *Pseudophsia inchdens* larvae than unparasitized larvae by parasitism of *Microplitis demolitor*. The control animals of *S. litura* ($12.85x10^5$ cell/ml) and *H. armigera* ($13.97x10^5$ cells/ml) were recorded, while the time and dose of VS were increased showed a decreased count of the THC. The less THC was due to the aggregation of haemocytes followed, this may play on the cytoskeleton of haemocytes and causes a pore formation and abnormalities which leads to the cell death (Richards and Edwards, 1999) which was observed in both *S. litura* and *H. armigera*. In the haemocytes, the cell apoptosis is also an important factor (Richards and Parkinson, 2000). Earlier from the venom of *O. sinensis* which actively suppresses the haemocyte immune response of the host, *Helicoverpa armigera* possibly by destroying the host haemocyte cytoskeleton (Li *et al*., 2009). In our studies, the VS of *R. fuscipes* involved in the haemocyte morphology and the cytoskeleton of the haemocytes were changed and made them to lyses by formation of nodule like appearance over the cell surface and leads to the pore formation. The GC initially encapsulated the invading foreign substances and it was coated by the plasmatocyte which led to phagocytosis of them (Pech and Strand, 1996), whereas the VS of *R. fuscipes* reacted with the PL and GC and made them disabling its function by altering its morphology. One possibility is that they lyses either by direct contact of biomoleclues (33.1 kDa) present in the VS of *R. fuscipes* reported in other predatory arthropods (Richards and Dani, 2008; Dani and Richards, 2009)

which was observed in the Tricine SDS-PAGE of the VS of *R. fuscipes* mentioned in Chapter 3. The viability of PL and GC was also decreased at high VS concentrations. This suggests that increased concentrations of venom proteins not only affected spreading but also viability after exposure over extended time periods.

Another possibility is that the haemocyte are not eliminated but merely become sensible by attaching to the host tissues (Parkinson *et al.,* 2002a). Abnormalities (Strand and Noda, 1991; Richards and Parkinson, 2000), aggregation (Amaya *et al*., 2005; Richards and Dani, 2008), encapsulation (Clark *et al*., 1997; Zhang and Wang, 2003; Turnbull *et al*., 2004) of haemocytes of *S. litura* and *H. armigera* are also featured due to the VS of *R. fuscipes.* Proteases and toxic metabolites produced by *Beauveria bassiana* cause reduced haemocyte spreading in the greater wax moth, *Galleria mellonella* (Griesch and Vilcinskas, 1998), and the beet armyworm, *S. exigua* (Hung *et al*., 1993; Mazet *et al*., 1994). Lysis of PL and GC was also observed indicating the success of these host-predator relationships.

Our *in vitro* spreading study indicates that the plasmatocyte (PL) and granular cells (GC) spread rapidly. This is due to functional impairment of PL and GC in their spreading behavior *in vitro*. The reduction in the number of circulating haemocytes is caused by the breakdown of the circulating haemocytes and of the hematopoietic organ which generates the circulating haemocytes. Changes in the behavior of capsule functioning cells have been reported in other lepidopteran host predator relationships (Parkinson *et al.,* 2002b; Cai *et al.,* 2004; Dani *et al.,* 2005). Most of the PL of *H. armigera* remained spindle shaped while the other assumes a bipolar morphology. It could reflect the existence of PL subpopulation or a variable response by the cells (Strand and Noda, 1991). Both PL and GC remain affected when host haemolymph was incubated along with VS of *R.* fuscipes. Interestingly, changes in the behaviors of encapsule–forming cells, altered morphology of PL, loss of adhesion property *in vitro*, apoptosis. The significance of haemocytes clumping *in vitro* (Plate 4.1 and Plate 4.2) is clear. Indeed, clumping as the basis of an *in vitro* encapsulation system (Ratner and Vinson, 1983). However clumping occurs, in this study suggesting that the suppression of encapsulation is not due to the inhibition of cell-cell adhesion phenomena and PL are involved in extending the pseudopodia formation (plate 4.3 and plate 4.4) by making interaction with the nearby cell to agglutinate. Previously, venom protein (VPr_3) of *Pimpla hypochondriaca* was shown to inhibit the spreading and aggregation of lepidopteron larvae haemocytes maintained *in vitro* (Richards and Dani, 2008) as observed here. Alternation in

haemocyte behavior could be due to direct contact of VS biomoleclues (protein, peptides or other molecules) with haemocytes, but additional studies are needed to determine the basis for the abnormal behavior of PL and GC.

4.6. CONCLUSION

The present study shows that hunter reduviid venomous salivary components had several effects on the host haemolymph, haemocyte, nutritional physiology, digestive physiology, detoxification mechanism and neuro muscular tissue. The microinjection bioassay was found to be more effective than the oral toxicity bioassay, caused more mortality, increased digestive enzyme activity, suppressed immune system, and decreased total haemocyte count. Along with anti-aggregation, anti-spreading behaviors, VS also made changes in the morphology of PL and GC, which may lead to their death.