Rhynocoris fuscipes fab venomous saliva biological immunomodulatory activity against insect pest and mice

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Doctor of philosophy in Biotechnology – Zoology

By

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CERTIFICATE

I certify that the thesis entitled **"*Rhynocoris fuscipes* (Fab.) venomous saliva: biological and immunomodulatory activity against insect pest and mice"** being submitted by **Mr. A. Vinoth Kanna** (Reg. No. 3176) for the award of the **Degree of Doctor of Philosophy in Biotechnology – Zoology** at Manonmaniam Sundaranar University is a Bonafide record of the research work carried out by him independently in the Crop Protection Research Centre, Department of Advanced Zoology and Biotechnology, **St. Xavier's College (Autonomous), Palayamkottai** under my guidance and supervisor. The details furnished in the thesis is the original work of the candidate and has not been submitted elsewhere in part or full for any other degree, diploma, associate ship or other similar titles. It is not the plagiarism of any other work either published or unpublished without acknowledgement.

Place: Palayamkottai
Date: 26.08.2011

Signature of the Guide

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CONTENTS

1. Sahayaraj, K. and Vinoth Kanna, A. 2009. Starvation impact on venom quantity of a reduviid predator, *Catamirus brevipennis* (Servile) (Hemiptera: Reduviidae). *Entomon,* 34(2): 119-121.

2. Sahayaraj, K., Kanna, A. V. and Kumar, S. M. 2010. Gross morphology of feeding canal, salivary apparatus and digestive enzymes of salivary gland of *Catamirus brevipennis* (Servile) (Hemiptera: Reduviidae). *Journal of Entomological Research Society,* 12: 37-50.

Preface

Reduviids belong to the family Reduviidae (Hemiptera). It comprises true bugs which contain 6224 species; most of them are zoophagous predators. These predators are found in the agro-ecosystems, semi-arid zones, scrub jungles and forests of different kinds. Under natural conditions, they hunt other insects for sustenance. Hence, enormous hunter predators have been identified as biological control agents against many economically important pests. So far enormous literatures are available about the distribution and diversity, biology, mass rearing, stage preference, host preference and biological control potential of hunter reduviids. Reduviid predators capture their prey, inject the toxic venomous saliva, partially digest the prey; flush out the content of the prey, leaving the empty cases of the prey and post-predatory behaviour. Though reduviids follow specific adaptations to paralyze and suck the prey content, no one has tried to study the gross morphology of the head in general and rostrum in particular and its associate organ, salivary gland complex. Furthermore, biochemistry of the venomous saliva is a very poorly understood area. To fulfill this existing lacuna, we consider this problem and provide basic and applied highlight through this Ph. D thesis entitled "*Rhynocoris fuscipes* (Fab.) venomous saliva: biological and immunomodulatory activity against insect pest and mice". The investigations were carried out at Crop Protection Research Centre, St. Xavier's College (Autonomous), Palayamkottai.

Rhynocoris fuscipes (Fab.) is a bright coloured, crepuscular, entomosuccivorous, polyphagous predator. It has a prey record of more than 42 insect species. It is mainly found in the agro-ecosystems, scrub jungles, semi-arid zones and tropical forests and is considered as a good biocontrol agent in many Asian countries. However, no one has studied the morphology and anatomy of head, rostrum and their parts; salivary gland complex (SGC) of this reduviid and hence an attempt was made to describe the mouthpart, SGC morphology and anatomy. Furthermore, chemical, biological and immunomodulatory activity were studied using standard procedure with sophisticated instruments.

The results of the above said problem has been documented in five chapters as follows: Gross morphology of the reduviid head and salivary gland complex, venomous saliva-optimization and utilization, biochemical characterization, biological characterization

including immunomodulatory activity and immunomodulatory activity on Swiss Albino mice.

The results concluded that the *R. fuscipes* head, mouthpart and salivary gland morphology and histology reveal that it is adaptive for zoophagy. The three-day starved predator yields a maximum quantity of VS by milking method when fed with *Spodoptera litura* (Fab.). The zootoxic enzymes such as phospholipase, hyalurindase, protease and trypsin like were high in the VS. The EDX spectra, FTIR spectra, HPLC, GC-MS analyses showed the presence of these enzymes. Similar types of molecular mass components were identified in both electrophoresis and MALDI-TOFMS analysis.

The VS causes mortality in *S. litura* and *Helicoverpa armigera* larvae by both microinjection and oral toxicity bioassay, and alters the gut digestive and detoxification enzymes. Venomous saliva has anti-agglutation, anti-spreading and cytolytic behaviour on *S. litura* and *H. armigera* haemocyte. However, VS doesn't cause mortality in Swiss Albino mice, while it induces edema formation and inflammation with infiltration of granular cells and tissue distraction. We propose further studies for a better understanding of VS nature and its biological impact on many insects.

CHAPTER 1. GROSS MORPHOLOGY OF THE REDUVIID HEAD AND SALIVARY GLAND COMPLEX

1.1. ABSTRACT

Rhynocoris fuscipes is a generalist predator found to be preys belong to various insect orders. The reduviid used its stylet to feed the prey. The gross morphology of the feeding canal and the salivary apparatus of *R. fuscipes* were investigated for the first time. *Rhynocoris fuscipes* bears a pair each of compound eyes and oceli; bears curved rostrum (2.73 ± 0.01 mm length). The salivary apparatus has a pair of maxillary and mandibular stylets each. The maxillary stylets are shorter than the mandibular stylet and are used to deliver the venomous saliva (VS) and suck the predigested food. The mandibular stylet bears 28 barbs in 3 rows useful for holding and rasping the prey. The salivary gland consists of a pair of principal gland (PG) and accessory gland (AG) each. The PG is further bifurcated into anterior lobe (ALPG) and posterior lobe (PLPG) and is joined at hilus (HI) region. The ALPG (1.16 ± 0.04 mm) is smaller than the PLPG (1.52 ± 0.30 mm). The accessory gland (AG) was attached to the gut binding with the trachea and tracholes. AG was interlinked with the PG through the accessory duct (AD). From HI, a salivary duct (SD) extends towards the head and ends with the maxillary stylet through adductor muscles (AM). Histologically the anterior and the posterior portion of principal gland has a clear distinction. The AG is typically of the vesicular type. From these results, by its gross morphological features, it is evident that the predator *R. fuscipes* is equipped strictly for zoophagy.

1.2. INTRODUCTION

Reduviidae is the largest family of predaceous land Heteroptera (Maldonado, 1990). They are abundant, voracious predators that consume not only more prey but also a wide array of prey (Schaefer, 1988). The head and the rostrum are designed to prey by piercing and sucking the prey tissues. The reduviids contain their elongated head with a transverse groove behind the compound eyes, and the short, prominent apparently three segmented rostrum curved outwards from the head. The tip of the rostrum in response fits into the groove. The head has a pair of large compound eyes, with two oceli and a pair of four segmented antennae (Capinera, 2008). The oceli or simple eyes have been the subject of numerous anatomical, physiological and behavioral investigations (Goodman, 1981; Wehrhahn, 1984; Mizunami, 1995). *Rhynocoris marginellus* (Fab.) (Fabricius, 1803); *Rhynocoris manticola* (Oshanin) (Murugan *et al.,* 1870); *Rhynocoris marginatus* (Fab.) (Ambrose, 1980; Kumar, 2011);

Rhynocoris fuscipes (Fabricius) (Ambrose, 1980); *Rhynocoris kumarii* (Ambrose and Livingstone, 1986); *Rhynocoris cruralis* (Bergroth) (Bergroth, 1915); *Rhynocoris nysiiphagus* and *Rhyncoris lapidicola* (Samuel and Joseph, 1953) head morphology were briefly elaborated. External morphological features have also been studied in details by various investigators for the past five decades (Lavoipierre *et al.*, 1959; Edwards, 1961; Cobben, 1978; Haridass and Ananthakrishnan, 1981; Sivaraj, 1986; Morrison, 1989; Santha, 1986; Udayakumar, 1986; Vellingirinathan, 1986; Sahayaraj *et al.,* 2010). However gross morphology, internal anatomy of head or rostrum has not been studied in detail, except in the work of Cohen (1990, 2000); Boyd *et al.* (2002); Boyd (2003); Sahayaraj *et al.* (2010) and Kumar (2011).

The reduviid predator salivary gland has been previously described by many investigators (Baptist, 1941; Barth, 1954; Southwood, 1955, Louis and Kumar, 1973; Haridass and Ananathakrishnan, 1981; Morrsion, 1989, Maran, 1999; Sahayaraj *et al.,* 2010). The morphology of salivary glands is diverse in different sub-families which could be utilized as reliable taxonomical tools (says Louis and Kumar, 1973). In general, the principal gland is unilobed or bilobed or multilobed, and the accessory gland is unilobed and vesicular, exhibiting distinct functional and histological differences. Further, it was reported that the principal gland is divided into anterior lobe and posterior lobe, suggesting the differential functions of the lobes involving division of labor (Haridass and Ananathakrishnan, 1981). The anterior lobe secretes zootoxic enzymes which are used to paralyze the prey, and the posterior lobe secretes the digestive enzymes. The accessory gland is typically vesicular (says Baptist, 1941; Southwood, 1955; Edwards, 1961). However, the detailed morphology, anatomy and histology of *R. fuscipes* are not available in the literature. The animal mouthpart and salivary gland morphology and its characteristic features give an idea of the toxicological, biological and biochemical characters. Thus, the focus of this study was to examine the structural morphology of *R. fuscipes* mouthparts including maxillary and mandibular stylets using light and scanning electron microscope (SEM). Furthermore the morphology and histology of salivary gland complex has also been studied using light-microscope.

1.3. MATERIALS AND METHODS

1.3.1. Insect collection and maintenance

Life stages of *R. fuscipes* were collected from cotton, bhendi agro-ecosystem of Tirunelveli district, Tamil Nadu, India. The collected animals were reared with factitious host *Corcyra cephalonica* (Stainton) fourth and fifth instar larvae under laboratory conditions at 30 ± 0.21 ºC temperature, 71.68 ± 0.41% RH and a photoperiod of 13L:11D h. Laboratory emerged adult male predators were used for the studies.

1.3.2. Head and stylet preparation

Fifteen to twenty alive adult male predators were kept in ice for 5 min and the animal were transferred into the dissection plate contain insect ringers solution (IRS) [1.09% NaCl, 0.16% KCl, 0.08% $CaCl_2$**.** $2H_2O$, 0.08% $MgCl_2.6H_2O$ (Li *et al.,* 2009)]. The excised head and the stylet samples were placed separately in the fixation solution (2.5% glutaraldehyde) for 24-48 h. Then the samples were rinsed with phosphate buffered saline (PBS) (0.2M Na_2HPo_4, 0.2M NaH_2Po_4, 0.8% NaCl) four times for 5 min and dehydrated in a gradient series of ethanol (75%, 80%, 85%, 90% and 99.9%-3 min in each gradient). Now the head, rostrum and stylets were air dried and coated by carbon particles using the carbon coater. The carbon coated samples were placed in the sample holding disc and it was loaded into the scanning electron microscope (SEM) (JSM – 6390) (Heng-Moss *et al.,* 2003). The images of the head and stylet were photographed and displayed in plate 1.1b, c, d and 1.2e.

For line diagram, the head of the animals were excised and placed in 70% ethanol for 24 h. After the incubation, the head was transferred to clean glass slide, and mounted using DPX moutant (Qualigens, India). The stylet of animals were dissected out longitudinally from the rostrum using the fine forceps, the head of the animal was held and using other fine forceps the rostral tip was gently and carefully removed without damaging the inner stylets using a dissection microscope. Removed stylets were prewashed with 70% ethanol and mounted on a clean glass slide using the DPX mountant (Qualigens, India). The specimens were then examined and photographed with a phase contrast microscope (Olympus CS 41, Japan) (Ambrose and Livingstone, 1986a; Fernández *et al.,* 2005).

1.3.3. Morphometry of head and stylet

The mounted head and stylet samples were analyzed for their morphometry by using a light microscope (Amba Optik – AE 11, India) equipped with ocular and stage micrometer (Erma Inc, Japan). The size of the various parts of head (anteoocular, postoccular, compound eyes, oceli and rostrum), mouthparts (base, mid and terminal) and stylets (maxillary and mandibular) were recorded. Ten male animals were used in each analysis. The line diagram of the head and the stylet were drawn using the Camera Lucida fitted to the light microscope and the diagram was drawn at 5X. Moreover, phase contrast photographs were taken using a phase contrast microscope (Olympus CS 41, Japan) equipped with a digital camera (Olympus E 240, Japan)

1.3.4. Gross morphology and histology of salivary gland

Ten alive adult predators were initially anaesthetized by placing them in ice as described by Sahayaraj *et al.* (2010) and transferred into the dissection tray coated with wax containing ice cold IRS. Using fine forceps, the animals were held, the wings, legs were cut off using scissors. A circular lateral incison around the first segment of abdomen was made with a sterile surgical blade. The main salivary duct was detached from the sclerotised mouthparts closer to the salivarium. The salivary gland complex was transferred to a clean watch glass containing ice cold IRS. The dissected glands were fixed for one hour in 0.1M sodium phosphate buffer (pH 7.2) containing 40% (v/v) paraformaldehyde at room temperature. The glands were settled onto micro slides and imaged using phase contrast microscope. The length and width of the principal gland and accessory gland were measured using the micrometer and the micro drawing (line diagram) was performed using the Camera Lucida (Kumar, 2011).

For histological studies, the salivary gland complex such as PG (ALPG and PLPG) and AG were separated in the IRS, which was drained off and washed with distilled water. The dissected glands were fixed in alcoholic Bovines fluid (Haridass and Ananthakrishnan, 1981; Moraes *et al.,* 1995). After the 24 h of the fixation, the glands were dehydrated in series of increasing alcohol dilutions (30, 50, 70, 90 and 100%), for 15–30 min of each concentration, embedded in the paraffin wax and cut in 5µm thin sliced segments using a rotary microtome (Microtome India, No, 1010-SMT-005, India). The sections were stained with Eosin followed by haematoxylin (sections were fixed on a clean glass slide which was preheated at 60 °C). After the staining, the slides were washed with distilled water. Stained

sections were dehydrated with absolute alcohol for 2 min, washed with xylene for removing the bounded in the glass slide. Now the sections were mounted on the glass slide using DPX mountant. The slides were viewed through the phase contrast microscope and image was captured using the digital camera fitted to the microscope.

1.4. RESULTS

1.4.1 Head

The entire head of *R. fuscipes* (Plate 1.1a) is a bright colored, finely pubescent, moderately elongated and shorter that the pronatum, anteriorly unarmed, a median transverse impression in between eyes dividing the head into almost four portions. Anteoccular portion (1.03 ± 0.02 mm) is significantly longer than the post ocular portion (0.62 ± 0.01 mm) ($t = 90.529$; $p < 0.05$) and ocular portion (0.42 ± 0.01 mm) ($t = 46.342$; $p < 0.05$). The reduviid possesses a pair of oceli (0.10 ± 0.00 mm) located at the anterior region (Plate 1.1b) of the head projecting backwards of the compound eyes. The rostrum (Plate 1.1c) is cylindrical and it was anteriorly bisected by a deep labial groove. The rostrum consists of base (B) (0.63 ± 0.01 mm), mid (M) (1.13 ± 0.01 mm) and terminal (T) (0.33 ± 0.00 mm) (Plate 1.1a, b). The base is shorter than the mid segment. The surface of the segment is covered with a few numbers of trichomes (Plate 1.1d). The rostrum contains different types of trichomes such as long spikes (LS) (117.33 ± 13.82 µm), short spikes (SP) (50.00 ± 5.00 µm), small spikes (SS) (12.66 ± 1.45 µm) and solei (S) (2.43 ± 0.14 µm) (Plate 1.1c).

1.4.2. Mandibular stylet

The stylet is located inside the rostrum; composed of a pair of mandibular stylet (Plate 1.2a) and a pair of maxillary stylet (Plate 1.3a). The average length of mandibular and maxillary stylet is 6.54 ± 0.00 mm and 5.26 ± 0.04 mm respectively. The mandibular stylet (MAS) is situated on each of the lateral side of the maxillary stylets (MS). MAS consists of three distinct parts namely base (B), mid (M) and terminal (T). The base is attached to the head by means of adductor muscles (AM) (Plate 1.2f, 1.3f). The base is continually up to salivary gland complex by a distinct duct called salivary canal (SC) (Plate 1.2e). There are three rows of numerous teeth like barbs (Ba) (Plate 1.2b, 2c) on the inner edges of the mandibular stylets. The barbs are in the form of concave shape (Plate 1.2d, 1.2e) facing outwards the head. In the mandibular stylet each row of the barbs is located adjacently with a distance of 5.25 ± 0.25 µm, whereas the space between the barbs is 4.90 ± 0.10 µm to 8.00 ± 0.50 µm (Plate 1.2f). SEM photograph showed that there are three types of barbs: long (6.57

µm), medium (5.26 µm) and small (4.47 µm) (Plate 1.2f). These barbs are helpful to the predator for holding and rasping the tissue of prey.

1.4.3. Maxillary stylet

The maxillary stylets (MS) are located in between the mandibular stylets. Structurally, they are similar to MAS as base (B), mid (M) and terminal (T) segments (Plate 1.3b, 1.3c). However anatomically, MS are smooth without any serrations and the tip of the stylet is highly pointed (To). The inner margin of the stylet contains a small finger like projection that seems to be a brush (Br) (Plate 1.3d, 1.3e). Along with the brush like structures, maxillary stylet possesses a hook (H) at the tip with furrow (F) for holding the prey (Plate 1.3b, 1.3c) and at the tip a terminal opening (TO) which helps in sucking the contents of predigested prey (PDF) (Plate 1.3c). During prey capturing both the MS interlock to form a canal like (FC) structure, which acts as a salivary canal for the delivery of the VS and also as a food canal for sucking the predigested prey contents. Generally, these maxillary stylets are inserted after the mechanical disruption of the prey tissue by the MAS.

The movements of stylets are supported by the adductor muscles (AM) (Plate 1.3a and 1.3f) located at the head. These adductor muscles support both the MAS and MS for their movement during the prey capture and ingesting the prey contents. Through the adductor muscles, the salivary and food canal pass in and out from the stylets.

1.4.4. Salivary gland-Morphology and Anatomy

The salivary gland complex (SGC) contains a pair of principal (PG) and accessory glands (AG) (Plate 1.4a and 1.4b). The PG is bilobed, comprising anterior and posterior lobes. The PG is started from the mesothorax and extended up to the abdomen (2.70 ± 0.11 mm long). It is divisible into anterior lobe (ALPG) and posterior lobe (PLPG). The anterior lobe (ALPG) (1.16 ± 0.04 mm) is smaller than the posterior lobe (PLPG) (1.52 ± 0.30 mm) (Plate 1.4a, 1.4b). They are situated in the thorax on either side of the gut. The ALPG is vesicular and is always filled with watery fluid. A very lobulated PLPG extends into the abdominal cavity located on either side of the gut. PLPG is highly nodulosus at posterior side rather than at the anterior side. These nodulosus is highly distinct as constrictions, attached to the foregut region and both the AG and PG are joined by the AD. The AG (1.15 ± 0.04 mm) is a vesicular unilobed having its base, mid and terminal region and the secretions are milky white in colour. The ALPG and PLPG join at hilus (HI) (Plate 1.4a, 1.b) which is well-

developed, compartmentalized, provided with valvular openings for their regulation of secretions sent out from the different lobe of principal gland and accessory glands. The outer chamber of hilus receives the incoming accessory duct (AD) and sends out the main salivary duct (SD); the respective openings of these ducts are being guarded by valve-like flaps. The openings of the outer chamber into the salivary and accessory ducts as well as into the inner one are provided with separate valves. The SD arises from the hilus moves to the head and finally it gets ended with the MS by the help of the AM. Both the PG and AG and their ducts receive a tracheal supply (1.4a, 1.4c, 1.4d). AG and both ALPG and PLPG are derived from a tracheal trunk of the cephalic and thoracic spiracular trachea respectively. A distinct nerve plexus (NP) is found on the principal gland of *R. fuscipes.* The nerve (N) which supplies this plexus was also seen, and is derived from the hypcerebral ganglion of the stomatogastric bervous system (Plate 1.4d, e).

1.4.5. Salivary gland-Histology

The principal gland of *R. fuscipes* is bilobed in nature having ALPG and PLPG. Both these lobes are interconnected by hilus. The PLPG (Plate 1.5A a) is surrounded by the thin layered membrane propia (MP) (Plate 1.5A b) followed by the epithelial cells (EC) (Plate 1.5A c) and beneath the inner membrane (IM). The EC possesses dense cytoplasm region having the secretory granules (Plate 1.5A b, c). These granules are located below the IM and form dense patches (DPSG). There are several number of collecting vacuoles (CV) (Plate 1.5A d) located in and around the EC, these CV help to accumulate the secretions and they become a larger one. The CV finally end with the lumen (L) which is lined by the columnar epithelial cells (CEC). The lumen is the place of the secretions which are stored and used when needed. The outer surface of the principal gland is occupied by the surface cells (Plate 1.5A f, g) which are in the dense form and the cytoplasm is highly viscous and dense in nature. And similar observations were seen in the ALPG (Plate 1.5B a), but the nature of the secretions are highly denser than the PLPG having a larger lumen for the storage of the secretions of the secretory materials. The ALPG possesses different types of cells such as uninucleated (UN), binucleated (BI) and polynucletaed (PN) (Plate 1.5B b), but in the PLPG only UN and BI cells were observed. And between each cell intercellular spacing (ICS) (Plate 1.5B f) was seen. Irregular shaped secretion granules (SG) are distributed both in anterior and posterior principal gland, which are concentrated in the ALPG (Plate 1.5B h) and more separately distributed in the PLPG (Plate 1.5Ae).

The AG (Plate 1.5C e) is of vesicular type having a minimum of secretory granules which is surrounded by the MP and EC which is almost UN in nature and produce watery saliva. The inner margins of the cells in AG exhibit distinguished striated or separate filaments with prominent nucleus. The hilus (Plate 1.5B g, h) is surrounded by the MP followed by the EC that shows the presence of UN and BN cells on the outer surface cells and devoid of rich CV where observed.

1.5. DISCUSSION

1.5.1. Head

The gross morphological features of the head and the mouthparts of *R. fuscipes* are similar to those that have been reported for other harpactorinae reduviids (Haridass and Anathakrishnan, 1981; Sivaraj, 1986; Morrison, 1989; Santha, 1986; Udayakumar, 1986; Vellingirinathan, 1986; Agnes, 1990; Ambrose, 1999; Sahayaraj *et al.,* 2010; Kumar, 2011). The head is elongated having a median transverse impression in between eyes dividing the head into almost two areas such as anteoccular and postoccular areas. Head gross morphology is similar in *R. kumarii* as described by Ambrose and Livingstone (1986b). The three segmented rostrum bears a short base, moderate median segement and a long terminal segment. It bears hair-like sensillae used in the orientation of the stylet fascile to the prey surface which is a typical pattern of *R. marginatus* (Kumar, 2011). This is aptly suited for the pin and jab type of feeding behavior, where, the rostrum and stylets are used to attack the soft bodied preys types without the necessity to actively chase, to grab, to hold or to pounce on them as done by the "chase and pounce" type of reduviids (Sahayaraj *et al.,* 2010). The oceli help the predator for the dorsal light response used during the flight (Gooddman, 1981; Wehrhah, 1984; Mizunami, 1995). The hair-like sensilla are mostly used to detect the worth or suitability or palatability or acceptability of the prey, and it is to assign a sensory function too. The trichomes present in the surface of the rostrum of *R. fuscipes* are used by this predator for the location of the prey and similarly types of the trichomes have been previously reported in *R. marginatus* (Kumar, 2011).

As in all other heteropterans, the stylet bundle of *R. fuscipes* has two maxillary stylets inside and two mandibular stylet outside. Both MAS and MS of *R. fuscipes* are typical of the Reduviidae as reported in *Catamirus brevipennis* (Sahayaraj *et al.,* 2010) and *R. marginatus* (Kumar, 2011). Many other heteropterans produce a salivary flange that is used as a fulcrum, among other functions, for stylet movement (Cohen, 1998b, 2000). Because reduviids do not

produce a salivary flange, the apical serrations on the MS (Plate 1.2d) are considered adaptive in holding onto tissues below the outer layer of the prey and in producing a fulcrum for the movement of the MAS (Cobben, 1978; Cohen, 2000; Wheeler, 2001; Boyd, 2003). The MS is longer than the MAS. This mandibular stylet surrounds the maxillary stylet (Lavoipierre *et al.*, 1959; Cobben, 1978). Boyd (2003) and Boyd *et al.* (2002) reported that the maxillary stylets of *Deraeocoris nigritulus* (Uhler) are more serrated than the phytophagous insects (e.g., Tingidae), but in *R. fuscipes,* the mandibular stylet is used to tear and wear the tissues of the prey by using the barbs (Ba) or serrations present on the outer edge of the stylet (Cohen, 1990, 2000; Kumar, 2011). Similarly, in the predaceous pentatomids the barbs on the mandibular stylets are found pointing towards the head of *R. fuscipes* (Cohen, 1996). The barbs point away from the head indicating that the cutting action occurs when the stylet is thrust forward, unlike predatory pentatomids, which have barbs on the MS pointing towards the head (Cohen, 1996). The barbs on the mandibular stylets of reduviids and other predacious heteropterans are more numerous than the barbs on the mandibular stylets of phytophagous Heteroptera (e.g., Lygaeidae, *Sensu lato*) (Cohen, 1990). *Rhynocoris fuscipes* has three rows of barbs on the surface of the mandibular stylet. The SEM photograph showed that the barbs (Plate 1.2f) are varied in the size and concave in shape ranging from large to small facing towards the head and so during the action the barbs move to and fro which helps to tear the prey tissues and help to form a pore to deliver the VS into the prey. The inner maxillary stylet is sharply pointed and has numerous brush (Br) like projections (Cobben, 1979) over the inner side of the stylet. The deeper serrations in the MAS of *R. fuscipes* are similar to those of *Deracoerous nebulosus* (Boyd *et al.,* 2002), *Deracoerous alivaceaous* (F.) (Cobben, 1978), *Deracoerous nigritulus* (Boyd, 2003), probably are used to disrupt prey by ripping and tearing tissues (Cohen, 2000). The mandibular stylets are considered adaptive in holding the prey tissues below the outer layer of prey and produce a fulcrum for maxillary stylet movement (Cobben, 1978; Cohen, 2000; Wheeler, 2001; Kumar, 2011). For both mandibular and maxillary stylet holds support by the adductor muscles (AM) (Plate 1.2e and 1.3f), because inside the rostrum (labium) there is no muscles are present and so far movement adductor muscles were used. And at each base of the stylet, the adductor muscles are present. Generally, these stylets serve multiple purposes, including prey anchoring, delivery of VS. Mechanical disruption of solid structures by laceration or rasping with stylet dentition, delivery of digestive secretions, uptake of partially digested food (Cobben, 1978; Cohen, 2000; Wheeler, 2001; Sahayaraj *et al.,* 2010; Kumar, 2011).

1.5.2. Salivary gland

The anatomical pattern of the salivary system of *R. fuscipes,* confirms to the general heteropteran plan in general (Baptist, 1941; Barth, 1954; Southwood, 1955) and reduviid in particular (Louis and Kumar, 1973; Haridass and Ananthakrishnan, 1981 and Morrison, 1989; Maran, 1999; Azevedo *et al.,* 2007; Sahayaraj *et al.,* 2010; Kumar, 2011). The under lying form of the salivary gland of *R. fuscipes* is obviously consisting of a principal part and an accessory part. The principal part is bilobed, and where it is further sub-divided to anterior and posterior glands with reference to the issuing salivary duct. The PG is found to be simply bilobed in the family Reduviidae (Baptist, 1941; Haridass and Anathakrishnanan, 1981; Maran, 1999; Sahayaraj *et al.,* 2010). However, Louis and Kumar (1973) suggested the trilobed condition of the salivary system as primitive type and an advanced character and unilobed was recorded in Triatominae of Reduviidae (Anhe and Oliveria, 2008). The principal salivary glands of *R. fuscipes* are elongated vesicles with tubular extensions as observed among the members of Reduviinae, Salyavatinae and a member of Harpactorinae (Haridass and Ananthakrishnan, 1981). Similar morphology was also reported in a sister species *R. marginatus* (Kumar, 2011). The differential functions of anterior and posterior lobes suggest division of labour. But Baptist (1941) believed that there is no such division in the functions of salivary glands of Heteroptera. In Pentatomomorphid families, the secretions of anterior lobes are primarily concerned with stylet – sheath formation, whereas those of posterior lobes involved in the production of digestive enzymes (Hori, 1969; Miles, 1972). Edwards (1961) found that in *Platymeris rhadhamanthus* (Gearstacker), zootoxic enzymes present both in the anterior and posterior lobes. The secretion in the anterior lobe is lesser in quantity, viscous and transparent, whereas the posterior lobe secretes larger quantity of highly viscous and milky white secretions as reported by Haridass and Ananthakrishnan (1981). In the forth coming chapter we analyzed, recorded and discussed in detail about various types of enzymes secreted by PG and AG.

Muscle layer associated with the lobes were not found in the principal salivary glands. This indicated that the saliva of *R. fuscipes* could be injected into the prey by extrinsic muscles. The presence of muscle fibers seems to be related with the predatory habit (Baptist, 1941), where a muscle sheath might be important to mobilize greater amounts of saliva. The nerve plexus, trachea and fine muscle fibres support and discharge the required quantity of saliva for paralysising or killing the prey. The tracheal supply of the salivary gland comes from the first visceral trachea which is generally the largest of the tracheae supplying the gut

as observed by Baptist (1941). The accessory salivary glands of *R. fuscipes* are typical of vesicular type as observed in other heteropterans (Baptist, 1941; Southwood, 1955) including reduviids (Edwards, 1961; Haridass and Ananthakrishnan, 1981; Vellingirinathan, 1986; Agnes, 1990; Kumar, 2011). However, it is an elongated vesicle with triradiate tubular branches in ectrichodiines, elongated vesicle in peiratines and saccular vesicle in triatomines (Haridass and Ananthakrishnan, 1981; Santha, 1986). Accessory glands are filled with watery fluid which helps the predator to flush out the predigested food from the body of the prey, very similar to the lacerate-flush mode of feeding of Pentatomomorpha in which the watery saliva is useful in flushing out the food from its source (Miles, 1972; Miles and Slowiak, 1976).

Hilus is distinct in *A. pedestris* (Morrison, 1989). The hilus provides a regulatory system for sending out secretions from different lobes of the salivary system. In *Lestomerus affinis* and *Haematorrhophus nigroviolaceous,* the valves in the hilus make it possible not only to send the secretions independently from the accessory glands, but also to send separately the secretions issued from the anterior and posterior lobes of the principal gland (Haridass and Ananthakrishnan, 1981). Such an independent flow for the anterior and posterior lobes of the principal gland is also observed in *R. fuscipes*. The salivary lobes open out individually by a small pore, guarded by thick circular muscles into a compartmentalized hilus as observed by Morrison (1989). A complex nervous supply was observed previously by Baptist (1941) and Miles and Slowiak (1976). A double nerve supplies separately to the anterior and posterior lobes facilitates independent discharge of saliva (Miles, 1972). Perhaps the nerve plexus is responsible for an acceleration of cellular activity, which seems to result in the production of a somewhat thinner secretion than that ordinarily found stored up in the lumen of the gland.

Different types of cells such as, mono, di, and poly nucleated cells were observed in ALPG and PLPG. Both the ALPG and PLPG possess bi-nucleated cells. But Morrison (1989) observed uni-nucleate cells in anterior lobe and bi-nucleate cells with highly viscous cytoplasm in posterior lobes of *A. pedestris.* Such variations are found among members of different subfamilies of Reduviidae (*Triatoma rubrofasiata*) (Haridass and Anathakrishnan, 1981). The cytoplasm is traversed by various sizes of collecting vacuoles (CV) containing secretion. These increase in size towards the inner parts of the cells. Regular rounded secretion granules are distributed to near or around the collecting vacuoles. Another characteristic feature is that the central lumen of the gland is lined by a special flattened

secretary epithelium, with irregular intercellular space around the central lumen. The cytoplasm possesses typical secretion granules, dense around the collecting vacuole. The cytoplasm of the anterior- and posterior-lobe cells differs slightly in texture. The cytoplasm is densely packed with large secretion granules and the collecting vacuoles are moderate in size and full of dense secretion. This characteristic feature of collecting vacuoles is to serve the purpose of storing up quite an appreciable quantity of secretion. The accessory glands attached to the lateral sides of the first midgut appear triangular with a tubular appendix opening into the common salivary duct. Accessory glands are thought to function as water recapturing organs, a function that has been underemphasized in an account of feeding by predaceous heteropterans, *R, fuscipes* (Miles, 1972). Accessory salivary glands are filled with watery fluid (Baptist, 1941), which recirculate water from the gut to ensure a copious flow of watery saliva and helps the predator to flush out the predigested food from the body of the prey. It is forwarded by a single layered epithelium as observed in other predatory bugs like *Brontocoris tabidus* (Pentatomidae) (Azevedo *et al.,* 2007). The accessory glands differ histologically from the lobes of the principal gland and secrete watery saliva, which has less protein fractions than the other lobes. Similar results were highlighted in Pentatomid and Coreid bugs (Miles and Slowiak, 1976), and in assassin bugs (Haridass and Anathakrishnan, 1981; Morrison, 1989). A well developed nerve plexus is always present on the surface of the principal gland. The histochemical analysis by Agnes (1990) suggests excretory function to the salivary system in addition to the salivary secretory function (Schuh and Slater, 1995). A poorly developed nervous plexus is always present on the surface of the accessory gland (Haridass and Ananthakrishnan, 1981).

1.6. CONCLUSION

The gross morphological features of *R. fuscipes* are strictly for the zoophagy by its presence of the stylets used for the rasping and holding of the prey tissues. The stylet movements are supported by the head adductor muscles. The salivary gland complex is made up of a pair of principal and accessory gland, where the principal gland is further bifurcated into anterior lobe and posterior lobe interconnected by the hilus. The histological features of the salivary gland showed the presence of the secretory cells and the also the secretory granules from the two lobes of the principal gland. Less or no secretion was observed in the accessory gland. Centralized lumen helps for the secretion and for it the release. The nerve plexus stimulated to secrete the secretions and are present on the surface of the salivary gland complex.

CHAPTER 2. VENOMOUS SALIVA: OPTIMIZATION AND UTILIZATION

2.1. ABSTRACT

Milking and electrical stimulation methods were used for the venomous saliva (VS) collection by subjecting the *Rhynocoris fuscipes* (Fab.) to continuous (CMM) (1, 2, 3, 4, 5 and 6 days) and discontinuous (DMM) (1, 3, 5 and 7 days) food deprivation levels. The quantity of VS production and utilization were examined by providing *Corcyra cephalonica* (Stainton), *Spodoptera litura* (Fab.) and *Helicoverpa armigera* (Hubner). The male predators significantly secreted more VS than the females both in the continuous and discontinuous prey deprivation of both manual milking and electrical stimulation methods. The 100% survival (SR) and venom milking rate (VMR) were observed in continuous milking method. In discontinuous manual milking (DMM), 20% each SR and VMR were recorded and similarly in the continuous electrical stimulation (CES) 30% SR and 20% VMR and in discontinuous electrical stimulation (DES), 20% SR and 30% VMR were recorded. The quantity of protein was high in the one day starved female VS milked in the DES. *Spodoptera litura* fed *R. fuscipes* secreted higher quantity of VS than *H. armigera* and *C. cephalonica* fed groups. The predator uses the maximum quantity of VS to paralyze the *S. litura* than the *C. cephalonica* and *H. armigera*. For the maximum VS yield, continuous manual milking method fed with *S. litura* can be used for the VS optimization and utilization.

2.2. INTRODUCTION

The heteropteran predators do not confine them to the body yields of their prey, as is so after or implied (Cohen, 1996). Instead, they use a solid to liquid feeding method by attaching the nutrient rich solid or semi-solid organs and tissues of prey (Cohen and Patana, 1984, 1985; Cohen, 1998a; Sahayaraj *et al.,* 2010). Venomous insects are known from the orders Lepidoptera (Bene *et al.,* 1999); Hemiptera (Maran, 1999; Ambrose and Maran, 1999b; Maran and Ambrose, 2000; Sahayaraj *et al.,* 2006 a, b; 2010; Sahayaraj and Vinoth Kanna, 2009) and Hymenoptera (Blum, 1981; Ućkan *et al.,* 2004; Rivers *et al.,* 2006). Venom consists of a complex mixture of toxic components that include protein and peptide toxins, enzymes and other active biomoleclues (Corzo *et al.,* 2001; Sahayaraj *et al.,* 2010; Sahayaraj and Muthukumar, 2011). These molecules serve the dual purposes of prey paralysis and digestion and / or defense against other predators (Bailey and Wilce, 2001;

Palma 2006; Rivers *et al.,* 2006; Sahayaraj *et al.,* 2006a; Turillazzi *et al.,* 2006; Sahayaraj and Vinoth Kanna, 2009; Sahayaraj *et al.,* 2010).

The venom milking is used for the collection of venom and preventing the venom contamination by non-venom enzymes (Rash and Hodgsen, 2002). Three different methods have been used for the VS/true venom collection: a) manual milking method in reduviids (Sahayaraj *et al.,* 2006 a, b; Sahayaraj and Vinoth Kanna, 2009; Kumar, 2011); in hymenoptera (Piek, 1986; Funari *et al.,* 2001; Deyrup and Matthews, 2003; Hisada *et al.,* 2005) and in spiders (Vonarx *et al.,* 2006); b) electric stimulation methods in reduviidae (Barbosa *et al.,* 1999; Corzo *et al.,* 2001; Sahayaraj *et al.,* 2006a; Kumar, 2011); in hymenoptera (Funari *et al.,* 2001); in spiders (Johnson *et al.,* 1998; Herzig and Hodgson, 2008; Herzig *et al.,* 2008; Rocha-e-Silva *et al.,* 2009); in scorpions (Possani *et al.,* 2000; Incesu *et al.,* 2007) and C) whole gland extraction in reduviids (Maran, 1999; Ambrose and Maran, 1999; Maran and Ambrose 2000); ants (Haight and Tschinkel, 2003; Chen *et al.,* 2009); in wasps (Uçkan *et al.,*2004; Rivers *et al.,* 2006); in spiders (Silva *et al.,* 2008).

Reduviids are mostly insectivorous predators which use their VS as a tool of aggression in order to paralyze or immobilize rather than kill their prey. Venomous saliva is synthesized in relatively small quantities (Maran, 1999) in the paired principal gland (Cohen, 2000; Sahayaraj *et al.,* 2010). Since reduviids capture several prey items per day (Ambrose, 1999; Sweet, 2000; Sahayaraj, 2007), one would expect, that they would strictly control the quantity of VS released according to prey type. Injection of too much VS into smaller preys could be metabolically expensive and may deplete venom reserves, leaving the reduviid vulnerable to predation or unable to deal with subsequent prey. No attempts have been made to quantify the amount of VS injected by reduviids into different prey items. It has been reported widely that reduviids take less time while they are in starvation (Maran, 1999, Ambrose and Maran, 1999; Maran and Ambrose, 1999) and provided with small preys. The gender of the animal also influences the quantity of VS released, the female predators secrete more quantity of VS by *R. marginatus* and *Catamirus brevipennis* (Sahayaraj *et al.,* 2006a) and a similar observation was made by Sahayaraj and Vinoth Kanna (2009) in *C. brevipennis* by discontinuous milking method. Very recently, Kumar (2011) took an attempt to quantify VS, when *Rhynocoris marginatus* (Fab.) subjected to continuous (1, 2, 3, 4, 5, 6 and 7 days) and discontinuous (1, 3, 5, 7 and 9 days) prey deprivation. However, no one has made an attempt to quantify the VS secreted in relation to manual milking, electrical stimulation by

combining while the *R. fuscipes* (Fab.) was subjected to continuous (1, 2, 3, 4, 5 and 6 days) and discontinuous (1, 3, 5 and 7 days) prey deprivation period. The evolution of venomous and poisonous organisms, whether toxic compounds are acquired through the development of a venom producing organ or through the external sources (says Mebs, 2001) is essential. The serving of the main nerve supply to the venom glands does not affect venom production, protein concentration or enzyme activity. It has been suggested previously that venom production is not under nervous control (Kochva, 1978). Maran (1999) has studied total body carbohydrate, protein and lipid of *R. fuscipes*, *R. marginatus* and *Rhynocoris kumarii* and has fed different pest such as *Spodoptera litura* (Fab.), *Dysdercus cingulatus* (Fab.) and *Mylabris pustulata* (Thungberg).

The objectives of this study were: 1) to optimize the VS secretion by different methods (manual milking and electrical stimulation) while *R. fuscipes* was subjected to continuous and discontinuous prey deprivation periods; 2) to evaluate the impact of preys on VS milking on different pests (*S. litura, H. armigera* and *C. cephalonica*) and 3) to evaluate the utilization of VS by different prey.

2.3. MATERIALS AND METHODS

2.3.1. Insects Collection

The adult male and female *Rhynocoris fuscipes* used for the study were taken from our permanent breeding stock. The animals were kept under the following 30 ± 0.21 ºC temperature, 71.68 ± 0.41% RH and a photoperiod of 13L: 11D h. The adults were kept individually in polystyrene boxes (5 x 4 cm) with *C. cephalonica.* All boxes have a layer of absorbent paper at the bottom. Also cleaning occurs weekly.

Life stages of armyworm, *Spodoptera litura* (Fab) and American bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuiidae) were collected from the castor, groundnut and bhendi agro-ecosystems of Tirunelveli District, Tamil Nadu. The pest life stages were reared using their natural host under the above mentioned laboratory conditions. The laboratory emerged F_1 or F_2 generation life stages were used for the studies.

2.3.2. Venomous saliva optimization

2.3.2.1. Starvation and collection method

The five-day old *R. fuscipes* male and female were subjected to continuous (1, 2, 3, 4, 5 and 6 days) (CPD) and discontinuous (1, 3, 5 and 7 days) (DPD) prey deprivations. For CPD category predators were fed *ad libitum* before the commencement of the experiment. After this, the animals of this group did not receive food for 6 days, whereas, DPD category, after 1 ,3, 5 and 7 days of starvation, the predators were subjected to VS milking, then the predators received food in *ad libitum.* After each prey deprivation period, the VS were collected once in the glass capillary tube (6 cm long, 2 mm outer diameter, 1 mm inner diameter) using milking and electrical stimulation method (Sahayaraj *et al.,* 2006a; Sahayaraj and Vinoth Kanna, 2009). The weight of the predators and a capillary tube were recorded before and after collection by milking method. To milk VS, *R. fuscipes* was held between two fingers – thumb on the ventral side of the abdomen and second finger on the dorsal side. Care was taken not to give too much stress to the animal during handling. Then a glass capillary tube was inserted into the tip of the rostrum. By gently pressing with the fingers, the insect was stimulated to insert the rostrum deeper into the capillary tube and eject the VS from the salivary gland. During this act, the venom flowed from the tip of the rostrum as thin drop of VS into the capillary tube. The act of gently pressing the abdomen of the animal was made twice or thrice, with an interval of 5-10 seconds each. The collection of the VS stimulated additional venom flow, as a result of either the pull on the capillary tube into the rostrum up to the second segment or the contact of the finger on abdominal hairs. Generally a milking lasts for a minute.

In the electric stimulation method, instead of holding the animals with fingers, the animals were held with a forceps attached with the electric shock inducer (Mahalakshmi Electric and Co., Tamil Nadu). After holding the animal, electric stimulus of 20 mV was passed into the abdominal sternal region either between fourth and fifth segment or fifth and sixth segments. The stimulation was given two or three times during one milking. After the VS collection, the weight of the predator and capillary tube was reweighed for quantifying the amount of VS milked. The survival rate (SR) and venom milking rate (VMR) were calculated using the following formulae:

$$SR\ (\%) = \frac{\text{No. of animals survived after the VS collected}}{\text{Total no. of animals subjected to VS milking}} \times 100$$

$$VMR\ (\%) = \frac{\text{No. of animals milked VS}}{\text{Total no. of animals subjected to VS milking}} \times 100$$

2.3.2.2. Prey type

Newly emerged *R. fuscipes* males were continuously fed for a period of 40-50 days with third stadium of *S. litura* (133.0 ± 1.9 mg), *H. armigera* (173.3 ± 2.6 mg) and fifth stadium of *C. cephalonica* (29.0 ± 0.4 mg) individually. In each category, 15 males were maintained. Then the predators were allowed to starve for a period of 3-day and VS was collected by manual milking methods as mentioned in the above section. The predatory efficiency of *R. fuscipes* have also been studied on the above three pest; by using the three days starved predators (pre-weighed) were released into the Petridish (9 cm) and followed by the individual prey item. Now the no. of sites pinned and paralyzing time were recorded.

2.3.3. Venomous saliva utilization

We performed this study with 10-days old *R. fuscipes* from our permanent breeding stock. Three different prey types were chosen for the bioassays: third instar larvae of *S. litura, H. armigera* and fifth instar larvae of *C. cephalonica*. We used the same culture which had been starved for 3-day and pre-weighed. In the feeding experiment, pre-weighed predator was released into a glass Petridish (9 cm diameter) (Borosil, India), then the pre weighed preys (2 each) were released. Now allow the predator to capture and inject the VS for exactly 5 min. Then both the predator and the prey were separated gently with a soft fine brush (Camalin, India) and weighed. The weight difference was considered as the amount of VS injected by a predator or the amount of VS utilized by a prey.

2.3.4. Quantification of VS for protein

The VS obtained from the predators in the prey deprivation and the different preys offered were used. The total protein content of different categories of VS was quantified using Lowry *et al.* (1951) method. In protein quantification, the VS was mixed with the 5 ml of reagent C (99 ml of reagent A: 2% Na_2Co_3 in 0.1 N NaOH and 1 ml of reagent B: 1% $Cu_2So_4.5H_2O$ in 1% potassium sodium tartarate). The mixture was incubated at room

temperature for 10 min, and then 0.5 ml of three fold diluted folin-ciocalteou's reagent was added and re-incubated at room temperature for 30 mins. The blue colour developed was read at 750 mm against a blank (replacing distilled water instead of VS) in a spectrophotometer (Elico, India). The values obtained were compared with the standard (Bovine Serum Albumin, 1 mg/1 ml).

2.3.5. Statistical analysis

The statistical comparison was made between different days of starvation in both continuous and discontinuous starvation; one way analysis of variance (ANOVA) and the post hoc Tukey's test were also performed. The Box plots were made for the protein quantity of the VS yield by the starvation. Line column on 2 axes were performed for the VS yield was fed by *R. fuscipes* on different prey items. Box plot were made for the VS utilization by different prey items was made by using SPSS statistical software package Ver. 11.5 (SPSS Inc., 2005) and Microsoft excel.

2.4. RESULTS

2.4.1. Venomous saliva optimization

In continuous starvation, 3-day starved male secreted more quantity of VS both in milking (2.61 ± 0.28 mg/100 mg of animal wet weight) ($df = 6, 3$; $F = 0.660$; $p > 0.05$) and in electric stimulation (1.32±0.06 mg/100 mg of animal wet weight) ($df = 6, 3$; $F = 5.17$; $p > 0.05$) method respectively (Table 2.1). In the discontinuous starvation in the milking method, five-day starved male milked (4.07 ± 0.60 mg/100 mg of animal weight) ($df = 7, 2$; $F=14.85$; $p > 0.05$) more quantity of VS, whereas in the electric stimulation method three-day starved male (3.17 ± 0.46 mg / 100 mg of animal weight) milked more VS ($df = 7, 2$; $F = 7.865$; $p > 0.05$) (Table 2.2).

2.4.2. Survival (SR) and venom milking rate (VMR)

In continuous starvation, 100% of the male and female survived up to the sixth day of starvation, whereas, the VMR was high during the third day of starvation and gradually decreased to 70% at day six of starvation (Figure 2.1. a, b). In the electrical stimulation method, the VMR was more on the 3rd day of starvation, and gradually decrease to sixth day (Figure 2.1. c, d.). In the discontinuous starvation of milking method, VMR gradually

increased from day one to day five day and decreased for the remaining starvation period. However, the survival rate was high on the third day of starvation, whereas in the discontinuous electrical stimulation method, the survival rate gradually decreased from the first day to seventh day of starvation (figure 2.1. g, h).

2.4.3. Quantification of protein of VS yielded in the prey deprivation

The protein quantity of *R. fuscipes* female was insignificantly high when VS was milked by electric stimulation method ($df = 1, 9$; $F = 0.017$; $p > 0.05$) whether the predator was subjected to continuous (Figure 2.2) or discontinuous ($df = 12, 3$; $F = 0.683$; $p > 0.05$) (Figure 2.3) starvation. In general, VS of female has more protein content than male.

2.4.4. Influence of prey on VS yield

Spodoptera litura fed *R. fuscipes* insignificantly milked more VS ($df = 1, 9$; $F = 0.239$; $p > 0.05$) than the *C. cephalonica* (1.15 ± 0.11 mg) and *H. armigera* ($df=1, 9$; $F = 0.001$; $p > 0.05$) (Figure 2.4). The protein content of VS obtained from *C. cephalonica* (48.80 ± 0.20 mg) was insignificantly higher than the *S. litura* ($df = 1, 4$; $F = 0.028$; $p > 0.05$) and *H. armigera* ($df = 1, 4$; $F = 0.041$; $p > 0.05$) (figure 2.4). From the predatory potential studies, it was found out that *R. fuscipes* took 32.38 ± 1.96 min to paralyze *H. armigera* and 8.00 ± 0.57 times pinned the prey. It was significantly higher than *S. litura* (16.19 ± 2.43 min; 5.20 ± 0.81) ($df = 1, 18$; $F = 26.89$; $p < 0.05$) and *C. cephalonica* (3.62 ± 0.43 mins and 3.10 ± 0.31) ($df = 1, 18$; $F = 205.60$; $p < 0.05$).

2.4.5. Utilization of VS by the prey

The quantity of VS injected into the three different prey types is shown in Figure 2.5. All prey were accepted prey types (100% attacked and paralyzed, $n = 10$). *Spodoptera litura* third instar larvae significantly received more quantity of VS (1.36 ± 0.53 mg) ($df = 7, 2$; $F=1.175$; $p < 0.05$) than the *C. cephalonica* (1.05 ± 0.22 mg) and *H. armigera* (0.59 ± 0.20 mg) ($df=7, 2$; $F=2.130$; $p < 0.05$).

2.5. Discussion

Reduviid venomous saliva has insecticidal (Corzo *et al.,* 2001; Kumar, 2011; Sahayaraj and Muthukumar, 2011), antimicrobial (Sahayaraj *et al.,* 2006b) and cytotoxic (Sahayaraj and Muthukumar, 2011) activities. However, major difficulty is in obtaining

sufficient amounts of high quality VS. Two methods have been used to obtain reduviid venomous saliva: salivary gland isolation and homogenization (Maran, 1999; Ambrose and Maran, 1999; Maran and Ambrose, 1999); manual milking method and electrical stimulation (Kumar, 2011; Sahayaraj and Muthukumar, 2011).

2.5.1. Prey deprivation

This is experimental evidence that spiders (Vapenik and Nentwig, 2000) and reduviid (Kumar, 2011; Sahayaraj and Muthukumar, 2011) are able to regulate the quantity of VS milked according to prey deprivation. In general, male secreted more VS than the female. This might be due to the factor that females utilize water from the salivary gland (reabsorption) for the body maintenance and fecundity (Miles, 1972; Miles and Slowiak, 1976). In the milking method, the male and the female showed 100% survival rate. The electric stimulation used to obtain VS from the reduviids through an electric stimulator was found to be stressful (such as: enhancing of VS release, leg contractions) or in extreme cases fatal. In the electric stimulation, the animal does not receive any markings due to the electric shock and while considering both milking and electrical stimulation, the milking method was found to be better which results in the high yield of VS and 100% animals survived up to 6 days of starvation. This was due to the limited stress given to the predator during the milking act. And also the third day of starved animals had high quantity of VS and it gradually decreased up to the sixth day of starvation. In contradiction, previously Ambrose and Maran (1999) and Maran and Ambrose (1999) demonstrated that the accumulation of VS in the salivary gland and increased starvation leads to loss in the size and quantity of VS and salivary gland. Size of the predator has not been considered in this study, because we converted all the value to 100 mg body weight of the predator.

Generally during starvation, predators do not use their VS and this leads to the accumulation of VS in the salivary gland. This indicates that the maximum quantity of VS is secreted by the predator in the third day of the starvation. Reabsorption of water makes the predator to live and this is provided during the sixth day of starvation with a very lower quantity of VS (0.02 ± 0.01 mg/100 mg of the animal weight). The third day of starvation was found to be an optimum period for the VS collection whereas previously Sahayaraj *et al.* (2006a) had shown that the female predators' *R. marginatus* and *Catamirus brevipennis* yield more VS than the male. Vapenik and Nentwig (2000) observed that in *Cupenis salei*, the venom quantity was decreased as the starvation period was increased. In our studies, the

quantity of VS yield by the male was high than that of the female, whereas in the *Bothrops insularis* females yield more quantity of venom than the male (Rocha-e-Silva *et al.,* 2009).

Protein play an important vole in VS toxicity and the role assumes differential importance in various species of venoms animals. And in general, the cost of venom production may increase dramatically in predators with high feeding frequencies (Secor and Diamond, 2000). When compared to manual milking method, the electrical stimulation method was a painful procedure gives more stress to secrete the VS and so the animals secrete a more quantity of VS (Rocha-e-Silva *et al.,* 2009). Previously, Miles (1972) reported that the reabsorbing of the water from the salivary gland during the prey deprivation period makes the animals to live for a few more days. But in the electrical stimulation, the stimulus causes an irritant to the animal which may stress the animal to secrete the total quantity of VS which may lead to earlier depletion of VS and leads to the shrinking of the salivary gland (Ambrose and Maran, 1999). This some times may lead to the death of the animals. The electric stimulation of an *R. fuscipes* causes a recoverable paralysis which does not allow them to feed (Rocha-e-Silva *et al.,* 2009). The prevention of VS contamination by non-venom enzymes is an important consideration during VS collection (see Rash and Hodgson, 2002).

The protein quantity of the VS of female obtained from the electric stimulation method was found to be high. The animals were motivated to produce more quantity along with the salivary gland secretions and thus the level of protein quantity was high in the electric stimulation. The sex related divergence in the venom yields was quite small in the *Atrax* species (Atkinson and Walker, 1985) and *Cupenis salei* (Kuhn-Nentwig *et al.,* 2004).

2.5.2. Prey type and VS yield

The *S. litura* fed *R. fuscipes* secrete more quantity of VS than the other two pest. This might be due to the high nutrient (Figure 2.4) and size of the prey. Previously, Maran (1999) demonstrated that the *S. litura* fed predators' body carbohydrate, protein and lipid were higher. Recently, Kumar (2011) has shown that the soft bodied prey, *S. litura* was more preferred than the hard cuticle prey from Coleoptera and Hemiptera.

2.5.3. Venomous saliva utilization

From a reduviid's point of view *S. litura* is unproblematic prey type. They are not protected by thick chtininaceous exoskeleton and cannot escape by fight. However,

H. armigera try to escape by rolling and splitting behaviors (Sahayaraj, 1991), but the *R. fuscipes* attacked in the head region of the *H. armigera*. Moreover, due to the defensive nature of the *H. armigera* during the predation it attains a maximum time for its paralysis. Boeve *et al.,* (1995) described that the spiders inject more venom into a bigger than into a smaller prey. *Spodoptera litura* on contact, *R. fuscipes* showed a greater response, by releasing more quantity of defensive fluid from its mouth to defend the predators and this prompts the predators to provide more quantity of VS injected.

Perret (1977) reported that the spiders inject more quantity of venom into the larger prey (Boeve *et al.,* 1995) and in scorpions the same was recorded by Bub and Bowerman (1979); Cushing and Matharne (1980); Casper (1985); Rein (1993). The potentially high costs of venom production are frequently assumed to have evolutionary, ecological and behavioral consequences, to quantify the actual energetic costs of venom production (Pintor *et al.,* 2010). The quantity of VS differs depending upon the place of injection. If it injects near the nervous system it requires a low quantity of venom/VS whereas the injection of VS into the abdomen by the predator requires more quantity (Kuhn Nentwig *et al.,* 1994, 2000). Maran (1999) has observed the nutrient ecology of *R. fuscipes, R. marginatus* and *R. kumarii* fed on different pest which showed a high intake of protein and lipid followed by carbohydrate and in our studies after the feeding on different pest, increased protein quantity of VS was observed in the predator fed with *S. litura* than *H. armigera* and *C. cephalonica*.

2.6. CONCLUSION

We concluded that three-day starved predators in the continuous milking method yield a high quantity of VS. And in this method the animals survive up to 6 days of the starvation period and on the third day the animal secretes the maximum quantity of VS. The prey type also determines the quantity of VS yield and the feeding of *R. fuscipes* with *S. litura* makes them to provide a high quality of VS. Such long fasting periods are a natural situation for reduviid. Different prey types receive different VS quantities according to the difficulty in overwhelming them.

CHAPTER 3. BIOCHEMICAL CHARACTERIZATION

3.1. ABSTRACT

Reduviids are found to paralyze and predate on the other insect prey by using their venomous saliva (VS). In this study, *Rhynocoris fuscipes* (Fab.) VS protein profile was qualified and quantified after lyophilization. It was characterized using EDX spectra, FTIR, HPLC, GC-MS and MALDI-TOFMS analysis. The enzyme profile and protein profile of salivary gland parts and guts were also analyzed. Results showed that the VS, salivary gland parts and gut showed the presence of amylase, lipase, protease and phosphatase, hyalurindase, phospholipase, and trypsin like enzymes. The SDS-PAGE profile showed that VS has 19 polypeptide bands (41.2 kDa to 6.4 kDa). Various parts of the salivary gland showed the 18 polypeptide bands (49.6 to 2.4 kDa). The combination of acetone and methanol (1:1) was found to be a system for the VS purification. The EDX spectrum results showed that potassium (29.21%) constituted the major element in the VS. Presence of proteins and their acidic nature and disulfide bridges were observed in the VS by FTIR spectroscopy, while in both HPLC and GC-MS analysis, the presence of phospholipase, hyalurindase and lysolecithin have been observed. About 69 components have been recorded in the VS through MALDI-TOFMS analysis ranging from 2.4 to 59 kDa and molecular mass at 3.8 kDa (RF1T1) have resulted in a higher intensity. In conclusion, the VS of *R. fuscipes* have potent of toxic nature resembling the higher analysis venom and yet the presence of disulfide bridges showed to non-toxic nature towards the mammals.

3.2. INTRODUCTION

Assassin bugs are the predatory bugs found to be predating on other insect using their VS for paralysis and pre-oral digestion (Cohen, 1998a; 2000). Generally predatory Hemipteran bugs use the solid to liquid feeding method (Cohen, 1995, Swart and Felgenhauer, 2003). The fundamental nature of the digestion is to render macromolecules into simple compounds that can be absorbed and circulated (Gilmour, 1961; House, 1974). The types of digestive enzymes, especially those of salivary origin, are highly correlated with the feeding habitats of Hemipteran (Miles, 1972; Hori, 1975; Agusti and Cohen, 2000; Hori, 2000; Zeng and Cohen, 2000a, b; Boyd *et al.,* 2002; Boyd, 2003). Proteinases are the most important liquefaction enzymes for predators (Rees and Offord, 1969; Miles, 1972; Cohen, 1993). Trypsin like enzymes acts on amino acid sites cleaving the protein at lysine and

arginine residues (Law *et al*., 1977; Cohen, 1993). Chymotrypsin like enzymes attack proteins at their aromatic sites (Gilmour, 1961; Law *et al*., 1977; Cohen, 1990, 1993). The presence of protease in the salivary secretions was reported in Blestomatid (Rastogi, 1962; Rees and Offord, 1969) and in the reduviid (Edwards, 1961).

The salivary gland as the source of proteolytic enzymes is injected by carnivorous insects' into the prey (Law *et al*., 1977). Trypsin like protease was the only protease detected in salivary gland complex of *Deracocoris nebulosis* (Boyd *et al.,* 2002). The presence of trypsin like enzymes demonstrates the insect's ability to access structural or other insoluble proteins (Cohen, 1993; 1998b, 2000). In the reduviid, *Zelus renardii* salivary gland trypsin like enzyme was found to be predominant and it has the ability to digest the protein in the prey (Cohen, 1993). The activity of carbohydrases in the salivary gland of the *Lygus disponsi*, *Lygus saundersi, Adelphocaris suturalus, Orthocephalus funestus, Palomera angulosa, Eurydena rigosum and Coreus marginatus* have been studied (Hori, 1975).. Takanona and Hori (1974) have studied the digestive enzymes of *Stenotus binotatus* salivary gland. Triacyl glycerol lipases were found in the saliva and salivary gland of heteropteran predators (Rastogi, 1962; Wigglesworth, 1972; Cohen, 1990).

In *L. rugulipennis* salivary gland, secretable enzymes such as polygalacturonase, amylase and alkaline proteinase and other enzymes such as acid proteinase, phosphatase, trehalase, invertase and phenol oxidase were found (Laurema *et al*., 1985). Phospholipase digests the phospholipids in cell membranes, disrupting neurons and muscle cells (Schmidt, 1982). Phospholipase were detected in the saliva of heteropteran and they digest the phospholipids (Edwards, 1961; Cohen, 1990). The extra-oral digestion secretions could reasonably be expected to contain hydrolases that digest these substances and only hyalurindase have been found (Edwards, 1961; Rees and Offord, 1969; Mommsen, 1978b). In insects, considerable amounts of proteoglycans, glycosano glycans, chondrotin sulfates, dermatan sulfates and hyaluronic acids that form connective tissues, basement membranes, peritrophic matrices, collagenous and fibrous tissues were found (Chippendale, 1978). Hyalurindase is also a spreading factor for venom (Edwards, 1961; Mommsen, 1978a; Foelix, 1982). The saliva of the hemipteran insects separates cells in intact tissue and reduces viscosity of hyaluronic acid and prey fluids (Edwards, 1961). There are only a few reports for the presence of acid phosphatase in the venom (Dani *et al*., 2005; Sahayaraj *et al*., 2010). In

Apis mellifera, acid phosphatase might be a vestigial enzyme, which may have served the function of predigestion of prey before it was eaten or fed to the young (Benton *et al.,* 1963).

Midgut digestive enzymes are similar to vertebrate digestive enzymes have been reported from a large number of insects (Day and Waterhouse, 1953; House, 1974, Dadd, 1970; Eguchi and Iwamoto, 1976; Law *et al*., 1977; Pritchett *et al*., 1981). Where the anterior midgut has low levels of trypsin like, elastase like and chymotrypsins like proteases were identified in *D. nebulosis* (Boyd *et al.,* 2002). The α–amylase is a hydrolytic enzyme that is widespread in nature. These enzymes catalyze the hydrolysis of α-D–(1, 4)–glucan linkages in glycogen and other related carbohydrates (Strobi *et al*., 1998; Franco *et al*., 2000). Amylase was found to be useful in the glycogen digestion (Rees and Offord, 1969). Salivary amylase was present more consistently in the pentamomorphs than in Cimicomorpha (Edwards, 1961; Laurema *et al*., 1985; Cohen, 1998). The high amylase activity not only indicates its potential to digest the starch, the major glycoside reserve found in plants, but also other polysaccharides and dextrin's from glycogen, the major cell glycoside reservoir (Cohen, 1990; Stamppoulos *et al*., 1993). The plant feeding mirids usually have high levels of amylase in their salivary glands (Agusti and Cohen, 2000). The amylase secretions are ingested by the mirid, along with the partially digested starch to be used in the midgut to continue the starch break down (Hori, 1973; Takanona and Hori, 1974; Wheeler, 2001). In insects, the abundance and activity of α-amylase are dependent on food sources (Slansky, 1982; Dow, 1986).

The activity of carbohydrases in midgut of *L. disponsi*, *L. saundersi, A. suturalus, O. funestus, P. angulosa, E. rigosum and C. marginatus* has been studied and all these bugs had amylase, phlenase, α and β–glucosidase and α and β–galactosidase in the midgut (Hori, 1975). Trehelase is an important enzyme that has all the functions throughout the life cycle in all tissues which rely upon glucose generated from trehalose reserves to meet their energy needs (Wyatt, 1967). The lipase action is believed to occur mainly in the midgut to digest triacyl glycerol from prey items (Terra, 1990). Acid phosphatase is also one of the major lysosomal enzymes found in the invertebrates and they are associated with degradation and cell death (Bowen and Lockshin, 1981; Anderson, 1981; Gregorc *et al*., 1998).

The electrophoretic profiles of the venom have shown the protein profiles and the possible venom peptides were identified (Munekiyo and Mackessy, 1998). Azevedo *et al.*

(2007) have analyzed the protein of the salivary gland homogenates using SDS–PAGE and they observed 13 polypeptides, with one of them exclusive of the posterior lobe and molecular weight < 60 kDa. The SDS–PAGE of the crude venom of Black widow spider, *Labrodectus tredecunguttatus* showed that the venom proteins are distributed through a wide range of molecular mass, with abundant protein bands at molecular weight greater than 31.0 kDa and few proteins distributed in the range of 14.4 kDa (Duan *et al*., 2006). The venom of *Pimpla turionellae* is composed of highly complex mixture of polypeptides. It primarily consists of components with molecular weight from 106 kDa to 20 kDa.

The FTIR spectroscopy has become a well accepted and widely used method to characterize biological tissues. A wide range of biological studies have been covered by FTIR analysis (Movasaghi *et al*., 2008). The infrared spectroscopy of endoparasitiod wasp *P. turionellae* has been analyzed and the possible frequencies of 3410, 2361, 1648, 1547, 1398, 1125, 1050 and 618 cm^{-1} were observed. And from their results they have characterized the venomous nature having proteinous nature and also the carboxylic nature of the venom (Uçkan *et al*., 2004). Similar observations were found in the venom of *Nasonia vitripennis* (Rivers *et al*., 2006). Scorpions and wandering spiders evolved their venoms to contain structurally compact peptides due to the presence of disulfide bonds; and, these peptides are characterized by their high affinity for ion channels and/or nervous receptors, causing activation or blockage of the ion flux through the cellular membranes (Escoubas, 2006; Sollod *et al*., 2005).

Shikata *et al*. (1995) characterized the venom of Funnel web spider using HPLC and they have purified a novel peptide isomerase found to be having 29 kDa polypeptide that consists of an 18-residue light chain and a 243–residue heavy chain connected by a single disulfide bridge. The venom of the *Boiga irregularis*, HPLC analysis showed about 8 to 10 protein peaks and they have their enzyme activity. Thirteen different compounds have been identified by gas chromatogaphy mass spectrometry in the Sydney funnel web spider, *Atrax robustus*. The Matrix assisted Laser desorption/ionization (MALDI) is used for the identification of proteins isolated through electrophoresis, affinity chromatography, strong/weak ion exchange (Wu *et al*., 1993). The venom components of *Liocheles austratiasiae* were characterized by MALDI-TOFMS analysis and its toxicity has been analyzed (Eitan *et al*., 1990; Zlotkin *et al*., 1991; Moskowitz *et al*., 1998; Sautrere *et al*., 1998; Hamon *et al*., 2002). In the venom of *Tityus discrepans*, 205 components were identified by MALDI-TOFMS analysis and 70% were having molecular mass less than 5000

kDa (Batista *et al*., 2006). The crude venom of *Lycosa singoriensis* analysis by MALDI showed 10000 to 1000 Da molecular mass distributions (Liu *et al*., 2009). Schwartz *et al*. (2008) have analyzed the venom components of Brazilian scorpion, *Opisthacanthus cayaparum* and they have obtained 221 distinct components.

Only a few reports are available on the characterization of reduviids VS. Previously no one had studied the VS of *R. fuscipes;* hence I had proposed these objectives to study the VS and parts of salivary gland components by enzyme profile and protein profile. The VS have been biochemicaly characterized by using EDX spectra, FTIR spectroscopy, HPLC chromatogram, GC-MS analysis and MALDI-TOFMS analysis.

3.3. MATERIALS AND METHODS

3.3.1. Venomous saliva collection

The laboratory emerged adult *R. fuscipes* (n=80) with three-day starved predators have been used for the VS collection and the collected VS was pooled and it was stored at 0 – 4 ºC for 2 months. Similarly in another set of predators, collected VS was pooled and it was air dried in a sterile condition under a laminar air flow chamber. The powdered VS sample was stored at 0 ^{o}C until further use.

3.3.2. Venomous saliva pH

The pH of the crude and lyophilized VS was determined using the discs of different pH paper (Himedia, India). And the animal VS was placed to find out the pH which was compared with a pH standard.

3.3.3. Total water content of the venomous saliva

The total water content (%) of VS of *R. fuscipes* was analyzed from three-day starved adult predators (n=10). The VS was collected by the method of Sahayaraj *et al*. (2006a) with slight modifications. The animal was allowed to milk in the glass slide and the weight of the crude VS was taken as VS initial weight (SIW) and after 24 h, glass slide weight was noted and the lyophilized VS weight was considered as VS final weight (SFW) and the water content was calculated using the formula:

$$\text{Total water content in VS (\%)} = \frac{\text{SFW}}{\text{SIW}} \times 100$$

3.3.4. Enzyme levels

3.3.4.1. Preparation of Enzyme extracts

Enzyme samples were prepared by the method of Cohen (1993) and later modified by Sahayaraj *et al.* (2010) using 20 adult insects (Male and female in 1:1 ratio) for each sample. Young adults (one week post adult eclosion) were starved for at least 15h before dissection to standardize the insects and allowed for the accumulation of the enzymes. The insects were placed at -20 ºC for 5 min and then dissected out in the ice cold insect Ringers solution (NaCl – 6.5 g, KCl – 0.25 g; $CaCl_2$ – 0.25 g, Na_2Co_3 – 0.25 g in one litre distilled water) under a dissection microscope (Everest, India). The salivary gland and its parts including the principal and accessory glands were removed away from the abdomen using a fine dissection forceps (Tiger, India). Followed by the insects gut was also dissected out and they were split into fore, mid and hindgut. All the samples, accessory gland (AG), anterior lobe of principal gland (ALPG), posterior lobe of principal gland (PLPG), foregut (FG), midgut (MG) and hindgut (HG) were weighed separately using a monopan balance (Dhona, India) (+ 0.1mg) and homogenized using a tissue homogenizer (Kemi, India) for 3 min at 4 ºC in the ice cold phosphate buffer (pH 7.0). Homogenates were centrifuged using a micro centrifuge (Kemi, India) at 10000 g for 15 min at 4 ºC . The supernatant was removed and made up to 10 ml with the above said buffer. Now the extracts were used for the protein estimation, qualitative and qualitative enzyme analyses.

3.3.4.2. Qualitative enzyme profile

3.3.4.2.1. Amylase

The reaction mixture containing 1ml of 1% starch solution with 100ml of enzyme source was incubated in a water bath at 25 ^{0}C for about 30 min. Every 10 min a drop of iodine was added. Reddish violet color appeared when starch was hydrolyzed to dextrin. No color appeared, when starch hydrolyzed to maltose (Nigam and Kumar, 2003).

3.3.4.2.2. Invertase

The reaction mixture containing 2 ml of 10% sucrose solution with 0.2 ml of enzyme extract was incubated at 37 ºC for about 24 h. One drop of Fehling's A and B were added. It was kept in a water bath at 45 ºC. At the end of reaction, brown or brick red precipitate appeared (Nigam and Omkumar, 2003).

3.3.4.2.3. Lipase

The reaction mixture was prepared by using one ml of boiled milk, one ml of 1% phenol red and 0.5ml of enzyme extract. Now a drop of 0.025% Na_2Co_3 was added. Appearance of pink color was observed. It was incubated at 37 ºC for 24h. The disappearance of pink color shows the presence of lipase (Nigam and Omkumar, 2003).

3.3.4.2.4. Protease

One ml of casein solution along with 100 µl of enzyme extract and 500 µl of TCA were centrifuged at 5000 rpm for 10 min. The supernatant was mixed with 0.5 ml of saturated Na_2Co_3 and a few drops of folin phenol reagent were added. Formation of blue color or precipitate indicates the digestion of casein (Balogun and Fisher, 1970).

3.3.4.2.5. Trypsin

The reaction mixture containing 0.5 ml of alkaline casein with 0.5 ml of enzyme extract was incubated at room temperature for about 6 h. 1% acetic acid was added. Increased turbidity indicates the tryptic activity (Balogun and Fisher, 1970).

3.3. 4.2.6. Pepsin

The reaction mixture containing 0.5 ml of casein (pH 2.0) with 0.5 ml of enzyme extract was incubated at room temperature for about 6 h. 10% sodium acetate was added. Increased turbidity indicates the presence of pepsin (Tonapi, 1996).

3.3.4.2.7. Polypeptidase

Incubate the reaction mixture containing 0.5 ml of 2% peptone solution with 0.5 ml of enzyme extract along with 0.5 ml of phosphate buffer, incubated in room temperature for

about 24 h. A few drops of diluted acetic acid and bromine water were added. Pink color developed indicating the presence of polypeptidase activity (Tonapi, 1996).

3.3.4.3. Quantitative enzyme studies

3.3.4.3.1. Amylase and Invertase

Amylase and invertase activity was studied (Bernfield, 1955 and later modified by Ishaaya and Suriski, 1970). The reaction mixture consisted of 1 ml of 0.2% soluble starch in borate buffer (amylase) and 0.2% sucrose in phosphate buffer (invertase). To the above mixture 250 µl of enzyme extract was added and incubated at 37 ºC for 60 min. The enzyme activity was terminated by adding 0.4 ml of DNS reagent. The reaction was maintained at 100 ºC for 5min. Absorbance of the sample was measured using optical density units at 575 nm (amylase) and 550 nm (invertase) against a blank containing distilled water. The enzyme activity was expressed in terms of the weight of the reducing sugars, glucose (amylase) and sucrose (invertase) produced by the enzyme action per unit weight of a sample per unit time.

3.3.4.3.2. Protease

Protease activity was qualified spectrophotometrically as described by Morihara and Tsuzuki (1977) and later modified by Soyelu (2007). The reaction mixture consisted of 1ml of 1% casein and 0.5 ml of the enzyme extract. This was incubated at 35 ºC for 30 min in a water bath. The reaction was terminated by adding 3 ml of ice cold 10% TCA. The mixture was then allowed to stand at 4 ºC for 30min, and centrifuged at 3000 rpm for 10min. Now the supernatant was collected for the determination of non-precipitated products of digestion. This was determined following the Folin Ciocalteu's phenol reagent (Lowry *et al.,* 1951). One ml of the TCA protein was mixed with 5 ml of Lowry's reagent of C, mixed thoroughly and incubated at room temperature for 5 min. 0.5 ml of three fold diluted Folin Ciocalteu's phenol reagent was added to the mixture with shaking and incubated at room temperature for 30 min. The optical density was taken at 670 nm in Elico spectrophotometer. The amount of non precipitated TCA protein was estimated as tyrosine from a standard curve known as concentrations of tyrosine. One unit of protease activity is defined as the quantity required producing 100 mg of tyrosine in 1ml of TCA filtrate under the above concentrations.

3.3.4.3.3. Lipase

The lipase activity was carried out titrometrically as described by Cherry and Crandall (1932). One ml of enzyme extract (the control tube was placed in a boiling water for 15 min to destroy the enzyme activity and then cooled. 500 µl of phosphate buffer solution (pH 8.0) and 2ml of olive oil emulsion were added, and placed in the shaker, incubated at 37 ºC. After 24h, 3 ml of 95% ethanol was added and two drops of phenolphthalein indicator was also added to each tube, the tubes were titrated separately with 0.05N NaOH and the end point was the formation of pink color. Lipase activity was calculated using the following formula:

$$\text{Lipase (mequ/min/g/sample)} = \frac{\text{Volume of NaOH consumed x alkali strength}}{\text{Sample weight in g x Time in min}}$$

3.3.4.3.4. Acid phosphatase

Acid phosphatase activity was quantified following Beaufay *et al*. (1954) method. The enzyme reaction mixture consists of 0.5 ml of substrate (ρ-nitro phenyl phosphate disodium), 1.1 ml of 0.2M sodium acetate buffer (pH 5.5) and 0.2ml of the enzyme extract was incubated for 10min at 37 ºC. And after incubation, the reaction was terminated by adding 4.5 ml of 0.01N NaOH. The product ρ-nitrophenol was measured by spectrophotometer; absorbance at 420 nm against an enzyme blank was taken. The amount of ρ-nitro phenyl phosphate released as a result of the enzyme activity was determined from a standard curve drawn using standard ρ-nitro phenyl (10 µmoles/ml).

3.3.4.3.5. Hyalurindase

Hyalurindase activity was determined tubidometrically by the method of Pukrittayakamee *et al*. (1988). The assay mixture consists of 0.2M acetate buffer (pH 6.0) containing 0.15M NaCl, 50mg hyaluronic acid (0.5 mg/ml in acetate buffer) and enzyme source having a final volume of 1.0ml. The mixture was incubated for 15 min at 37 ^{o}C. Then the reaction was stopped by the addition of 2 ml of 2.25% acetyl trimethyl ammonium bromide in 2% NaOH. The absorbance of each reaction mixture was read at 410 nm against a blank containing 1 ml of acetate buffer and 2 ml of 2.25% acetyl trimethyl ammonium bromide in 2% NaOH. Turbidity reducing activity was expressed as a percentage of the remaining hyaluranic acid, taking the absorbance of a tube as 100% in which no enzyme

added. Turbidity reducing unit is expressed as the amount of enzyme required to hydrolyze 50% (25 mg) of the hyaluronic acid. Specific activity is expressed as turbidity reducing units/mg enzyme per min. One unit is defined as the amount of enzyme that will cause 50% turbidity reduction as 1.0 unit of international standard preparation.

3.3.4.3.6. Phospholipase A_2 activity

Phospholipase activity was determined as described by Santoro *et al.* (1999). Fifteen µl enzyme source was added to 1.5 ml of reaction solution (100 mM NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.265% egg lecithin, 98.8 µm phenol red, pH 7.6) in a spectrophotometer cuvette. The mixture was read at 558 nm. One unit of activity was defined as the quantity of venom protein (mg of protein/assay) producing a decrease of 0.001 units of absorbance in the conditions described.

3.3.4.3.7. Trypsin-like-enzyme

The assay of trypsin-like-enzyme activity was carried out as described by the methods of Stewart (1993) and Zheng *et al.* (2002). The substrate solution (1 mg/ml) was prepared by dissolving 100 mg of N α-benzoyl-L-arginine-p-nitroanilide (BApNA) (Sigma, India) in 5 ml of dimethyl sulfoxide (DMSO) and then 95 ml of 0.05M Tris–HCL buffer (pH 8.2) was added. Trypsin-like activity was monitored as BApNA hydrolysis by adding 100 µL of extract (diluted to 100 µl with buffer for the crude sample before the assay) to a well in an assay plate, and by adding 100 µl of the substrate solution. The absorbance, which is proportional to protease activity, was measured with a plate reader ELISA strip reader (SR 601-Qualisystems). The plate was read at 420 nm after incubation for 10 min at 37 ºC. The endogenous control (substrate solution and buffer only) was used. The reading was corrected by subtracting the reading from that of the endogenous control sample.

3.3.5. Total protein quantification

Total protein content (Lowry *et al.* (1951) of the venomous saliva and parts of the salivary gland were done using Bovine Serum Albumin (BSA) as standard which was previously described in Chapter 2 (page no.)**.**

3.3.6. Electrophoresis (Tricine SDS-PAGE)

Tricine sodium dodocyl sulfate polyacyrlamide gel electrophorsis (Tricine SDS PAGE) was performed by the method described by Schägger and Von Jagow (1987) using 16% separating gel and 5% stacking gel in a mini electrophoresis unit (Biotech, India). The separating gel (16%) contained acrylamide stock (29:1 ratio of acrylamide and bisacrylamide), 1.5M Tris HCl (pH 8.25), glycerol, 10% APS and TEMED. The above mixture was mixed and poured in the gel cast and one ml of the overlaying solution (70% n-butanol) was poured. After the polymerization, the excess overlaying solution was poured off and washed with distilled water. Now the stacking gel (5%) containing acrylamide stock (29:1 ratio), Tris HCL (pH 8.25), 10% APS and TEMED was added and after the polymerization, the gel was placed in the tank containing two buffer systems (cathode buffer and anode buffer). To the 30 µl of the sample, an equal quantity of sample buffer was added and it was loaded in the gel. And ten µl of reference standard marker (Low molecular weight protein marker, Genei, India) was also added. Now the current was set at 30 mv and later it was raised to 90 mv until the dye front reached the end of the gel.

After the electrophoresis, the gel was stained with the staining solution (0.1% Commasie brilliant blue, 40% methanol and 10% glacial acetic acid) for 24 h followed by destaining (40% methanol and 10% glacial acetic acid). Gel image was captured using Biotech image analyzer system (Biotech, India) connected to an Intel Pentium computer (Samsung, India) equipped with DGel system (Biotech, India). Molecular weight of protein bands were estimated with reference to molecular weight marker (Genei, India).

3.3.7. Fourier Transfer Infrared (FTIR) spectroscopy

Infrared spectroscopic analysis was performed essentially as described previously (Uçkan *et al.,* 2004). In brief, 15 µg of lyophilized VS was homogeneously ground with potassium bromide (Hi Media, India) before the infrared spectroscopic analyses. The FTIR was carried out at room temperature using Schimadzu FTIR (Model 8400S, Japan). The spectra were interpreted with available literature (Stuart, 1997; Kalsi, 1998; Uçkan *et al.,* 2004).

3.3.8. High Performance Liquid Chromatography (HPLC)

The lyophilized VS was analyzed on a HPLC (Shimadzu LC/10AD, Japan) equipped with an injector – 20 µl loop. Experiments were conducted using a Shimadzu C-18 column (5 µm particle size, 250 mm x 4.6 mm I.D) with a flow rate of 1ml min^{-1} at room temperature. The mobile phase used was CH_3CN/H_2O (1:1, v/v) with elution isocratic and UV detection (SPD-10A/UV-Vis) at 280 nm. Acetonitrile used was HPLC grade (Sigma Aldrich, India) and degassed in an ultrasonic bath before use. The water was distilled using a Milli-Q system (Millipore) and the mobile phase was filtered through a 0.49 µm nylon filter (Corzo *et al.,* 2001).

3.3.9. Gas Chromatography-Mass spectrometer (GC-MS)

Lyophilized fractions from HPLC were mixed in 10 µl of HPLC grade water. The fractions were further analyzed in gas chromatography. For GC/MS a Hewlett Packard gas chromatograph 6390 series II Plus was linked to Hewlett Packard mass spectrometer system equipped with a capillary column HP5-MS (64 m\0.34 mm, 0.21 µm film thickness) was used. The temperature was programmed from 240 ^{o}C to 350 ^{o}C at rate of 6 ^{o}C min D1 with 10 min hold. Helium was used as a carrier gas with a constant flow at 0.8 mL minD1. The ionization voltage was 80 eV. Fraction also was analyzed after silylation at the conditions given for the silylated polar compounds mentioned below. Quantitative analysis of sterols was performed on a Hewlett Packard gas chromatograph (Zhu *et al.,* 2008) 5890 equipped with FID and capillary column HP5-MS (64 m\0.34 mm, 0.21 µm film thickness), at 230 $^{\circ}$C and programmed to 300 ^{o}C at 4 ^{o}C minD1 and5 10 min hold (Munyiri and Ishikawa, 2004; Zhu *et al.,* 2008). Injector and detector were at 280 ^{o}C. One µl of each sample was injected triplicate split/splitless and quantities represented as relative area percent as derived from the integrator. Calibration was established using hexane as standards.

3.3.10. Matrix Assisted Laser Desorption/Ionization –Time of Flight Mass spectrometry (MALDI-TOFMS)

MALDI-TOFMS spectra were obtained on a The Voyager-DE™ PRO Biospectrometry™ spectrometer (Applied BioSystems, Framingham, MA, USA) equipped with a model VSL-337ND nitrogen laser (Laser Science, USA). The accelerating voltage was 20 KV. The matrix was α-Cyano-4hydroxycinnamic acid (Sigma Aldrich, India), which

was prepared at a concentration of 10 mg/ml in 1:1 CH_3 CN/0.1% TFA. Lyophilized VS was analyzed as described in assassin bugs by Corzo *et al.* (2001) to get the exact molecular weight of the component. An equal amount of the sample and matrix were dropped onto the MALDI sample plate and allowed to dry at room temperature. Time-to-mass conversion was achieved by external and /or internal calibration using standards of bovine pancreatic beta insulin (m/z 3496.9), bovine pancreatic insulin (m/z 5734.6), and apomyoglobin (m/z 16,952.6) (Sigma Aldrich, India). Experiments were facilitated by the Voyager Version 5 with Data Explorer™ software.

3.4. RESULTS

3.4.1. pH and water content

The pH of VS immediately collected from *R. fuscipes* was 7.3 ± 0.1, whereas the lyophilized VS was 6.24 ± 0.08. The water content of crude VS of *R. fuscipes* was 24.3 ± 2%.

3.4.2. Qualitative enzyme profile

In the qualitative enzyme studies, the VS and the salivary gland complex (SGC) consist of amylase, invertase, lipase, protease and trypsin. In the SGC and VS do not show pepsin (except in male) and polypeptidase level activities (Table 3.1). In the gut, presence of amylase, invertase, protease, trypsin and pepsin activities was recorded, whereas lipase activity was not recorded in the foregut of the predator. In the VS, salivary gland complex and gut no polypeptidase activity was observed.

3.4.3. Quantitative enzyme levels

The amylase level was maximum in the female accessory gland ($df = 2, 9$; $F = 8.376$; $p > 0.05$) and female midgut ($df = 2, 9$; $F = 218.49$; $p < 0.05$). Higher level of lipase activity was recorded in the accessory gland of both male (41.68 mequ/min/g) and female (42.81 mequ/min/g) and foregut (49.70 mequ/min/g) possess maximum enzyme activity. The invertase activity was found to be higher in the posterior lobe of the principal gland ($df = 2, 9$; $F=1.385$, $p < 0.05$; $df =2, 9$; $F=3.178$; $p < 0.05$) for male and female respectively and the foregut of both male and female have enzyme activity. The posterior lobe of the principal gland possess high level of trehalase activity ($df = 2, 9$; $F = 4.504$; $p < 0.05$) and foregut possesses high trehalose activity (Table 3.2).

The high level of protease activity was recorded in the VS (23.41 ± 0.00 mg tyrosine/g protein/min), and in hindgut (df = 2, 9; F = 8.879; $p < 0.05$). The AG of both male (df=2, 9; F=1.009; $p > 0.05$) and female (df = 2, 9; F = 2.429; $p < 0.05$) have maximum acid phosphatase activity. The foregut of both male and female have high level of enzyme of about 7.62 + 0.05 and 7.85 + 0.02 mmol/g1hr respectively. The VS of female possesses maximum phospholipase A_2 enzyme activity of 18.32 ± 0.73 nm/min/mg protein. The anterior lobe of principal gland (df = 2, 9; F = 1.877; $p < 0.05$) has a high level of hyalurindase enzyme activity. The higher level of trypsin like enzyme activity was observed in the female VS (18.08 ± 0.03 units/mg) (Table 3.3).

3.4.4. Total protein in the VS and salivary gland complex

The mean protein content of venom reservoirs, whole SGC samples were significantly higher (df = 3, 36; F =18.00; $p < 0.05$) than that of VS, PG and AG (Figure 3.1). Female always possesses more protein than the males.

3.4.5. Tricine SDS-PAGE of VS and salivary gland complex

The electrophoretic analysis of VS of *R. fuscipes* showed a complex protein composition with molecular weight ranging from 41.2-6.4 kDa. The most characteristic protein band profile was observed in the region between 41.2 and 26.0 kDa. This region contains 14% of the total applied protein distributed in 19 bands as determined by densitometry (Plate 3.1). The band at 41.2 kDa showed a high intensity of occurrence. Regarding the number of bands in AG, ALPG, PLPG and WSG; 17, 18, 16 and 18 and their molecular weights ranged from 48.6 to 2.4 kDa, 48.6 to 7.8 kDa, 49.4 to 5.0 kDa and 49.6 to 5.0 kDa respectively (Table 3.4). There was no significant difference in the band pattern between VS and AG (df = 1, 34; F = 1.745, $p > 0.05$), ALPG (df = 1, 34; F = 0.004; $p > 0.05$), PLPG (df = 1, 34; F=1.875; $p > 0.05$) and WSG (df = 1, 34; F = 0.564; $p > 0.05$).

The mixture of acetone and methanol (1:1) showed that the VS have been purified and it showed about 11 polypeptide bands ranging from 33.1 to 9.4 kDa and considering the other solvent systems such as pure methanol which showed 8 polypeptide bands ranging from 27.7 to 10.3 kDa and for the acetone 9 polypeptide bands ranging from 31.1 to 10.5 kDa (Plate 3.2). The action of methanol + acetone was found to be insignificant to acetone (df = 1, 16; F =0.649; $p > 0.05$) and methanol (df = 1, 16; F = 0.028; $p > 0.05$) respectively (Table 3.5).

3.4.6. EDX spectra

The EDX spectra of lyophilized VS showed the presence of inorganic elements (Silicon – 2.20%, Sulphur – 21.27% Chlorine – 23.35%); alkaline earth metal ions (Calcium – 2.29%); transition metals (Copper-6.46%, Zinc – 3.47% and alkali metals (Potassium – 29.23%, Zirconium – 11.72%). Potassium was found to be high (29.23%) (Figure 3.2).

3.4.7. FTIR spectroscopy

An infrared spectrum analysis of the VS is shown in the Figure 3.3 and characteristic absorption bands are interrupted in Table 3.6. The absorption bands at 3441.12, 1647.26, 1545.03 cm^{-1} can be interrupted to indicate the presence of secondary amines and amide groups and to confirm showed the proteinous nature of VS. The observed band at 2964.69 cm^{-1} is characteristic for the presence of carbohydrate moiety. The vibration at 2378.31 and 522.73 cm^{-1} showed the presence of sulphur. The acidic nature of the VS was supported by the presence of vibration 1398.44 cm^{-1} (Table 3.6). The absorption bands at 1085.96, 983.73cm^{-1} suggest the presence of some venom components, possibly enzymes with phosphorous. The presence of alkyl halides (675.11 cm^{-1}) has found interpretation of potassium bromide during the experimental analyses.

3.4.8. HPLC analysis

The HPLC analysis of lyophilized VS of *R. fuscipes* (Figure 3.4) showed the presence hyalurindase (30%) lysolecithin (20%) phospholipase and masto 2, 2'- pranol (89%). (Table 3.7)

3.4.9. GC.MS analysis

GC – MS analysis shows (Figure 3.5) the presence of metho 5-primol (80%), hyal-2, urindase (30%), phospho-lipase (22%) and lysolecithin 2, 2'-ol (20%) were identified (Table 3.7).

3.4.10. MALDI – TOFMS analysis

The lyophilized VS of *R. fuscipes* contain a wide variety of components with molecular masses ranging from 2357 to 58261 Da (Figure 3.6). About 69 molecular mass

components were identified. From the molecular mass components, the peptide at 3802.2Da showed a maximum absorbance of 10039.3 mAU (RFITI) and two minor peptides were recorded, they were at 2358Da (2608 mAU) and 3423 Da (2836 mAU) and named as RFIT2 and RFIT3 respectively (Figure 3.7). From the total molecular mass components, the percentage of distribution data showed that about 17.39% components were located between 20.0 to 25.0 kDa when considering the maximum absorbance they were located between 1000 to 5000Da and they had a percentage distribution of 7.24% (Table 3.8).

3.5. DISCUSSION

This is the first time that VS and salivary gland complex of *R. fuscipes* have been chemically characterized. The prey paralysis may results from damage by certain digestive enzymes in neurons, muscles and storage tissues of the prey. Structural proteins are hydrolyzed and liquefied inside the prey by endopeptidases such as trypsin like (14.64 and 18.1 μ/g) for male and female respectively) injected by the *R. fuscipes*, while cell membranes, storage tissues and reproductive system are affected by phospholipase (12.4 and 18.3 g/min/g protein for male and female respectively), lipase and amylase (Azevedo *et al.,* 2007). The enzymes such as phospholipase and hyalurindase are important enzymes which were responsible for the toxin nature of the VS (Cohen, 2000) of *R. fuscipes*. The enzymatic action of phospholipase was already detected in the heteropteran saliva (Edwards, 1961; Cohen, 1990), and also in the spiders (Mommsen, 1978a). *R. fuscipes* use their VS for their extra oral digestion for liquefaction the solid prey into a liquid one and reduce the viscosity of intractable liquid (Cohen, 2000). Similar observation was made in the reduviid, *Catamirus brevipennis*, higher level of protease activity was observed in the salivary gland complex than the other enzymes (Sahayaraj *et al*., 2007, 2010). Later, the hydrolyzed material was ingested by the predator and additional processing occurs in the gut allowing its absorption by the predator (Cohen, 1993, 1995). The maximum protease activity in the gut showed that the several arthropods use the EOD, whose digestive enzymes all originate from the gut rather than the salivary glands (Cohen, 2000). Boyd (2003) observed that trypsin like; chymotrypsin like activity was high in the salivary gland of *Deraecois nigritulus* rather than the gut, having a high level of α-amylase, α–glyocosidase and elastase activity. Moreover, carbohydrates are as evident from the presence of significantly high trehalose and invertase here (Table 3.2). Protein and lipids are digested in the hindgut and foregut respectively.

Nuorteva (1954) reported that five species of miridae and two species of pentatomidae had amylase and protease in their salivary gland and in *Lygus disponsi* (Hori, 1970 a, b, c) and in the *Dydercus koeingii,* amylase, sucrase, protease and esterase (Saxena, 1955), and in the *D. fasciatus,* α-glucosidose, β-glucosidase, α–glactosidase, aminopeptidase, carboxy peptidase and lipase (Ford, 1962; Khan and Ford, 1967). Presence of invertase, amylase, protease and lipase indicates VS has digestive function. In Heteroptera, proteolytic activity has been found in the salivary glands of several carnivorous species, (Baptist, 1941; Kretovich *et al*., 1943; Nuorteva, 1954; Edwards, 1961). The alkaline proteinase in the salivary glands of *Lygus* is certainly a secretable salivary enzyme, which facilitates the utilization of food proteins (Laurema *et al*., 1985). In *R. fuscipes*, the enzyme activity was found to be high in the female than the male. This might be due to the egg production, higher predation rate, and larger size of animal. Salivary gland complex is an important source of general proteinase and endopeptidase activity, as observed in *Zelus renardii* by Cohen (1993). Trypsin like endopeptidase was observed in the salivary glands of the terrestrial heteropterans (Cohen, 1993; Cohen and Wheeler, 1998). Similarly in *R. fuscipes*, the general protease and trypsin like activity and phospholipase activity were found to be high in the VS. These enzymes help the animal to liquefy the prey (Cohen, 1995; Swart *et al.,* 2006; Silva Cardoso *et al.,* 2010). The spreading behavior of phospholipase makes the other EOD enzymes to spread very rapidly.

The SDS-PAGE of VS and SGC of *R. fuscipes* showed molecular mass ranged from 41 to 2.4 kDa. These small peptides may generally be neurotoxins in higher Hymenoptera (Schmidt, 1982). This is consistent with the paralyzing function of *Pimpla turionellae* (Kilincer, 1975; Uçkan *et al.,* 2004). However, these molecular weight proteins/peptides are not recorded from the MALDI-TOF MS studies, indicates that during lyophilization process these molecular weight components are denatured. Posterior lobe of *Acanthispis pedestris* had more number of protein fractions (11) than the anterior lobe (6) and accessory gland (2) (Morrison, 1989). An opposite trend has been recorded in this study. The presence of identical protein in the VS as well as anterior (41231 Da), posterior (35244 Da) and accessory gland (26068 and 18179 Da) presumably indicate the transport of salivary gland protein/peptides into the VS. SDS-PAGE analysis of snake venom showed that the molecular weight of phospholipases is 16-17 kDa (Ramírez-Avila *et al.,* 2003). Similar molecular weight protein was detected in VS (16724 Da) and ALPG (17452 Da) of *R. fuscipes* which is

supported by our HPLC (Figure 3.4), GC-MS (Figure 3.5) and enzyme quantification (Table 3.2) studies. However, MALDI-TOFMS does not support these observations.

The EDX spectra results of *R. fuscipes* revealed that about 29.23% of potassium and 21.27% of sulphur showed the disulfide bridges in the VS. Similar observations were recorded in the cobra venom (Miyashita *et al.,* 2007) and also in reduviids (Corzo *et al*., 2001). The presence of sulphur indicates the disulfide bridges in the VS of *R. fuscipes* which was confirmed in the FTIR spectroscopy. The FTIR spectrum of the VS of *R. fuscipes* indicates that it is acidic in nature (1398 cm^{-1}) and it was confirmed by Leonard (1972) in the sawfly toxins. Absorption bands at 3441, 1639 and 1545cm^{-1} are expected for the proteinaceous nature of the VS (Stuart, 1997; Rivers *et al*., 2006) Alkanes characters was observed (2964cm^{-1}). The presences of phosphorous in the VS confirm the presence of enzymes, as suggested by Schimdt (1982); Piek and Spanjier (1986); Parkinson *et al*. (2001, 2002 a, b); Moreau *et al*. (2004); Uçkan *et al*., (2004) from the venom of endoparastie species and social Hymenoptera species. The Silicon observed in the EDX spectra of VS of *R. fuscipes* was also characterized in the FTIR spectra (2378 cm^{-1}). The disulfide nature of VS in *R. fuscipes* has been previously observed in the cobra venom and also in the reduviids by Corzo *et al*. (2001).

The HPLC and GC–MS analysis of VS of *R. fuscipes* showed the presence of compounds such as hyalurindase, lysolecithin, phospholipase which were found common in both the analyses. Metho 5 –primol (80%) and Mastro 2, 2'-pranol (89%) was found to be abundant in the VS of *R. fuscipes*. The MALDI–TOFMS analyze of VS of *R. fuscipes* indicated that VS consists of 69 components predominantly composed of components with molecular mass of 20 to 25 kDa (17.4%), 25 to 30 kDa (16%), 30 to 35 kDa (13%) and 50 to 66 kDa (6%). Corzo *et al*. (2001) observed that molecular mass of less than 30 kDa (56.5%) in reduviids like *Peirates turpis, Agriosphodrus dohrni* and *Isyndus obscurus.* However, low molecular weight proteins and peptides are typical of VS of *R. fuscipes* 3802 Da (10039) (RF1T1). The peptides such as 3802 Da (RF1T1) (10039 mAU) major one and two minor bards RF1T2 (3423 Da) and RF1T3 (2358 Da) were identified. The venom of *Lycosa sugouensis* was characterized by MALDI-TOF and it was characterized into three groups based on their molecular weight group ranging from 8.0 to 2.0 kDa (Liu *et al.,* 2009).

3.6. CONCLUSION

The VS of *R. fuscipes* has been characterized by using enzyme studies, protein profiling and also using various biochemical markers such as EDX spectra, FTIR, HPLC, GC-MS and MALDI–TOFMS analysis. In this study, the VS of *R. fuscipes* has the toxic nature of the venom and due to the presence of disulfide bridges it might not be toxic towards the mammals. The VS has the components like hyalurindase, phospholipase and lysolecithin. Hence, this can be used in the study for insecticidal activity. Clearly, additional studies are needed to further characterization and isolation of VS proteins.

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.

CHAPTER 5. IMMUNOMODULATORY ACTIVITY ON MICE

5.1. ABSTRACT

Rhynocoris fuscipes is considered as a biological control agent in Asian countries. Occasional sting of this reduviid causes local pain, erythema and edema. However, it depends upon the health status of the person. In this study, we proved that this hunter reduviid venomous saliva (VS) injection causes edema on mice model and also on human beings. Moreover, VS alter blood serum by increasing the total carbohydrate and protein levels and decrease lipid level, asparate aminotransferase (AAT), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and acid phosphatase (AP) levels in mice blood sera. It causes inflammatory response on mice skin; however, no mammalian mortality was observed.

5.2. INTRODUCTION

Venomous animal possesses a wide range of toxins for predation and defense (Kapoor, 2010). In many features, these toxins are found to be acting as therapeutic tools in curing several diseases. For instance, venom of honey bee, *Apis mellifera* acts upon the sodium potassium (SK) channel blocker (insulin releasing cells of the pancreases) (Stocker, 2004). The VS of *Rhynocoris marginatus* and *Catamirus brevipennis* have shown antibacterial activity against the human pathogens (Sahayaraj *et al.,* 2006b). Insect toxins are found to be acting against other arthropods (Zlotkin *et al.,* 1975) and rabbit (Ospedal *et al.,* 2002), the taxonomically related groups of arthropods have direct action towards the nervous system of the prey (D'Ajeilo *et al.,* 1972). Previously, Corzo *et al.* (2001) had studied the toxicity of VS of *Peirates turpis, Agriosphodrus dohrni* and *Isyndus obscurus* towards Swiss Albino mice. Other than the zoophagous reduviids, haematophagous reduviids cause several reactions in mammals. Several reactions such as anaphylaxis and delayed hypersensitivity reactions have been identified on the bite of *Triatoma* insects (Riley and Howard, 1894). The saliva (Triatominae) may cause sensitization during a blood meal (Pinnas *et al.,* 1978; Lindberg *et al.,* 1981; Marshall and Street, 1982). The anaphylactic reactions to saliva from the bites of kissing bugs are found to be species specific (Nicholas and Green, 1963; Lynch and Pinnas, 1978; Pinnas *et al.,* 1986; Klotz *et al.*, 2009). The saliva of aquatic predator *Belostoma* causes cardiac erythrythaneous hypotension and death in animals (Pereira *et al.,* 1989) and these saliva cause intense pain and paralysis in invertebrates (Haddad *et al*., 2010).

Vetter and Visscher (1998) demonstrated that the string of *A. mellifera* induces local pain and edema. The pain and inflammatory responses caused by the wasp venom may involve components like neuropeptides and phospholipases (Costa *et al.,* 2000; 2003, Zanchet and Cury, 2003; Chen and Lariviere, 2010; Ferreira Jr *et al.*, 2010). This is true in the case of scorpion venom too (Valdez- Cruz *et al.,* 2007; Feng *et al.*, 2008; Kanoo and Deshpande, 2008). Potier *et al.* (2009) have stated that the venom of Hymenoptera causes reactions ranging from mild local reactions with painful erythromatous swelling to severe life threading anaphylaxis (Dkhil *et al.,* 2010). The toxicity of scorpion venom was studied towards the mammals and insects (Zlotkin *et al.,* 1972). The venom of *Polybia parlista* causes muscle degeneration in the treated Balb\c mice (Rocha *et al.,* 2007). The long lasting edema has been observed in the mouse on injection of *Polistes lario* venom (Yshii *et al.,* 2009) and it results in microvascular permeability in the mouse dorsal skin.

Mirakabadi *et al*. (2006) have studied the osmotic fragility of red blood cells and serum biochemical (glucose, blood urea nitrogen, creatinine, uric acid, triglycerides, cholesterol, AAT and AST) changes in the rabbits using the venom of *Odonthobuthus doriae* and *Hemiscorplus lepturus* (Mirakabadi *et al*., 2007). Mirakabadi *et al.* (2010) have studied the serum of rabbits injected with venom of *Hemiscorpius lepturus* by using the biochemical parameters such as ALT, AST, LDH, urea, creatinine, blood urea nitrogen, creatine phosphokinase and creatine kinase isoenzyme MB. Similarly, Salman (2010) has studied the serum ALT, AST, alkaline phosphatase (ALP), glucose, urea, uric acid, creatinine and potassium ions from the Guinea pigs injected with the venom of *Buthus occitanus*

The occasional sting of reduviid might cause allogenic effects in humans, whereas previously the VS of the reduviid toxicity have not been clearly discussed on mammals. Hence in my study, I had made an attempt to study the toxicity of VS on Swiss Albino mice. Hence, we proposed to study the impact of VS on the macromolecular and enzyme profile of blood sera and also by using intra peritoneal injection and intra dermal injection in the Swiss Albino mice.

5.3. MATERIALS AND METHOD

5.3.1. Reduviid bite in humans

The bites of the reduviids have occurred occasionally during the time of collection, feeding and handling process. During the collection and maintenance of the reduviids the bite

may occur and these data have been obtained from the 29 reduviid researchers through the personal interview made regarding the type of species, location of bite, immediate pain nature etc. (see Table 5.1).

5.3.2. Animals

Adult male Swiss Albino mice (Strain no. HA1075) (23.10 ± 3.04 gm) were purchased from the Venture Institute of Biotechnology, Madurai and were maintained in the laboratory conditions as mentioned in the first chapter (Page No. 03). They were fed with standard mice pellets (Kisan feeds, India) with free access to water in mice cage (33 x 16 cm) and they was allowed for acclimatization in the laboratory for 15 days. The VS concentrations were prepared as described in the previous chapter 4 (page no. 45).

5.3.3. Immunization

For the experiment, mice (30 days old; 23.10 ± 3.04 gm) were divided into six groups having six animals in each group. The mice were anaesthetized with the help of chloroform soaked cotton for 1 min. Using the insulin syringe (Glaxo, India) 15 µl of the VS (200, 400, 600, 800 and 1000 ppm) concentrations were injected *via* intramuscularly in the hind leg and the control animals received an equal quantity of phosphate buffer (pH 7.2). After four days of the initial dose, the secondary dose of double the volume (30 µl) was injected as described by Aguiji *et al*. (1999).

Five days after the secondary dose, bleeding was carried out by the method of Hoff (2000) through heart puncture technique (Retro orbital method). The blood collected (2 ml) was immediately transferred into an eppendorf tube and it was placed in the refrigerator for overnight for the separation of sera. Then the accumulated sera was collected and transferred carefully into a sterile glass vial. Again the blood samples were centrifuged at 5000 rpm for 20 min and the sera was separated and stored in the respective glass vials. The blood biochemical parameters such as total carbohydrates, total lipid and total protein and enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AAT), lactate dehydrogenase (LDH) and acid phosphatase (AP) were estimated using the standard protocols.

5.3.4. Serum macromolecular profile

5.3.4.1. Total carbohydrate

To the 0.5 ml of the serum, 2.5ml of the arthrone reagent was added and the reaction mixture was kept in the ice bath for 30 min. It was then boiled in the water bath at 100 °C for 10 min. After cooling at room temperature, the absorbance was recorded at 620 nm using a spectrophotometer (ELICO, India). The distilled water replaced the serum act as a blank (Dubois *et al.,* 1956).

5.3.4.2. Total lipid estimation

0.5 ml of the serum was taken along with 2.0 ml of chloroform. This was centrifuged at 3000 rpm for 30 min. The supernatant was transferred to the test tube and evaporated to dryness. This was stored up to 2 days. To the above, 3 ml of water and equal amount of freshly prepared potassium dichromate solution was added. The intensity of the color developed was read at 640 nm in a spectrophotometer. The OD of the sample was compared with standard graph to estimate lipid (mg/l).

5.3.4.3. Total protein estimation

Total protein estimation was previously mentioned in the chapter 3 (Page no. 17) as described by Lowry *et al.* (1951).

5.3.5. Serum enzyme profile

The enzymes such as AAT, ALT (Bergmeyer and Bernt, 1965), LDH (King, 1965) and AP (Beaufay *et al.,* 1954) were previously mentioned in chapter 4 (Page nos. 46-47) done using the standard protocols.

5.3.6. Edema forming activity

Paw edema was induced by the injection of 20 µl of 1.5 mg/kg of VS of *R. fuscipes* into the subplantar tissue of the Swiss Albino mice (30 days old; 23.10 ± 3.04 gm) right hind paw (Nunes *et al.,* 2007). And for the control, animals received an equal quantity of phosphate buffer (pH 7.2). Paw edema was determined by measuring the paw thickness using vernier calipers. The size of edema was measured for every two h until the edema disappears. Results were expressed in the mean length calculated (in mm). This experiment

was repeated as mentioned above in the same animals after 45 days of the primary treatment to know the persistence of the VS antigens. The paw edema was also measured.

5.3.7. Anti-inflammatory activity

Anaesthetized Swiss Albino mice were used for this experiment. The animal hair on the dorsal skin above the abdomen was removed using a razor, then the skin region was wiped with absolute alcohol and was injected with VS (@ 1.5 mg/kg) of *R. fuscipes*. The animals were allowed to feed continuously. And after the formation of inflammation, the inflammated region was dissected out then the skin was directly transferred to Bovine's fluid, the dissected region was wiped with alcohol and bandaged with antiseptics. And for the control, the animals received an equal quantity of phosphate buffer (pH 7.2) in the same region as in the case of the test animals. Samples were obtained from the control animals too (Dkhil *et al.,* 2010). Now the samples were examined for the histopathological analysis (given in chapter 1 Page No. 7).

5.3.8. Statistical analysis

The results obtained from the biochemical and enzyme studied parameters were compared using student't' test. Error bars were performed for the edema formation activity using the SPSS statistical package Ver 11.5 (SPSS Inc., 2005).

5.4. RESULTS

5.4.1. Reduviid bite in human beings

Reduviid predator biting and its response were analyzed from 29 volunteers, who have been working on reduviid predators from one to 34 years. The personal interview response revealed that reduviid predator did not bite 13 of the researchers. Totally, 17 reduviid predators bite the researchers (Table 5.1). Among them, *Rhynocoris marginatus* bite maximum number of human beings (22.4%) followed by *Rhynocoris fuscipes* (20.41%), *Catamirus brevipennis* and *Acanthaspis siva* (10.20%), *Ectomocoris tibialis* (8.16%), *Rhynocoris longifrons* (6.12%) and *Rhynocoris kumarii* (4.08) (Table 5.2). Most of the reduviid predators bite the human being under the laboratory conditions and they prefer to bite forefinger (10.20%), index finger (16.33%) and thumb (4.08%). The biting causes severe pain which lasts for one to more than 10 min. It further prolongs from 0.001 to (*Endocus*

inoratus) to 120 h (*A. siva*). *R. fuscipes, R. longifrons, A. quinspenosa, Sycanus colaris, Sycanus rectatus, Cornaus siva, E. inoratus* and *Eocoris flageatus* did not cause any impact on any of the researcher. However, reduviid predators commonly cause erythema (12.24%), edema (12.24%), itching (8.16%), reddening (2.04%), headache (2.04%), swelling (2.04%), slight fever (2.04%), induration (2.04%), fluid accumulation (2.04%) and bleeding (2.04%).

5.4.2. Blood collection

The injection of VS doesn't cause mortality and hence there is no mammalian toxicity recorded. The blood samples were collected through retro orbital method (Hoff, 2000) caused no mortality.

5.4.3. Serum macromolecular profile

The total carbohydrate level was significantly high at the 1000 ppm ($t = 30.82$; $p < 0.05$) and in total protein at 800 ppm ($t = 52.26$; $p < 0.05$) high level was recorded. The total lipid was significantly reduced by the VS of *R. fuscipes* (40.93 + 0.28 mg/l) (Table 5.3).

5.4.4. Serum enzyme profile

Serum AAT ($t = 4.0$; $p > 0.05$), ALT ($t = 7.0$; $P < 0.05$), LDH ($t = 19.89$; $p < 0.05$) and AP ($t = 13.50$; $p < 0.05$) in the 400, 600 and 1000 ppm of VS of *R. fuscipes* respectively (Table 5.4).

5.4.5. Edema formation

Edema formation in the mice appeared within 10 min of the injection. Edema formation in VS injected mice was 4.56 times significantly higher than the phosphate buffer injected mice ($df = 6, 8$; $F = 6.712$; $p < 0.05$). The control category mice took 12 h to recover from edema (Figure 5.1a). However, VS injection needs 31 h to recover from edema. These times were prolonged (26 and 35 h for control and experimental category respectively) (Figure 5.1b) when the same mice were injected with VS after 45 days of the first injection (Figure 5.1b).

5.4.6. Inflammatory response

Hemorrhage and acute infiltration of inflammatory cells in the dermis and necrosis of some muscle fibres were observed (Plate 5.1d). They exhibit a slight inflammation in the

upper dermis region. The injected region in the skin of the mice still didn't show any muscular damage, except that affected cells were less esonophillic than the healthy ones. A mixed population of cells had infiltrated in and around the epithelial region and the degeneration of muscle cells was also observed (Plate 5.1c). The treated skin showed an increased cellular infiltration. A group of neutrophill, eosinophills, macrophages and mast cells were observed around the myoaxotic region.

5.5. DISCUSSION

The VS of *R. fuscipes* has shown no mammalian toxicity as evident in the Swiss Albino Mice. Previously, Corzo *et al.* (2001) studied mammalian toxicity of VS of *Periates turpis*, *Agriosphodrus dohrni* and *Isyndus obscurs* against mice. Yet from the oral review of the researchers in reduviid predators, we have evidence that the bite of the reduviid predators causes several impacts such as erythema, edema, itching and very slight fever associated with headache and bleeding. This might be due to the presence of the disulfide bridges in the VS (EDX spectra and FTIR spectroscopy). The dose used for the study of toxicity from *R. fuscipes* was found to be highly toxic to the invertebrate animals like *Spodoptera litura* and *Helicoverpa armigera*, the toxicity of VS was already studied in other reduviids such as *Rhynocoris marginatus* (Kumar, 2011), *Acanthispis pedestris* (Ambrose and Maran, 1999), *Catamirus brevipennis* (Maran and Ambrose, 1999). Inflammation is a protective response which serves to dilute, remove or destroy the inciting agent in animal (Gallin *et al.,* 1988). The acute form is characterized by signs of erytherma, fever, pain and edema (Ho *et al*., 1993). In our studies, paw edema appeared within 10 min of injection (8.33 + 0.88 mm in size), and it recovered after 24 h, 75.99% of edema disappeared and 100% edema disappeared after 31 h. The persistence of VS in the mice immune system have been evoked by the re-injection of VS which evokes the edema very fast (4 min) with the same size as the first injection. However, the edema persists for 35 h. The biochemical parameters of blood sera of mice showed that the level of both total carbohydrate and total proteins were high in the treated animals. The increased levels of blood glucose observed in the sera of VS injected animals in the present study could be due to increased glycogenolysis stimulated by increased catecholamines levels (Sofer and Gueron, 1992; Amaral *et al.,* 1994; Amaral and Rezende, 1997). The decreased level of total lipid was observed. The decreased levels of triglycerides in sera of VS injected animals were also previously reported by Radha *et al.* (1990; 1992). This effect could be due to hepatic tissue destruction, which consequently increased

cholesterol excretion through the bile secretion or due to hepatic failure in biosynthesis of cholesterol (Salman, 2010). The enzyme such as AAT, AST, LDH and AP activities were also high in the treated category of animals and this was shown in the previous studies by Mirakabadi *et al.* (2010). The VS of *R. fuscipes* evoke the immune system and also increase the enzyme level. It has been reported that AST is found in liver and heart at high concentrations; therefore, the increase in its levels is attributed to myocardial infarction and hepatic failure (Omran and Abd-EL-Rahman, 1992; Patel *et al*, 1992; Zare *et al*., 1994; Altinkaynak *et al*., 2002; Bahloul *et al*., 2004; Ribeiro *et al.,* 2010). Therefore, the increase in AAT, ALP and ALT levels may be due to the direct effect of the venom on the liver and the heart (Salman, 2010; Mirakabadi *et al.,* 2010). Mady (2002) have also shown the increased enzyme activity against the venom of *Echis coloratus* in the Ehrlich ascites carcinoma cells. While Salman (2010) has shown the increased enzyme profile and decreased biochemical profile in the serum of the Guinea pigs injected with the venom of *Buthus occitanus*

The inflammatory activity of VS of *R. fuscipes* against the mice induces the inflammation on the skin. Atkinson and Wright (1991) have showed the necrotic actions of spider venom on mice and human beings. The venom of Hymenoptera causes mild local reaction with painful anaphylaxis (Potier *et al*., 2009).

5.6. CONCLUSION

The venomous saliva of *R. fuscipes* had shown no mortality on injection, yet it had increased the blood biochemical parameters such as total carbohydrate and total protein and also the serum enzyme (AAT, AST, LDH and AP). The bite causes erythema, edema and itching associated with bleeding and slight fever in human beings in the occasional bite of the reduviid predators. The persistence of edema lasts 36 h which was 4 h more than the primary. The inflammatory response on the dorsal skin results in hemorrhage and acute infiltration.

SUMMARY AND CONCLUSION

Rhynocoris fuscipes (Fab.) is a reduviid predator found in many agro-ecosystems that predates on different types of insect pest including *Spodoptera litura, Helicoverpa armigera, Mylabris pustulata, Dysdercus cingulatus*. In this study, we described and characterized the mouthpart morphology and the venomous saliva, respectively. Furthermore, the impact of the venomous saliva has been evaluated on *S. litura* and *H. armigera* by using the microinjection bioassay and oral toxicity bioassay, anti-agglutination, anti-spreading and cytolytic effects. Other than the zootoxic nature of the venomous saliva of *R. fuscipes,* the mammalian toxicity has been evaluated on the Swiss Albino mice. The results are summarized here as follows:

1. *Rhynocoris fuscipes* possess three segmented curved rostrum bearing maxillary and mandibular stylets used for rasping, holding, ingesting and flushing out partially digested prey tissues. Stylet movements are supported by the presence of adductor muscles of the head. The salivary gland complex is made up of a pair each of the principal and accessory gland. The histological features of the salivary gland showed the presence of the secretory cells, secretory granules in the principal gland and the secretion is collected at the centralized lumen. The nerve plexus present on the surface of the gland was stimulated towards the secretion.

2. During milking method, three-day starved *R. fuscipes* milked more quantity of VS with 100% survival and venom milking rate. The *Spodoptera litura* fed *R. fuscipes* milked a high quantity of VS. At the same time *S. litura* utilized more quantity of VS for its paralysis.

3. The quantitative enzyme profile of VS and salivary gland complex of *R. fuscipes* showed the presence of hyalurindase, phospholipase A_2, protease and trypsin like enzymes. It was evident through SDS-PAGE, EDX spectra, FTIR, HPLC, GC-MS and MALDI–TOFMS analyses.

4. The insecticidal activity of VS of *R.* fuscipes had shown its effects (mortality) against *S. litura* and *H. armigera.* Furthermore, VS has also altered the larval gut digestive and detoxification enzyme levels. In another study, VS alters the total haemocyte count and haemolymph protein profile of *S. litura* and *H. armigera*. The plasmatocyte and

granulocyte cells respond against the VS- any change in their morphological features lead to death and abnormalities.

5. The VS of *R. fuscipes* showed no mortality in Swiss Albino mice. However VS, increased the serum total carbohydrate, total protein, AAT, AST, LDH and AP. The persistence of edema was 31 and 35 h primary and secondary dose. The anti-inflammatory responses on the dorsal skin results in the tissue degeneration followed by granular infiltration.

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ABSTRACT

Reduviidae is the largest family of predaceous land Hemiptera. They are abundant, cosmopolitan, entomophagous and voracious predators that consume not only more prey but also a wider array of prey. Reduviid predators are found to feed on various pests of agriculturally importance. The augmentative release of the reduviid predator is widely in practice. Reduviids use their venomous saliva (VS) for the paralysis and kill their prey for feeding. In this thesis, I studied the gross morphological features of the head and mouthparts of *Rhynocoris fuscipes* (Fab.) (Hemiptera: Reduviidae). In addition, biological, biochemical and immunomodulatory activities were carried out.

The gross morphology of the feeding canal and the salivary apparatus of *R. fuscipes* were investigated for the first time. *Rhynocoris fuscipes* bears a pair each of compound eyes and a pair of oceli. The curved rostrum is about 2.73 ± 0.01 mm long. The salivary apparatus has a pair of maxillary and mandibular stylets each. The maxillary stylets are shorter than the mandibular stylets that deliver the VS and suck the pre-digested food. The mandibular stylet bears barbs useful for holding and rasping the prey. The salivary gland complex (SGC) consists of a pair of principal gland (PG), which is further bifurcated into anterior lobe (ALPG) and posterior lobe (PLPG). The SGC has also a pair of accessory gland (AG). They are joined at the region called hilus (HI). The histological analysis of the salivary gland showed the presence of more number of secretory granules in the anterior than the posterior lobe. The lumen is the place where the secretions are stored and used when needed. The PG possesses uninucleated, binucleated and polynucleated cells. The AG is of vesicular type cells having a minimum of secretory granules.

Rhynocoris fuscipes was subjected to continuous (1, 2, 3, 4, 5 and 6 days) and discontinuous (1, 3, 5 and 7 days) starvation and the VS was milked by manual method and electrical stimulation method. The three day starved *R. fuscipes* milked more VS (2.61±0.28 mg/100 mg of animal weight) in manual milking during the continuous starvation. In the discontinuous starvation, the mortality was high while the starvation period was prolonged and simultaneously the venom milking rate also decreased. The protein quantity was found to be high in the electrical stimulation method, when the predator was subjected to one day starvation. The utilization of VS was found to be higher by the *Spodoptera litura* third stadium larvae. At the same time, the *S. litura* fed predator secreted more VS than other pests fed predator (*Heicoverpa armigera* and *Corcyra cephalonica*).

The extra oral digestive enzymes such as phospholipase, trypsin-like and protease were high in the VS of *R. fuscipes.* The protein profile results showed that the VS has more number of polypeptide bands (19 numbers) than the SGC (16). However, similar molecular weight bands were observed in both SGC and VS (13 no). The elemental characterization of VS showed the presence of more per cent of potassium (29.23%) followed by sulphur (21.27%) in the energy dispersive X ray (EDX) spectra. The FTIR spectrum shows the presence of amines, amides, which have proved the enzymatic and proteinious nature of VS and also the presence of aromatics (C-C stretching) and carboxylic (O-H bending) nature of the VS. In both HPLC and GC-MS analyses showed the presence of phospholipase, lysolecithin and hyalruindase. About 69 molecular mass peptides were recorded from the MALDI-TOFMS analysis of the VS. Among them, one major intensity at 10039 mAU (3802 kDa -RFIT1) and two minor intensity 2836 mAU (3423 kDa - RFIT2) and 2608 mAU (2358 kDa - RFIT3) were identified as predominant polypeptides.

The *R. fuscipes* VS impact was tested against *Spodoptera litura* and *Helicoverpa armigera* through the microinjection and oral toxicity bioassays. The results showed that the administration of VS causes mortality to *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 for microinjection and oral toxicity bioassay respectively) was less than the *H. armigera* (913.00 and 928.12 ppm for microinjection and oral toxicity bioassay respectively) at 96 hrs observations. Furthermore, VS changes the digestive and detoxification enzymes level of *S. litura* and *H. armigera*. 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 shows that the VS altered the protein profile of *S. litura* and *H. armigera*. The VS shows anti-spreading and anti–agglutination behaviour under *in* vitro condition. In addition, VS causes morphological changes in haemocyte which leads to cell death.

About 29 reduviid biologists responded that reduviid predator bites, while we disturbed them during feeding and mating. It caused pain, erythema, induration and edema. To confirm these oral responses, we studied the impact of the VS of *R. fuscipes* on Swiss Albino Mice. Results showed that total carbohydrate, total proteins and total lipids, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and acid phosphatase

activities were altered. While the venomous saliva evoked the paw edema it would last for a period of 31 h and 35 h in the primary and secondary dose, respectively. Local inflammation was recorded on the dorsal skin when VS injected. However, VS doesn't cause mortality in the Swiss Albino mice.

In conclusion, *Rhynocoris fuscipes* mouthpart morphology revealed the predatory nature of this reduviid for it piercing and sucking the prey tissues. Venomous saliva can be collected from 3-days continuously starved predator fed with *S. litura*. The biochemical characterization of VS showed the presence of hydrolytic enzymes, digestive enzymes, peptides, protein, aromatic compounds and inorganic elements. The VS has insecticidal, cytolytic, anti–agglutination, anti–spreading activity against *S. litura* and *H. armigera.* It also causes swelling, irritation and inflammation at the point of biting in human being and Swiss Albino mice. However, it should be confirmed by specific bioassays.