STUDIES ON THE ROLE OF HORMONES IN THE REGULATION OF PROTEIN METABOLISM IN THE MANGO LEAF WEBBER *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)

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By

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DECLARATION

I, **Remya S**., do hereby declare that the thesis entitled **"Studies on the role of hormones in the regulation of protein metabolism in the mango leaf webber *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae)"** is an authentic record of the research work carried out by me in the Department of Zoology, University of Calicut, under the guidance of **Dr. M. Gokuldas.** I further declare that no part of this thesis has been submitted previously for any other Degree/Diploma of this or any other university.

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CHAPTER I
INTRODUCTION

Insects, the hexapod arthropoda, is the most diverse group of animals on the planet. It includes more than a million described species and represent more than half of all known living organisms. They play a remarkable role in the world of living things. Many species are extremely valuable to man. An endless variety of structural and physiological peculiarities and adaptations to different living conditions are found among these animals. For these reasons, insects provide an ideal model for conducting studies on almost all the problems in physiology. They have shorter life spans and require less time and money for maintenance.

Investigations in entomology become important primarily because many species of insects are pests of agriculture, domestic animals and vectors of many human and animal diseases. Pest insects are those that damage or kill agricultural crops, ornamental plants or native plants. Some insects are beneficial at one stage of life and a pest at another stage, for example many lepidopterans may be serious pests as larvae, while they may be pollinators in adulthood. There are several species of productive insects such as honey bee (bees wax and honey), silk moths (silk) and lac insect (lac). Various aspects of insect biology such as their ecology, behaviour, physiology, biochemistry, and endocrinology thus have been the focus of many investigators.

The major metabolic pathways in insects involve the degradation or biosynthesis of proteins, carbohydrates, lipids and several other secondary metabolites specific to individual species are also known. The regulatory mechanisms of these pathways are also similar to those in vertebrates and other higher organisms. Fat body which is the major organ found in an insect's body cavity is functionally homologous to the vertebrate liver and adipose tissue combined. Almost all the metabolic activities take place in the fat body. Besides, fat body is also the main storage organ of an insect. It stores lipids in the form of triacylglycerols, carbohydrates as glycogen and proteins as glycoprotein and lipoprotein granules. The stored lipids and proteins are released from the fat body at times of demand such as locomotion, reproduction and such other activities. This mobilization is under the control of hormones secreted from the brain and other endocrine glands.

Various stages in the metabolism and metamorphosis of insects are regulated by different hormones secreted by brain and its associated glands such as the corpora allata (CA) and the corpora cardiac (CC) and also other secretory glands. Insect hormones are of three chemical types. They are: lipid hormones such as juvenile hormone and ecdysone secreted into the hemolymph by corpora allata (CA) and prothoracic glands (PTG) respectively; peptide hormones which are synthesized by the neurosecretory cells and secreted into the hemolymph via neurohaemal organs and amines such as octopamine and 5HT which are released from nerve endings.

Ecdysteroids are molting hormones in both insects (Sehnal, 1989) and crustaceans (Chang and O'Connor, 1988). They are polyhydroxyl sterol compounds that play an important role during insect development, such as induction of ecdysis, termination of diapause and regulation of reproduction and embryonic development (Wang and Gong, 1997; Haegele and Wang, 2004; Bownes, 1989). Now many types of ecdysteroids have been found in insects, for example, α-ecdysone (E), 20-hydroxyecdysone (20E), 26-hydroxyecdysone (26E), 2-deoxyecdysone (2dE), but the main type of ecdysteroids detected in most insects is E or 20E (Tawfik *et al*., 1999; Cao and Jiang, 2002).

Juvenile hormones (JHs) are members of a family of acyclic sesquiterpenoids unique to insects. They are major effector hormones in insects, whose primary role is the regulation of metamorphosis and reproduction. In some species, JHs are also associated with the regulation of polyphenisms including color morphs and caste differentiation of social insects. JH biosynthesis by the *corpora allata* (CA) is tightly regulated to ensure proper timing of developmental stages and gonadotropic cycles (Goodman and Cusson, 2012). JH is produced through a biosynthetic pathway related to that of cholesterol. Unlike the cholesterol pathway, the precursor, farnesyl pyrophosphate, is converted to farnesol, not squalene, resulting instead in the synthesis of sesquiterpenoids. In general, a high JH titre maintains a larval form, whereas the absence of JH allows for imaginal

differentiation (Kikukawa and Tobe, 1986 a, b). Early in larval stage, JH is necessary to trigger release of prothoracicotropic hormone, and thereby ecdysteroid secretion required for ecdysis. However, in final instars, ecdysteroid titre becomes elevated only after JH release declines, suggesting continuously high JH titre may also block ecdysteroid release (or synthesis) (Kikukawa and Tobe, 1986 a).

Peptide hormones are produced by neurosecretory cells, the glandular cells associated with the neuroendocrine system. These peptides may either get released in to the hemolymph as circulating neurohormones or at specific sites as neurotransmitters. Peptide hormones are involved in the control of a wide range of physiological, biochemical and developmental functions, including cuticle tanning (Fraenkal *et al*., 1966), carbohydrate metabolism (Steele, 1961; Friedman, 1967; Goldsworthy, 1969), muscle contraction (Davey, 1964; Mordue and Goldsworthy, 1969) and lipid metabolism (Mayer and Candy, 1969; Beenakkers, 1969; Gokuldas *et al*., 1988). Peptide hormones include diuretic factors, chloride transport stimulating hormone, proctolin, hyperglycaemic factor, heart-accelerating factors, eclosion factors and adipokinetic factors.

Besides the above-mentioned hormones, various biogenic amines such as octopamine, dopamine, 5-hydroxy tryptamine and noradrenaline have been reported in the hemolymph and nervous system of insects. These amines

function either as neurotransmitters, neuromodulators or as neurohormones (Evans, 1980).

Insects require constant supply of energy for their activities, such as reproduction, flight and locomotion. Flight requires rapid mobilization of energy sources, transport and transformation of food energy in to ATP. The disaccharide trehalose and the polysaccharide glycogen are the most common carbohydrate stored reserves in insects. The hemolymph, fat body and gut tissues are the major sources of stored carbohydrate, but small amount of trehalose and glycogen occur in muscle. When energy is needed, trehalose is the first metabolite used. Glycogen, is a second form of storage energy. Flight muscles of insects contain glycogen in small quantities. Most of the glycogen occurs in the fat body, some glycogen is stored in gut epithelial cells, and to a slight extent in muscle cells.

Some insects such as lepidopteran adults, Orthoptera etc. use fatty acid as a fuel for flight. Fatty acids are stored in the fat body cells as triacylglyceroles and they are released from the fat body cells and transported into the muscle before utilization. This release of lipids from fat body cells is under the control of a peptide hormone the adipokinetic hormone (AKH), secreted from the corpora cardiaca. Cyclic AMP is involved in this process. Activation of a lipase causes the release of diacylglycerols from fat body cells.

Ecdysteroids as well as juvenile hormones, the hormones that regulate the overall development and metamorphosis, are collectively referred to as insect growth regulators (IGRs). They decide the time and type of growth, moulting and development in insects. The titre of these hormones are counteracting. The juvenile hormone allows the insect to grow and develop whereas the ecdysone give signal to moult to the next stage. At the same time they are involved in many ways in the protein metabolism and egg maturation or vitellogenesis. Although there are marked differences between different insect groups on the hormone titres in the tissue, mainly the hemolymph, the general pattern of titre change during growth and reproduction follow almost the same pattern. Ecdysone also has marked influence on yolk protein synthesis in the fat body (Bownes, 1989). There has been several reports of switching on and off a gene responsible for silk biosynthesis by the circulating hormones. Silk synthesis is very crucial for several insects that undergo pupation (holometabolous insects). The same hormone keeps these glands in regressed state during larval moults. Steroids also affect embryogenesis and have indirect effects on juvenile hormone synthesis.

Juvenile hormones are involved in many physiological and behavioural processes such as caste determination, foraging behaviour, diapause, vitellogenesis besides its major role in metamorphosis and related synthesis of metabolites. Generally, this hormone maintains the larval form and its

absence initiates moulting. It also promote protein (vitellogenin) uptake by ovaries.

The ecdysone and juvenile hormone are considered as prospective candidates for developing mimics for the control of insect pests. Any changes in the homeostasis of these hormones with exogenous sources of hormones or with synthetic analogues would result in the disruption of the normal course of development and reproduction of the target insect (Hoffmann and Lorenz, 1998). In *M. Sexta* larvae, administration of ecdysteroid analogue RH-5849 showed an increase in the level of this compound in the hemolymph 6 h after administration and a slight decrease thereafter, which was stable over next 36 h. RH-5849 was found to have powerful ecdysonergic activity by initiating premature lethal moults (Wing *et al.*, 1988). In the spruce budworm *Choristoneura fumiferana* and *M. sexta*, a failure in endocuticle production was reported for the RH-5992 treated larvae (Retnakaran and Oberlander, 1993). The strategy thus involves the use of steroid analogues to tamper with the hormonal balance in the insect thereby interrupting normal moulting and growth and associated metabolic pathways leading ultimately to the death of the insect. Several steroidal and non-steroidal analogues have been attempted successfully for the control of pest insects (Chandler *et al.*, 1992; Heller *et al.*, 1992).

The major objectives of the present study are:

- Detect and identify the different proteins in the fat body, hemolymph, silk glands and ovary of the insect *Orthaga exvinacea,* the mango leaf webber.

- Correlate the presence and the quantity of proteins with the stages of development of insect.

- Correlate the protein profiles with the titre of growth regulating hormones, i.e., juvenile hormone and ecdysteroids

- Structural studies of ecdysteroid present in the hemolymph of insect using FTIR.

- Explore the possibilities of using the information on the role of hormones in the protein synthesis, release, transport and sequestration into oocytes etc. for designing molecular biological techniques in insect pest management.

CHAPTER II
REVIEW OF LITERATURE

Insects, the group of animals with the highest degree morphological and physiological adaptations have essentially all the metabolic pathways and the regulatory mechanisms similar to those in higher animals such as vertebrates. Studies on insect biology, behaviour and biochemistry has therefore become very intense as a model organism. This has been useful in two ways; one to set a thorough knowledge of insects helping to formulate strategies in pest management and two to get information on animal physiology, biochemistry etc. to design high end animal experiments.

Insect pests consists of less than one per cent of all insect species. Pest insects have adverse and damaging impacts on agricultural production and market access, the natural environment and our lifestyle. They feed on, compete for food with, or transmit diseases to humans and livestock. The classification of an insect as a pest is a subjective one, based on its potential damage to human needs and/or natural habitats and ecosystem. Pest insects cause problems by damaging crops and food products, parasitizing livestock or being nuisance and health hazard to humans.

Pest insect can be controlled by using various methods such as mechanical, chemical biological and physical, but in all these methods, both the pest and the hosts get affected. In the present time insect hormones

collectively referred to as insect growth regulators (IGRs) are considered as prospective candidates for developing mimics for the control of insect pests. Any changes in the homeostasis of these hormones using exogenous sources of hormones or with synthetic analogues would result in the disruption or abnormal course of development and reproduction of the target insect (Hoffmann and Lorenz, 1998). So development of IGR based methods instead of conventional methods is very important in the field of insect pest control.

Insect fat body and metabolism

Insect fat body is the centre of all metabolic activities comparable to vertebrate liver. It also stores large quantities of proteins, lipids and glycogen, just like the liver of vertebrates. The structure of this loosely organized tissue changes cyclically in relation to nutrition, metabolic demand, stress, metamorphosis and reproduction (Dean *et al*., 1985). Fat body reserves are utilized for tissue reorganization during pupation in holometabolous insects, egg production and occasionally for other functions such as sustained flight and diapause. The activity pattern of fat body cells depends on nutritional, hormonal and developmental signals. The biochemical changes taking place in the fat body may vary according to the specific stages of the insects' development.

Fat body lies freely bathed in the hemolymph and there is a direct exchange of materials between them. They exchange proteins, lipids, carbohydrates and other materials for synthetic and degradative purposes. A functional shift of the fat body takes place at the end of the last larval stage from the biosynthetic organ to storage organ and this has been supported by electron microscopic studies (Dean *et al*.,1985). Towards the end of larval period in holometabolous insects and maturity in hemimetabolous insects, fat body gets crammed with reserve materials, mainly protein granules and lipid droplets.

Fat body and protein synthesis

Insect fat body produces many unique and physiologically significant proteins such as vitellogenins for oocyte maturation, stage specific amino acids, storage proteins such as calliphorin, drosophillin, manducin, diapause proteins, lipid binding proteins, JH binding proteins and even haemoglobins (Keely, 1985; Levenbook, 1985). In insects as in other animals, protein synthesis is a major biochemical event resulting from cellular activation. In recent years, study of insect systems has begun to make significant contributions to a number of aspects of protein synthesis, which remain to be totally defined. Continued contributions are required in areas such as mRNA and tRNA structure and population dynamics, ribosomal membrane interactions and ribosome assembly.

In insect protein synthesis, there are several unique features, which need special mentioning. The metabolic status and cellular conditions in insects of each species show remarkable specialization. The activation process leading to initiation as well as production of proteins also appears to be highly specific and these are all hormone mediated. Tissue and stage specific patterns of protein synthesis are correlated with changes in gene activity at different phases of the insect life cycle. Various intrinsic and extrinsic factors such as hormones, feeding, copulation, photoperiod, temperature and humidity, control the cellular activation and deactivation of protein synthesis.

The synthesis and release of specific proteins by the fat body of the growing larvae and the removal and storage of these proteins in the same tissue during later development are apparently under hormonal control. Hormonal control of protein synthesis had been studied in a large number of insects (Chippendale and Beck, 1967; Luscher, 1968; Engelmann, 1969; 1971; Scheurer, 1969; Wilkens, 1969). Cautery of the cerebral neurosecretory cells of the desert locust resulted in a low incorporation rate of ^{14}C-glycine in to the fat body protein (Hill, 1965). Similar observations were made on the influence of neurosecretory cells on protein synthesis in the American cockroach (Mills *et al.*, 1966). Studies by Raghavan and Muraleedharan (1985) on *Dysdercus cingulatus* have shown that hormonal principles from pars intercerebralis neurosecretory cells (PINSC) and corpora

allata have stimulatory effects on the synthesis of proteins in the fat body. Carlisle and Loughton (1979, 1986) reported the inhibitory activity of locust adipokinetic hormone on protein synthesis from labelled leucine, when the hormone was used at concentrations lower than those used for studying lipid release. Keely (1985) has pointed out that the ovaries may act as sinks to absorb vitellogenin and release a feedback inhibition on its further synthesis or release by the fat body. The levels of circulating vitellogenin inhibit the release of further vitellogenin from the fat body of *Leucophaea maderae* but do not suppress its continued synthesis.

Hill (1965) has noted that fat body protein synthesis in *Schistocerca*, low before oocyte production, increases during yolk deposition and drop again at the end of oocyte development. In *Tenebrio molitor*, fat body synthesises proteins in a cyclic pattern, which may be related to ovarian development (Pemrick and Butz, 1970).

Insect proteins and lipoproteins

Proteins are macromolecules made up of amino acid residues. It performs a variety of functions within living organisms, including DNA replication, catalyzing metabolic reactions, responding to stimuli, transporting molecule from one location to another. Proteins differ from one another primarily in their sequence of amino acid residues. A protein contains at least one long polypeptide. Short polypeptide chain made up of linear amino acid

chains. The individual amino acids are bonded together by means of peptide bonds. Sequence of amino acid in the protein is defined by the sequence of gene, which is encoded in the genetic code. Proteins are essential part of organisms and participate in virtually every process within the cell. Many proteins are enzymes that catalyse biological reactions, and are vital to metabolism. Proteins also have structural and mechanical functions, such as actin and myosin in the muscle and the proteins in the cytoskeleton. Some other proteins are important in cell signalling, immune responses, cell adhesion, cell cycle etc. Structural proteins confer stiffness and rigidity to biological components. Most structural proteins are fibrous proteins; for example collagen and elastin in connective tissues, such as cartilage, and keratin found in the nail, hair, hooves and some animal cells etc.

Storage proteins

The accumulation and utilization of storage proteins are prominent events linked to the metamorphosis of holometabolous insects. Storage proteins are synthesized in fat body, secreted into the larval hemolymph and taken up by fat body shortly before pupation. Within the pupal fat body, proteins are initially stored in the protein granules, and later under gone proteolytic cleavage to supply amino acid residues necessary for the completion of adult development. Most of the storage proteins, but not

all,belongs to a super family of hexameric larval serum proteins that are evolutionarily related to hemocyanin (Haunerland, 1996).

Arthropod and mollusc hemocyanin do not show any similarities in structure, copper binding site etc. Arthropod hemocyanins consists of hexamers or multihexamers of polypeptide chains of approximately 75 kD and molluscs hemocyanins consists of long polypeptide chains with seven or eight functional units of approximately 50 kD per chain (Muller *et al*., 1988). Proteins exhibiting an unambiguous sequence similarity within arthropod hemocyanins have not yet been found outside this phylum. Sequence of storage proteins occurring in the hemolymph of insects show a significant homology with that of arthropod hemocyanins (Sakurai *et al*., 1988; Fujii *et al*., 1989; Willott *et al*., 1989; Jones *et al*., 1990, 1993; Corpuz *et al*., 1991; Naumann and Scheller, 1991). These proteins are synthesized and secreted by the fat body during larval–to–pupal molt and reaches a maximum concentration in hemolymph just before metamorphosis (Telfer and Kunkel, 1991). In case of holometabolous insects, they are partially recaptured by the fat body during larval-to-pupal molt and disappear during adult development. In adults, these storage proteins are used for synthesis of new proteins, but they also may be incorporated into the cuticle as intact protein.

Insect hemolymph

Hemolymph is the circulating fluid in insects which is clear, often tinged with green or yellow pigment and is the medium through which all the chemical exchanges between the organs are effected, messages of the hormones conveyed, nutrients absorbed from the gut are received and waste products sent to the exterior. Hemolymph fills the body cavity, or haemocoel. It is separated from the cellular tissue by only a thin permeable connective tissue membrane and is maintained in circulation by a tubular, dorsal heart which is sometimes assisted by accessory organs in various parts of the body (Wyatt, 1961; Chapman, 1969; Jeuniaux, 1971; Florkin and Jeuniaux, 1974). Hemolymph comprises about 10-40% of the total body volume. Insect hemolymph contains a large number of inorganic and organic constituents. Inorganic materials include ions such as K^+, Na^+, Ca^{2+}, Cl_2^-, PO_4^{2-}etc. and the main organic constituents include carbohydrates, lipids, proteins, amino acids, vitamins, pigments etc. Of these various components proteins usually make up nearly 50-80%. Proteins comparable to those in mammalian blood are usually present in insect hemolymph.

Hemolymph proteins

Hemolymph proteins are generally referred to as interstitial proteins. It has been studied by a large number of workers for taxonomic purposes as well as for determining the origin and function of several protein components.

The protein components of hemolymph comprises a functionally and structurally heterogeneous array of macromolecules such as arylphorins, clotting proteins, storage proteins, enzymes, immunoproteins, lysozymes, tanning proteins, hormone binding proteins etc. (Wyatt and Pan, 1978; Riddiford and Law, 1983). The protein pattern of hemolymph is influenced by the complex relations of metabolism and the synthesis and uptake of proteins by the body tissue (Laufer, 1960 a; Salkeld, 1969; Doira and Kawaguchi, 1972).

Fat body is considered as the major source of hemolymph proteins. This was demonstrated in *Bombyx mori* (Shigematsu, 1958) and *C. erythrocephala* (Price and Bosman, 1966). A dynamic relationship exists between hemolymph and fat body proteins by the fat body cells have been observed in a number of insects (Tobe and Loughton, 1969; Chippendale, 1970). Hemolymph proteins are found to be selectively sequestered by the fat body at the time of pupation. The fat body of *Calpodes ethlius* takes up hemolymph proteins at the time of pupation and stores them as protein storage granules (Locke and Collins, 1968). Hemolymph proteins may be used directly as a source of material for the synthesis of protein by developing adult tissues (Heller, 1932).

Low molecular weight JH-specific binding proteins have been identified in larval hemolymph of Indian mealmoth, *Plodia interpunctella*

(Ferkovich *et al*., 1975). Synthesis of JH binding protein has been demonstrated in isolated *M. sexta* fat body (Kramer *et al*., 1976). Hemolymph may contain a variety of enzymes as an important part of the blood proteins (Laufer, 1960 b; Wyatt, 1961; Jeuniaux, 1971; Florkin and Jeuniaux, 1974; Agosin, 1978; Wyatt and Pan, 1978). It has been suggested that most of the enzymes present in the hemolymph of insects result due to leakage from tissues. O-Diphenol oxidases are found universally in the hemolymph of insects and are responsible for the darkening of the blood when it is exposed to air.

Trehalase forms a permanent constituent of the protein pool of the hemolymph (Wyatt, 1961). Other hemolymph proteins of the insects include those which are apparently involved in clotting and defence, frost protection, haemoglobins, and chromoproteins (Wyatt and Pan, 1978; Crossley, 1979; Wyatt, 1980; Anderson, 1985; Sekeris and Fragoulis, 1985). Several of the hemolymph proteins are conjugated molecules. Glycoproteins, lipoproteins and ribonucleoproteins have also been described in the hemolymph of several insects. Glycoproteins serve as the carrier of carbohydrates required during vitellogenesis (Siakotos, 1960) and lipoproteins have been implicated in the transport of lipids from fat body to other tissues (Chino and Gilbert, 1965).

Storage proteins are an important class of proteins present in the hemolymph. The best known example of storage proteins is arylphorins

(Telfer *et al*., 1983) which possess a high content of aminoacids. There are other storage proteins with a less conspicuous amino acid composition and immunologically distinct from the arylphorins, but with the same shape and molecular dimensions (Tojo *et al*., 1980; Ryan *et al*., 1985). *Chironomus* haemoglobins are storage proteins having a respiratory function too (Thomson, 1975).

Developmental changes

During development of insects, many qualitative and quantitative changes occur in the protein content (Chen, 1966; Agosin, 1978; Wyatt, 1980). The hemolymph protein levels generally increase during each instar, but decline during moulting. In *M. domestica* protein synthesis begins at the cleavage stage and increases rapidly at the time of blastoderm formation (Chen, 1971; 1978). The protein concentration in the hemolymph increases rapidly during the later half of larval development, falls at metamorphosis and declines to very low levels in early adult life. Qualitative and quantitative changes in the protein pattern in *Pieris brassicae* were reported during development (Van der Geest and Borgsteede, 1969). Certain protein bands may be absent temporarily due to the very low concentration. The intensity of a number of protein bands increases rapidly towards the end of the larval life. Hemolymph protein pattern may also change during molting process, (Steinhauer and Stephen, 1959; Fox and Mills, 1969; Duhamel and Kunkel,

1978) and diapause (Chippendale and Beck, 1966). Hemolymph protein concentration fluctuates during metamorphosis due to the direct uptake of some of these proteins into various tissues. Studies on the amount and distribution of the nutrient reserves in the larvae, during spinning revealed that hemolymph proteins must be the main source for cocoon protein production (Terra and Bianchi de, 1975).

The hemolymph protein pattern of male insect is more or less constant. Nevertheless, it shows variations in relation to the physiological state of the individuals. The hemolymph of females on the other hand, may contain more proteins in the vitellogenic and previtellogenic stages (Whitemore and Gilbert, 1974), and in the adult there may be fluctuations correlated with reproductive cycles. The female insect utilizes the available hemolymph proteins for vitellogenesis (Engelmann, 1970). Quantitative differences in the hemolymph protein pattern existing between male and female insects, as well as hemolymph protein pattern of female during oocyte development have been confirmed through observation on *Gesonula punctifrons* (Engelmann and Penny, 1966; Thomas and Nation, 1966).

The presence of female specific larval hemolymph protein (FSP) in various insects has been clearly demonstrated and the concerned FSP genes are predominantly expressed in female larvae during the last instar (Ryan *et al.,* 1985). Electrophoretic and immunological data indicate that there are two

major and three minor male specific serum proteins in *Ceratitis capitata* adults (Katsoris *et al*., 1990). A maximum of 12 and 10 protein bands was observed in female and male hemolymph samples by polyacrylamide gel electrophoresis in the desert locust, *S. gregaria* (Kulkarni and Mehrotra, 1970).

Species specific variation in proteins present in insects has been useful as a parameter in understanding phylogenetic relationships (Paranjape *et al*., 1987). Stage specific pattern of protein synthesis which has been noticed in a number of holometabolous insects, is correlated with changes in gene activity at different phases of insect life cycle (Chen and Levenbook, 1966; Rockstein, 1978). Absence of such stage specific protein during metamorphosis is characteristic of hemimetabolous insects (Terendo and Feir, 1967; Wyatt, 1968; Gupta, 1977). The hemolymph protein concentration is under a direct neurosecretory control. Slama (1964) has reported that in *Pyrrhocoris apterus,* the corpora allata regulate the utilization of protein by ovaries and not the synthesis of proteins. Corpora allata have been shown to be responsible for the uptake of hemolymph proteins into the oocytes (Pfeiffer, 1945; Hill, 1962). In the desert locust *Schistocerca*, a high hemolymph protein concentration was found to be correlated with an active neurosecretory system and developing ovaries (Hill, 1962). The protein content of the hemolymph has been shown to decrease after allatectomy in *Leucophaea maderae* (Engelmann and Penny, 1966) and *Rhodnius prolixus*

(Pratt and Davey, 1972). While its level increased in *S. gregaria* (Highnam *et al*., 1963), *L. Migratoria migratorioides* (Strong, 1967) and *Leptinotarsa decemlineata* (DeLoof and DeWilde, 1970). Qualitative changes in the concentration of hemolymph protein of *Plodia* have been partially correlated with temporal increase in the endogenous titre of ecdysone (Pentz and Kling, 1972).

Vitellogenin

Insect vitellogenins are synthesized in the fat body and released into the hemolymph, usually only in the adult, close to the time of yolk deposition during oogenesis for utilization in the oocytes. Although vitellogenesis is regarded as specific to females, Telfer (1954) reported traces of vitellogenins in the hemolymph of male cecropia pupae. It has been found that copulation leads to the activation of corpora allata, which inturn induces the synthesis and release of these specific proteins from the fat body (Chen, 1985). In *Melanoplus sanguinipes* vitellogenic proteins are found to be present in the fat body, which supports the view that most ovarian proteins are synthesized in the fatbody (Elliott and Gillott, 1979). In *Hyalophora*, the vitellogenin is synthesized at first in the pharate pupa shortly after the coccon is spun (Pan *et al.*, 1969). In mosquitoes (Hagedorn and Judson, 1972), locusts (Hartmann *et al*., 1972) and *Leucophaea* (Brookes, 1976; Koeppe and Ofengand, 1976), it has been found that vitellogenin comprised the bulk of the proteins secreted

by fat body during ovarian development. However, Engelmann (1969, 1970) has reported an increased rate of synthesis of proteins other than vitellogenin during normal maturation of the oocytes or after JH treatment. It has been reported that three hormonal factors, juvenile hormone, adipokinetic hormone and a brain factor regulate the biosynthesis of vitellogenin in *Locusta migratoria* (Glinka *et al*., 1995). In adult females of *Locusta migratoria*, synthesis and secretion of protein by the fat body increases 3-4 fold in the previtellogenic stages, a further increase of nearly 5-fold in the post-vitellogenic stages and the subsequent decrease to the previtellogenic level at the end of the cycle have been correlated with the production of vitellogenin (Gellissen and Wyatt, 1981).

Insect hormones

Various biochemical and physiological process in insects are under the regulation of hormones secreted by endocrine glands. Endocrine structures in insects include neurosecretory cells in the brain and associated glands and prothoracic glands. Insect hormones are of three chemical types. They are: lipid hormones such as juvenile hormone and ecdysone secreted into the hemolymph by corpora allata (CA) and prothoracic glands (PTG) respectively. Peptide hormones which are synthesized by the neurosecretory cells and secreted into hemolymph via neurohaemal organs and amines such as octopamine, which are released from nerve endings.

Lipid hormones

The early work of Plagge (1938) and Fukuda (1940) led to the identification of thoracic centre secreting the moulting hormone, ecdysone which was later identified as the prothoracic glands, that get stimulated by the "brain hormone" or the prothoracicotropic hormone (PTTH). Ecdysone is also responsible for the regulation of growth and development. The hormone is released as α-ecdysone from the prothoracic gland. Ecdysone controls specific vitellogenin synthesis as reported in *Musca domestica* (Adams *et al*., 1985), *Lucilia caesar*, *Calliphora erythrocephala* (deLoof, 1982). Ecdysteroids are also reported to have some effects on lipid flux (Arnold and Regnier, 1975).

Pfeiffer (1945) noticed that the corpora allata (CA) in female grasshoppers were essential for egg development and maturation. The role of CA as a source of hormones regulating lipid metabolism was confirmed in *Periplaneta americana* (Bodenstein, 1953), *Schistocerca gregaria* (Odhiambo, 1966; Walker and Bailey, 1971) and *Locusta migratoria* (Strong, 1968). Implantation of active CA in to intact female *S. gregaria* one day after the final moult caused a reduction in fat body lipid to one third after thirty days (Hill and Izatt, 1974). Steele (1976) proposed a relationship of lipid accumulation with the synthesis proteins and lipid. In the absence of JH, the apoprotein moieties that normally facilitate the release and transport of lipid

from the fat body are not produced which results in accumulation of lipids. It also seems likely that availability of additional non-lipid precursors in allatectomised insects contributes to the increased levels of fat body lipids. It is also probable that the absence juvenile hormone from corpora allata inhibits vitellogenesis and consequent lipid transfer from the fat body to ovaries in association with the diacylglycerol carrying lipoprotein, lipophorin (Chino *et al*., 1977, 1981). The metabolic switch from the synthesis of lipid for migratory purposes to the synthesis of lipovitellins for oogenesis appears to be triggered by juvenile hormone (Hill and Izatt, 1974).

Peptide hormone

Peptide hormones are produced by neurosecretory cells the grandular cells associated with the neuroendocrine system. These neurosecretory cells are situated in the brain, corpora cardiaca and the medial nervous system (Goldsworthy and Modure, 1974). These peptides may either get released in to the hemolymph as circulating neurohormones or at specific sites as neurotransmitters. Peptide hormones are involved in the control of wide range of physiological, biochemical and developmental functions, including cuticle tanning (Fraenkal *et al*., 1966), carbohydrate metabolism (Steele, 1961; Friedman, 1967; Goldsworthy, 1969), muscle contraction (Davey, 1964; Mordue and Goldsworthy, 1969) and lipid metabolism (Mayer and

Candy, 1969; Beenakkers, 1969; Rasheed and Gokuldas, 2002; Ajaykumar and Gokuldas, 2011).

Peptide hormones include diuretic factors, chloride transport stimulating hormone, proctolin, hyperglycaemic factor, heart-accelerating factors, eclosion factors and adipokinetic factors. Proctolin was first isolated from the cockroach, *P. americana* (Brown and Starratt, 1975) and identified as a pentapeptide (Arg-Tyr-Lue-Pro-Thr) (Starratt and Brown, 1975) is widely distributed among insects (Bishop *et al*., 1981). It is an excitatory neuromuscular transmitter in the hindgut of the cockroach also exhibiting potent activity in muscle preparation like hindgut, heart extensor tibiae and oviduct (Cook and Holman, 1979; Lange *et al*., 1987). Hyperglycaemic hormone was the first reported peptide hormone from the corpora cardiac of the cockroach *P. americana* (Steele, 1961). It is comparable to glucagon of vertebrates. The hyperglycemic or hypertrehalosaemic hormone causes the activation of fat body glycogen phosphorylase (Steele, 1963). Holwerda *et al*. (1977) found that CC extract from cockroaches elicited hyperglycaemia in cockroaches but not in locusts. In locusts the extract exhibited hyperlipaemia. There is also evidence for the presence of hypoglycemic factor released from the neurosecretory cells of the brain in insects such as honeybee (Dixit and Patel, 1964) and blowfly (Norman, 1975). Insulin like material is identified from the extract of the CC-CA complex from the tobacco hornworm, *Manduca sexta* (Tager *et al.,* 1976).

Adipokinetic hormone is a major group of neuropeptides which regulate physiological homeostasis. The first member were discovered in the 1960s in the American cockroach and in locusts where they are involved in the control of carbohydrate and lipid breakdown. It is now known that these peptides are members of a large family of structurally related peptides which are found in crustaceans and insects (Gaede, 1996). Such peptides become known under acronym AKH/RPCH family peptides on the basis of the first members of this family to be fully characterised, viz., an adipokinetic hormone from locusts (Stone *et al*., 1976), now called Locmi-AKH-I according to the nomenclature proposed by Raina and Gaede (1988) and a chromatotropic peptide from prawns (Fernlund and Josefsson, 1972), the red pigment concentrating hormone (code name: Panbo-RPCH). These peptides are present in the neurosecretory X-organ/sinus gland complex in the eyestalks of crustaceans and in the intrinsic neurosecretory cells of the corpora cardiaca of insects. Both of these structures are neurohaemal organs, analogous to the vertebrate hypothalamo/hypophyseal system and it can thus be inferred that the peptides can be released from the neurohaemal organs into the circulation and thus act as true hormones. However, release has been demonstrated only in a few cases, for example, during flight in locusts, blowflies and *Manduca sexta* (Gaede, 1992). Adipokinetic hormone (AKH), located in the corpora cardiaca, mobilises lipids from the fat body and facilitates its transport and oxidation in flight muscles, is reported from desert

locust *S. gregaria* (Mayer and Candy, 1969) and *L. migratoria* (Beenakkers,1969). Besides the above- mentioned hormones, various biogenic amines such as octopamine, dopamine, 5-hydroxy tryptamine and noradrenaline have been reported in the hemolymph and nervous system of insects. These amines function either as neurotransmitters, neuromodulators or as neurohormones (Evans, 1980). Octopamine, the mono-phenolic amine, appears to be the most widely distributed and studied amine (Robortson and Jourio, 1976). Other amines include catecholamines and indole alkamines.

Octopamine has been shown to affect a variety of functions in insects. In the fat body of cockroach, Downer (1979) reported rapid elevation of hemolymph trehalose levels due to octopamine. Octopamine was found to stimulate an elevation of cyclic AMP levels in isolated fat body of *P. americana* (Gole and Downer, 1979; Downer, 1980) and *L. migratoria* (Orchard *et al*., 1982). Octopamine-mediated regulation of hypertrehalosaemic factor release from cockroach CC resembles the octopamine mediated control of AKH release from locust CC (Orchard and Loughton, 1981). In locusts, octopamine stimulates the release of lipid during the first 10-20 minutes of flight (Orchard *et al*., 1982; Orchard and Lange, 1983) and also acts as a neurotransmitter mediating hormone release (Orchard and Loughton, 1981) and stimulate substrate oxidation in locust flight muscle (Candy, 1978). Lipid synthesis from leucine by fat cells of *Glossina*

morsitans, in vitro, was inhibited by dopamine, adrenaline, nor adrenaline and octopamine (Pimley, 1984).

Ecdysteroids

Ecdysteroids are molting hormones in both insects (Sehnal, 1989) and crustaceans (Chang and O'Connor, 1988), which are polyhydroxyl sterol compounds that play an important role during insect development, such as induction of ecdysis, termination of diapause and regulation of reproduction and embryonic development (Wang and Gong, 1997; Haegele and Wang, 2004).

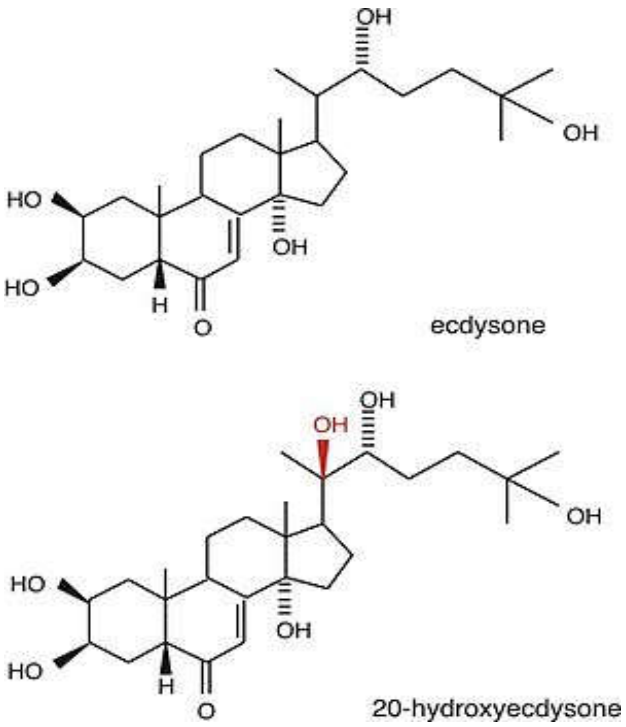

Figure 1. Structures of different Ecdysone

In insects, the moulting hormone ecdysone is synthesized in the prothoracic glands or in corresponding organs and secreted into hemolymph at particular times of development. Secreted ecdysone get oxidized in the fat body and several other tissues to 20-hydroxyecdysone, which exert moulting hormone activity on the target tissues. Synthesis and secretion of ecdysone by the prothoracic gland are under the control of brain hormone, PTTH, then accelerated by a small quantity of juvenile hormone and the nervous system also involve in the control mechanism. These control mechanisms are responsible for the precise timing and suitable amount of the ecdysteroid secretion (Calvez *et al*., 1976; Koolman, 1978; Gande *et al*., 1979; Schaller and Charlet, 1980). Now many structural variations of ecdysteroids have been found in insects, for example, α-ecdysone (E), 20-hydroxyecdysone (20E), 26-hydroxyecdysone (26E), 2-deoxyecdysone (2dE). However the main type of ecdysteroids detected in most insects is E or 20E. (Tawfik *et al*., 1999; Cao and Jiang, 2002). In the silkworm *Bombyx mori*, basically ecdysteroid induces molting and metamorphosis in larvae and exogenous administration hastens the silk synthetic activity and cocoon spinning process but it strictly depends on the time of administration (Miao *et al*., 2004)

Quantitative and qualitative differences were observed in the ecdysteroid levels of different insect species like *Pieris brassicae* (Lafont, 1975), *Bombyx mori (*Calvez *et al*., 1976*)*, *Heliothis zea* (Holman and Melola,

1978*), Manduca sexta* (Bollenbacher, 1981), *Heliothis armigera* (Zhu *et al*., 1986) and social bees (Pinto *et al*., 2002) during development.

Ecdysteroids in larvae were studied in many insects (Smith *et al*., 1985). The main types of ecdysteroids in larvae are α-ecdysone and 20E, they also contain compounds like 26E, 2dE and 20, 26-hydroxyecdysone (Qiu, 2005). According to Wang *et al*. (2007), the main types of ecdysteroids in the larvae of *Opogona sacchari* is 20E besides a little 26E. There are two peaks of ecdysteroids showing in the last instar larvae for most insects. The first peak is non-ecdysis peak which can induce the larval metamorphism, the second one is ecdysis peak which has something to do with prepupal development and ecdysis of larvae (Wang *et al*., 2007). Bownes' studies in *Drosophila,* reveals that both the fat body and ovary synthesize yolk protein, and the fat body yolk protein gene transcription is stimulated by 20-Hydroxyecdysone (Bownes, 1989). Ovarian and hemolymph ecdysteroid concentrations increases during ovarian development in the majority of the insects studied. At pre-adult stages, an ecdysteroid peak is present at the time of germ band shortening in embryos and relatively small peaks are present at larval moults followed by a larger peak during the larval-pupal moult (Harshman *et al*., 1998).

According to Hodgetts *et al*. (1977) there will be an increase in whole-body ecdysteroid levels after eclosion which apparently corresponds to the

progression of ovary maturation. A decrease in ecdysteroid titres during the first 36 h after female eclosion and an increase through the fourth day after eclosion were observed by Handler, which roughly corresponds to the age at which maturation is complete (Handler, 1982).

In the silk gland, the genes responsible for the silk biosynthesis are switched on or switched off at particular times (Maekawa and Suzuki, 1980). This switch on and switch off is controlled by the circulating hormones and can be altered by exogenous administration of pure hormone or its close mimics. It is widely known that the development and function of silk glands are under the control of juvenile hormones, ecdysteroids and neurohormones (Prudhome *et al*., 1985). As per Sehnal and Akai (1990) high concentration of ecdysteroids causes silk gland regression during larval molts and degeneration during pupal molt. The growth parameters of silkworm larvae in general and that of silk gland in particular on the administration of ecdysteroid give a fairly clear indication that the compound exerts considerable influence in the physiological activities and silk protein biosynthesis (Miao *et al*., 2004). The exposure time of the cells plays an important role in differentiation of many target tissues. Akai (1979) had reported precocious autolysins of the posterior silk gland on massive injection of ecdysone in the middle of the 5th instar. This is considered as evidence to believe that ecdysteroid has got only a general effect on the metabolic pathways of the silk gland cells without any specificity on the silk production.

Ecdysteroids in ovarian development

Changes in ovarian and hemolymph ecdysteroid concentration and its composition during vitellogenesis have been investigated in the fresh water prawn *Macrobrachium rosenbergii* and found that free ecdysteroids (20-hydroxyecdysone and ecdysone) in hemolymph increased in concentration during vitellogenesis from zero at stage 0 to1.5 ng/ml at stage I to 7.3 ng/ml in mature, stage IV animals. In the stage IV hemolymph, 20-hydroxy ecdysonic acid (1.2 ng/ml) was detected (Young *et al*., 1993). Studies on the role of ecdysteroids in ovarian development in insects has been studied in detail in *Drosophila melanogaster* and *Aedes aegypti.* In *Aedes*, a blood meal triggers the release of egg-development neurosecretory hormone from the brain, which in turn stimulates the ovarian production of 20-hydroxyecdysone to, in turn, stimulate yolk protein synthesis by the fat body (Hagedorn, 1985, 1989). Ovarian and hemolymph free ecdysteroid, predominantly 20-hydroxyecdysone, was maximal in animals with immature (stage 0) ovary, decreased gradually during early vitellogenesis (stage 0 to stage1) and then fell sharply during later vitellogenesis (stage I to stage IV) (Young *et al*., 1993). Quite different trends in ecdysteroid concentration were observed in the ovary and hemolymph of *M. rosenbergii,* during vitellogenesis. The free ecdysteroid concentrations were generally much higher in ovaries than in hemolymph (Young *et al*., 1993). An increase in ovarian ecdysteroid content during the first three days post eclosion was described by Bownes *et al.*

(1984). Maximal Free ecdysteroid levels were observed in stage 0 non-pigmented ovaries and fell during early vitellogenesis. Levels then increased gradually in stage IV tissue. Polar conjugates of ecdysteroids were detected in the more mature tissue (Young *et al*., 1993). According to Wang *et.al* (2008), in *Opogona* during the development of ovaries, the titre of ecdysteroids was low on the first two days, and high in the late period. During the development of eggs, the titre of ecdysteroids was about 0.010 ng/egg from day 1 to day3, and then decreased to 0.006 ng/egg on day 4. In both adults and eggs, three main components of ecdysteroids were found. They were 20-hydroxyecdysone, 26-hydroxyecdysone, and an unidentified compound (Wang *et al*., 2008).

The role of ecdysteroid in embryogenesis or oocyte maturation is partially inferred from a correspondence between hormone titres and biological events (Hagedorn, 1983; Lanot *et al*., 1989). Moderately high levels of ecdysteroid are closely related to morphogenetic and anabolic processes ordered in space and time like oocyte maturation, pre-embryonic diapause etc. (Guo, 1989; Tanaka and Takeda, 1993). Bownes'studies in *Drosophila,* explains that both the fat body and ovary synthesize yolk protein, and the fat body yolk protein gene transcription is stimulated by 20-hydroxyecdysone (Bownes, 1989). Ovarian and hemolymph ecdysteroid concentrations increases during ovarian development in the majority of the insects studied. Female whole body ecdysteroid titres tended to increase after

an interval of the time following mating (Lawrence *et al.,* 1999). Yolk protein mediated oocyte uptake of hemolymph ecdysteroids, combined with continuing hormone secretion into the hemolymph by *de novo* synthesis, may underlie the increase in female whole body ecdysteroid titres after mating.

Effect of mating on female whole body ecdysteroid titres is mediated by the action of male accessory gland proteins (Lawrence *et al*., 1999). Accessory gland proteins increases female whole body ecdysteroid titres as a by product of stimulating juvenile hormone synthesis and subsequently stimulating vitellogenesis and oogenesis which results in increased yolk protein mediated uptake of ecdysteroids into vitellogenic oocytes.

Ecdysone administration is known to suppress vitellogenic oocyte stages (Handler and Postlethwait, 1978; Soller *et al.,* 1998) and this may be physiologically analogous to the high ecdysteroid titre state in virgin females. Mating stimulates juvenile hormone biosynthesis, yolk protein synthesis and yolk protein uptake into the developing oocytes. Yolk protein uptake into the developing oocytes may have the effect of decreasing hemolymph ecdysteroid titres which facilitates the progression of oocyte maturation by removing the suppressive effect of high titres.

Ecdysteroid in hemolymph

The hemolymph ecdysteroid titre at any instant of development of an insect reflects the balance between the entry of ecdysteroids into the

hemolymph due to synthesis or release from storage, and their removal from the hemolymph by excretion, sequestration or uptake into storage. Such changes may also lead to changes in the relative proportions of different ecdysteroid molecules in the hemolymph (Gerstenlauer *et al*., 1995).

A decrease in hemolymph ecdysteroid titres after mating may result from yolk protein uptake of ecdysteroids into developing vitellogenic oocytes as a consequence of male accessory gland protein stimulation of female oocyte maturation and yolk protein synthesis following mating (Lawrence *et al*., 1999). Mating status is observed to have an effect on hemolymph ecdysteroid titres. Mating was observed to result in lower female hemolymph ecdysteroid titre than found in virgin females of the same age (Lawrence *et al*., 1999). In last larval instar the level of ecdysteroids in the hemolymph varied. The hemolymph showed a small peak of ecdysteroid on day 1 and a large peak on day 7. After this large peak, the titre declined drastically prior to the imaginal moult (Gerstenlauer *et al.,* 1995).

During vitellogenesis in *Orchestia gammarellus*, a low ecdysteroid level in whole body extracts, ovary, and hemolymph were observed indicating that vitellogenin synthesis is independent of ecdysteroid levels (Blanchet *et al*., 1979). Remarkable changes are seen in the hemolymph ecdysteroid titre throughout the life of an insect, indicating a temporal correlation between moults and peaks in ecdysteroid titres (Fugo *et al*., 1996). A single massive

peak were observed in larval moults, generally display prior to moulting, and in hemimetabolous insects the larval-adult molt likewise displays a single peak. Ecdysteroids in the hemolymph closely paralleled that of the testes, although the titres were much higher than the amount in testes just after the 4th ecdysis (150 pg/pair) and rapidly decreased by day 2. Afterwards, ecdysteroids titre increased gradually. An abrupt increase of ecdysteroids titre was observed during the wandering stage. After pupation, the level of ecdysteroid became increasingly detectable on day 3 of the pupal stage and it decreased rapidly thereafter (Fugo *et al*., 1996).

In eukaryotic cells, a number of genes regulated by hormones were described (Yamamoto, 1985; Evans, 1988). Genes coding silk proteins are also regulated by hormones (Tripoulas and samols, 1986; Hamada *et al*., 1987; Sehnal and Akai, 1990; Michalik *et al*., 1992; Kodrik and Sehnal, 1994). Like steroids of vertebrates, ecdysteroids regulate gene expression by binding, in association with their specific receptor, to promoter elements of their target genes (Koelle *et al*., 1991). Low titres of ecdysteroids are necessary for proper silk gland function (Shigemastu and Moriyama, 1970; Kodrik and Sehnal, 1994). On the other hand, high concentrations of ecdysteroid cause silk gland regression during the larval moults or degeneration during the pupal moult (Sehnal and Akai, 1990). The level of RNA transcripts of the low molecular weight silk protein gene in the isolated posterior silk gland was influenced by exogenous ecdysterone and these

RNAs were more abundant in the hormone-treated than in the control (Grzelak *et al*., 1993). Dai *et al*. (1985) indicated that ecdysone plays a significant role in nucleic acid metabolism and the related protein synthesis in silkworm.

The titre of ecdysteroids in both male and female pupae of *Opogona sacchari* get higher gradually and then decreased slowly after reaching a peak (80 ng/pupa). The titre of ecdysteroids was about 30 ng/pupa in the later stage of pupae. The peak of male pupae appeared on the third day, but the peak of female pupae lasted from the second day to the fourth day during pupal developments (Wang *et al*., 2006). One major peak of ecdysteroids was observed in pupae of Indian meal moth that were kept in a 16 h light: 8h dark (long day) photo cycle. The ecdysteroids reached a maximum of 2000-2500 pg/mg wet weight between 36 h after pupation. The ecdysteroids declined to 40 pg/mg wet weight by 68 h after pupation. For pupae that were kept in continuous darkness, the ecdysteroid peak became broader with a maximum at 24 h (3130 pg/mg wet weight) (Shaaya *et al*., 1993). In *Gryllus bimaculatus* during the penultimate larval instar, hemolymph ecdysteroid titre increased slightly from about 3 ng/10 µl at day 1 to 11.2 ng/10 µl at day 3. On day 4 a single peak of ecdysteroid was observed with 54.7 ng/10 µl. After that the titre declined prior to the final larval moult. The pattern of *in vitro* prothoracic gland ecdysteroid release was similar to that of hemolymph ecdysteroid titre, starting at a low rate of 0.07 ng/16 h on day one, peaking at

0.69 ng/16 h on day 4 and dropping again prior to moulting. Hemolymph ecdysteroid titre of the fifth (last) larval instar of the *Rhodnius prolixus* has been determined by radioimmunoassay. During the last larval stadium the ecdysteroid titre increases from a negligible level in the unfed insect to a detectable level within minutes following a blood meal. The titre reaches a plateau of 50-70 ng/ml at 3-4 h and this level is maintained until day 5-6. Qualitative analysis of the hemolymph ecdysteroid RIA activity revealed the presence of only ecdysone and 20 hydroxyecdysone. For the large peak preceding larval-adult ecdysis, 20 hydroxyecdysone was the predominant hormone (Steel *et al.*, 1982).

Juvenile hormone

Juvenile Hormones (JHs) are a family of acyclic sesquiterpenoids unique to insects. They are major effector hormones in insects, whose primary role is the regulation of metamorphosis and reproduction (Noriega, 2004; Goodman and Cusson, 2012; Riddiford, 2012) . In some species, JHs are also associated with the regulation of polyphenisms including color morphs and caste differentiation of social insects. JH biosynthesis by the *corpora allata* (CA) is tightly regulated to ensure proper timing of developmental stages and gonadotropic cycles (Goodman and Cusson, 2012). JH is produced through a biosynthetic pathway related to that of cholesterol. Unlike the cholesterol pathway, the precursor, farnesyl pyrophosphate, is

converted to farnesol, not squalene, resulting instead in the synthesis of sesquiterpenoids. Farnesyl pyrophosphate is converted to JH by a series of two dehydration steps, a methylation reaction and an epoxidation reaction (Tobe and Bendena, 1999; Tobe and Stay, 1985). Among insects JH is involved in a broad range of physiological processes including, pheromone production, caste determination of social insects, foraging behaviour, phasepolyphenism, diapause, vitellogenesis and metamorphosis (Dale and Tobe, 1986, Smith and Schal, 1990; Glinka and Wyatt, 1996; Jassim *et al.*, 2000; Breuer *et al*., 2003; Shiga *et al.*, 2003; Park and Raina, 2004; Truman *et al*., 2006). In Crustacea, sesquiterpenoids are involved in similar functions including metamorphosis and reproduction (Borst *et al.*, 1987; Nagaraju, 2007). In general, a high JH titre maintains a larval form, where as the absence of JH allows for imaginal differentiation (Kikukawa and Tobe, 1986 a, b).

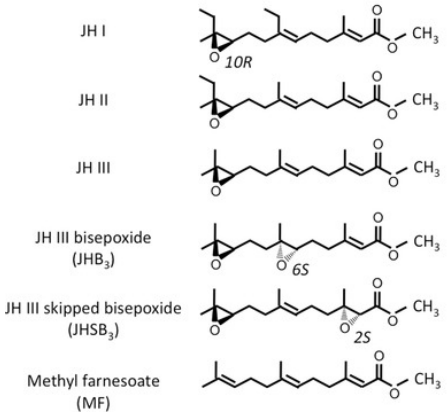

Figure 2. Structures of different JHs.

Early in larval stage, JH is necessary to trigger release of prothoracicotropic hormone, and thereby ecdysteroid secretion required for ecdysis. However, in final instars, ecdysteroid titre becomes elevated only after JH release declines, suggesting continuously high JH titre may also block ecdysteroid release (or synthesis) (Kikukawa and Tobe, 1986 a).

Discovery of Juvenile Hormones

The role of JH in regulation of metamorphosis was first discovered in the 1930s by V.B. Wigglesworth (Wigglesworth, 1934). Through a series of surgical manipulations on his model animal *Rhodnius prolixus*, commonly known as the kissing bug, Wigglesworth was able to conclude that circulating hormones regulate metamorphosis. In particular, he used a technique known as parabiosis in which a developmentally arrested animal was surgically joined with a normally developing one and the circulatory systems joined (Wigglesworth, 1934). Wigglesworth observed that a circulating hormone in young nymphs prevented a fifth (last) stage nymph from progressing to the adult stage. In essence, this hormone with antimetamorphic function promoted the retention of juvenile characteristics in *Rhodnius* and Wigglesworth coined the name Juvenile Hormone (Wigglesworth, 1934). The sole site of synthesis for JH was found to be a pair of neuroendocrine glands with neural connections to the brain called the *corpora allata* (CA).

Juvenile hormone homologues

Since its discovery, several natural occurring JHs have been characterized. Examples of some of the JH homologues characterized to date are: JH-I, JH-II, JH-III JH-0, 4-methyl JH-I, and JHSB3; a common feature of all is their farnesoid structure. The major JHs found in insects are JH 0, JH I, JH II, JH III (Goodman and Cusson, 2012; Riddiford, 2012). Unique to Lepidoptera are JH-I and JH-II, which were the first of the JHs to be discovered during the 1960s. A third homologue, JH-III, is the most ubiquitous and is present in most insects, including the Lepidoptera (Adams, 2009; Riddiford, 2012; Jindra *et al*., 2013). Additional JH homologues have been found in the eggs of the tobacco hornworm, *Manduca sexta*: JH-0 and 4-methyl JH-I (Adams, 2009). Lastly, another JH structure found in higher Diptera is JH bisepoxide (JHSB3) (Riddiford, 2012). Moreover, there are JH analogs which are non-insect derived compounds possessing JH-like biological activity in insects such as: fenoxycarb, pyriproxifen, and methoprene. The latter has exceptional biological activity in insects and has been used commercially as a control agent for insect pests, including mosquitos, since 1975 (Goodman and Cusson, 2012; Riddiford, 2012).

Site of juvenile hormone biosynthesis: corpora allata complex

Juvenile hormone is synthesized and secreted by the CA, a pair of endocrine glands, which are located posterior to the brain. The CA has neural

5 connections to brain and is part of the retrocerebal complex along with the *corpora cardiaca* (CC). The paired CC is a neurohemal organ that produces neurohormones and serves as a storage area for peptide hormones produced in the brain (Goodman and Cusson, 2012). Each corpus cadiacum is made up of only six neurosecretory cells (Clements, 1992). In the *Aedes* adult female, the CA is approximately 40-50 μm in length (Li *et al*., 2003). In the female, the CA is comprised of approximately 48 cells in the pupal stage and 60 in adult (Clements, 1992). Hence, it is not surprising that handling and dissection of these small glands proves challenging. Juvenile Hormone works in concert with ecdysteroids to modulate both metamorphosis and gonadotropic functions. In larval insects, the neuropeptide prothoracicotropic hormone (PTTH) stimulates the release of α-ecdysone by the prothoracic glands (PGs). However, in the adult these glands degrade and the ovaries become the source of ecdysone (Raikhel, 2005). Once secreted, ecdysone is converted by peripheral tissues to its active ecdysteroid form, 20-hydroxyecdysone (20E) (Riddiford, 2012).

Molecular basis of action of juvenile hormone

Upon secretion from the CA, JH is transported in the hemolymph by high affinity binding proteins to act at distant peripheral sites. Termed hemolymph JH binding proteins (hJHBPs), these proteins also protect JH from degradation by enzymes (Trowell, 1992; Goodman and Cusson, 2012).

Only recently was the receptor for JH identified as Methoprene-tolerant (Met). The intracellular hormone receptor is a basic helix-loophelix (bHLH) protein a member of the Period (Per)-Aryl hydrocarbon nuclear translocator (Arnt) – Single-minded (Sim) (PAS) domain family of transcription factors (Riddiford, 2012; 2013; Jindra *et al*., 2013). Upon binding JH the Met homo or heterodimer undergoes a conformational change that allows it to bind to another bHLH-PAS protein known as Ftz-F1 interacting steroid receptor coactivator (FISC) in *A.aegypti* (Jindra *et al*., 2013).

Role of juvenile hormone in metamorphosis

In holometabolous insects such as *Aedes aegypti*, metamorphosis takes place in two stages: larval-pupal and pupal-adult. After hatching, *Aedes* undergoes four larval instar molts known as the "wrigglers" during which feeding takes place. Hence, JH dictates the nature of the molts which occur in response to 20E (Goodman and Cusson, 2012). For example, in the course of larval development JH ensures that molts are from one larval instar to another larger larval instar. However, in the last larval stage a rise in 20E coupled with the absence of JH signals a cessation of feeding and onset of pupation. Following is the pupal stage, in which the non-feeding insect undergoes a major developmental restructuring. During this stage a pharate adult, that is, an adult encased in the pupal cuticle, develops until the appropriate time to emerge (eclosion). The pupal stage takes approximately 50 h. Application of

fenoxycarb in European corn borer, *Ostrina nubilalis* during the second to fourth larval stadium will not affect the duration of these instars, but application of fenoxycarb showed an increase in the duration fifth larval stadium. In the case of *C. fumiferana*, application of fenoxycarb results in precocious invagination of wing disk during larval-pupal intermediates and deformed pupae production (Mulye and Gordon, 1989; Hicks and Gordon, 1992). Same results were observed in fenoxycarb treated light brown apple moth, *Epiphyas postvittana* (Mcghi and Tomkins, 1988). *Heliothis virescens* (Mauchamp *et al*., 1989) and leafroller, *Adoxophyes orana* (Charmillot *et al*., 1994). Application of pyriproxyfen in *Spodoptera litura* results in production of supernumerary larvae (Hatakoshi *et al*., 1986). In lesser mealworm, *Alphitobius diaperinus* adult emergence was prevented completely when it was fed on poultry food containing 0.05 ppm fenoxycarb (Edwards and Abraham, 1985). In the German cockroach, *Blattella germanica* application of fenoxycarb during fifth instar results in morphological deformities and sterility in adults. It seems that sterility is being transferred from treated males to untreated females, which suggests effects on sperm (King and Bennett, 1990). Similar effects were observed for *Choristoneura fumiferana* (Hicks and Gordon, 1992). In *Solenopsis invicta* queens suppression in egg production were observed due to the topical application of fenoxycarb, similarly a reduction in egg production and hatching were observed in California five-spinedips, *Ips para confusus* (Chen and Borden, 1989).

Juvenile hormone biosynthesis

The biosynthetic pathway of JH-III involves 13 sequential enzymatic steps and is best divided into early and late steps (Bellés *et al*., 2005). The early steps follow the mevalonate pathway (MP); where two acetyl-CoAs are reductively polymerized into five carbon (5C) isoprenoid units. These isoprene units are then sequentially condensed to form farnesyl diphosphate (FPP). Up to the formation of FPP, the MP of insects shares homology with all organisms that use the mevalonate pathway to synthesize cholesterol or other biomolecules. In insects, which do not produce cholesterol, FPP is instead used for production of compounds such as ubiquinone, for proteinprenylation, and for JH synthesis (Goodman and Cusson, 2012). In

the late steps, FPP is transformed sequentially to: farnesol, farnesal, farnesoic acid (FA), methylfarnesoate (MF), and ultimately JH III.

Regulation of juvenile hormone biosynthesis: Allatoregulatory factors

Considering the important developmental and gonadotropic roles of JH, it is not surprising that biosynthesis of the hormone by the CA is under strict regulation. In essence, the rate of biosynthetic activity of the CA determines the JH titres. To ensure proper titres of JH, the CA is tightly regulated by allatoregulatory factors; such that information on nutritional and developmental status is transduced to the CA by them. These factors are both allatostatic (inhibitory) and allatotropic (stimulatory) in nature. These include

neuropeptides, a class of signalling molecules that have been shown to regulate endocrine functions in a vast array of organisms ranging from insects to vertebrates (Woodhead *et al*., 1989). Two examples of allatoregulatory neuropeptides are the pleiotropic neuropeptides, allatotropin (AT) and allatostatin (AS). Allatotropins are known to be stimulatory to JH synthesis and have been characterized in *A. aegypti* (Li *et al.*,2004). Conversely, allatostatins have been demonstrated to have an inhibitory effect in JH synthesis and have also been described in *Aedes* (Li *et al*., 2006). The allatoregulatory potential of these peptides underscores the importance of investigating the role of other neuropeptide hormones as possible regulators of JH synthesis in the mosquito. Five JH variants are known, JH I, JHII, JH III, JH 0 and iso JH 0. JH III has most often been found to be the principal or only JH molecule in many insects. However, analysis with GC –MS has shown multiple JHs in some insects, particularly in Lepidoptera (Bergot *et al*., 1981). JH is only detectable as a trace or sometimes not at all in some Lepidoptera (Edward *et al*., 1995; Ramaswamy *et al*., 1997; Park *et al*., 1998), and JH I and JH II are often the principal JHs. JH I, II, and III are released from the isolated CA of 10-day old *Actebia fennica* moth females, but JH II is the principal JH (Everaerts *et al*., 2000). JH II is the principal JH in the 4th and 5th instars of tomato moth larvae *Lacanobia oleracea*, and in the pupa, along with some JH I (Edwards *et al*., 1995). Audsley *et al.* (2000) found that 90% of the JH released by isolated CA is JH II.

Role of juvenile hormone in reproductive maturation

After metamorphosis, JH plays a different role in the adult female mosquito as a regulator of reproductive maturation. In autogenous mosquitoes such as *Aedes*, reproduction is cyclic and a blood meal is required by the female prior to oviposition (Riddiford, 2013). In short, their reproductive cycle has two distinct phases: a previtellogenic stage regulated by JH and a vitellogenic stage regulated by 20E (Raikhel *et al*., 2002). Newly emerged female mosquitoes feed on a nectar diet and synthesize low amounts of JH which, during the first 12 h post eclosion, increase to 90 fmol/CA/h. In sugar-fed females JH levels remain high but rapidly decrease after ingestion of a blood meal. Juvenile Hormone levels reach their lowest point 24 h later post blood meal at: < 5 (fmol/CA/h) (Li *et al*., 2003). Forty-eight hours post blood meal, JH levels begin to rise and return to a relatively high level on the third day (Noriega, 2004). Prior to taking a blood meal, the mosquito is in a resting stage in oogenesis which is known as the previtellogenic stage. Here, JH exposure leads to competence of the fat body (FB), the equivalent to the vertebrate liver, to synthesize massive amounts of yolk protein precursors upon later exposure to 20E (Adams, 2005; Riddiford, 2012). Another way JH regulates reproductive output is by inducing competence of the ovaries to uptake Vitellogenin (Vg), a major yolk protein, into developing oocytes. Moreover, JH also prepares the mosquito for blood digestion by regulating transcription of the early trypsin gene in the midgut (Noriega *et al.*, 1997). In response to a blood meal, 20E levels rise and the vitellogenic phase of egg

development begins. At this time, under the control of 20E, the oocyte completes a process of yolk deposition called vitellogenesis. Three days after blood feeding, 20E levels decrease and JH synthesis begins a new, repeating the preparatory steps necessary for another gonadotrophic cycle (Li *et al*., 2003)

Ecdysteroids as well as juvenile hormones, the hormones that regulate the overall development and metamorphosis, collectively referred to as insect growth regulators (IGRs) are considered as prospective candidates for developing mimics for the control of insect pests. Any changes in the homeostasis of these hormones with exogenous sources of hormones or with synthetic analogues would result in the disruption or abnormal course of development and reproduction of the target insect (Hoffmann and Lorenz, 1998). Studies on *Hyalophora cecropia* silkworm by Williams (1967) suggested that compounds that can mimic juvenile hormone can act as a good insecticide. After that numerous JH analogues were discovered and have been used as commercial insecticides, e.g.: methoprene, but the use of these analogues in agriculture is limited due to the lack of outdoor stability, limited insect control spectrum and slow toxic action. Most of the early analogues of juvenile hormone showed resemblance in their basic terpenoid structure. Farnesol and Farnesal were the first juvenoids isolated from the insects (Schmialek, 1963). Methoprene and hydroprene are active JH analogues, they lack an epoxide function present in JH. They are mainly used for controlling household pests and dipteran insects. During 1998, many

compounds with less apparent similarity with JH have been discovered e.g.: fenoxycarb, pyriproxifen and diofenolan. For controlling lepidopteran and coleopteran insects different formulations of fenoxycarb have been used.Pyriproxifen is used to control mosquitoes and other dipterans and insects of other orders. Another JH analogue diofenolan shown to have a high activity against lepidopteran pests and scale insects in orchards (Couillaud *et al*., 1985). Rohm and Hass company in 1983 discovered the first bisacylhydrazine ecdysteroid agonist RH-5849. This compound showed an ecdysteroid like action at cellular level in kc cells of *D. melanogaster* (Wing, 1988). In *M. Sexta* larvae, administration of ecdysteroid analogue RH- 5849 showed an increase in the level of this compound in the hemolymph 6 h after administration and a slight decrease thereafter, which was stable over next 36 h. In addition to these effects, RH- 5849 was showing a powerful ecdysonergic activity by initiating premature lethal moults (Wing *et al.*, 1988). In the spruce budworm *Choristoneura fumiferana* and *M. sexta*, a failure in endocuticle production was reported for the RH- 5992 treated larvae (Retnakaran and Oberlander, 1993). The strategy thus involves the use of steroid analogues to tamper with the hormonal balance in the insect there by interrupting normal moulting and growth and associated metabolic pathways leading ultimately to the death of the insect. Several steroidal and non-steroidal analogues have been attempted successfully for the control of pest insects (Chandler *et al.*, 1992; Heller *et al.*, 1992).

CHAPTER III
MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Experimental insects

The experiments were carried out on different tissues of the mango leaf webber, *Orthaga exvinacea* (Pyralidae: Lepidoptera), a seasonal and serious pest of mango trees infesting during the period from December to April. They were collected from their natural habitat.

3.1.2. Chemicals and reagents

Acetonitrile (SRL)

Acrylamide (SRL)

Ammonium persulphate (APS) (SRL)

Bis acrylamide (SRL)

Bromophenol blue (Qualigens)

Coomassie brilliant blue G-250 (Sigma)

Copper sulphate (SRL)

Disodium hydrogen phosphate (SRL)

Ecdysone (A gift from Central Silk Board, Bangalore, India)

Folin's reagent (SRL)

Formic acid (SRL)

Glacial acetic acid (SRL)

Glycerol (Qualigens)

Glycine (SRL)

Hexane (SRL)

Isooctane (Merck)

Juvenile hormone III (Sigma)

Potassium bromide (KBr) (Sigma Aldrich)

Potassium chloride (SRL)

Potassium dihydrogen phosphate (SRL)

Methanol (SRL)

Methoprene (A gift from Central Silk Board, Bangalore, India)

Petroleum jelly (Vaseline)

Sodium dodecyl sulphate (SDS) (BDH)

Sodium chloride (SRL)

Sodium hydroxide (SRL)

Sodium potassium tartarate (SRL)

Standard protein molecular weight marker (Bangalore Genei)

Tetramethylethylenediamine (TEMED) (SRL)

Triethyl amine (SRL)

Tris base (SRL)

Trifluoro acetic acid (SRL)

Tris-HCl (SRL)

3.1.3. Equipment

Distillation unit (Lab SI)

Desiccator

Electronic microbalance (Sartorius)

Electrophoretic apparatus (BangloreGenei)

Gel scanner (Bio Rad)

Hamilton syringe (Agilent)

Heating mantle (Rotek)

Homogenizer (Bangalore Genei)

Hot air oven (Adco)

HPLC (Shimadzu)

LC-Q-TOF-MS (Waters)

Micropipettes (P'fact)

pH meter (Bangalore Genei)

Polyacrylamide Gel Electrophoresis unit (Bangalore Genei)

Refrigerated centrifuge (Rotek)

Refrigerator (LG)

Stereozoom microscope (Carl Zeiss)

UV-VIS spectrophotometer (Shimadzu, JASCO)

Water bath incubator (Promega)

3.2. METHODS

3.2.1. Preparation of reagents/solutions

Alkaline copper reagent

Sodium carbonate (Na_2CO_3) 20 g in 260 ml distilled water was mixed with 0.4 g of cupric sulphate in 20 ml distilled water and 0.2 g of sodium potassium tartarate in 20 ml distilled water.

Folin's reagent

Folin's reagent 0.2 N was prepared by mixing 10 ml 2 N Folin's reagent with 90.00 ml distilled water and used afresh.

Sodium dodecyl sulphate solution (SDS)

Sodium dodecyl sulphate solutions (1%, 4% and 20%) were prepared by dissolving 1 g, 4 g and 20 g SDS each in 100 ml distilled water and were stored at room temperature.

Acrylamide solution

Acrylamide 30 g and 0.8 g of bisacrylamide were dissolved in 100 ml of distilled water. The solution was filtered and stored at 4^{o}C in an amber coloured bottle.

Ammonium persulphate solution

Ammonium persulphate solution (10%) was prepared by dissolving 10 g ammonium per sulphate solution in 100 ml distilled water and used afresh.

1.0 M Tris-HCl (pH 8.8)

Tris-HCl, 15.76 g was dissolved in 50 ml distilled water. The pH was adjusted to 8.8 with 1.0 M sodium hydroxide solution. This was made up to 100 ml with distilled water and stored at 4^{o}C in an amber coloured bottle.

1.0 M Tris-HCl (pH 6.8)

Tris-HCl, 15.76 g was dissolved in 50 ml distilled water. The pH was adjusted to 6.8 with 1.0 M sodium hydroxide solution. The solution was made up to 100 ml with distilled water and stored at 4^{o}C in an amber coloured bottle.

Running buffer (10x)/chamber buffer

Tris base 30.3 g, 144 g glycine and 10 g sodium dodecyl sulphate were dissolved in 100 ml distilled water. The pH was adjusted to 8.3 with 30%

HCl and the solution was made up to 1.0 l with distilled water (10x). Running buffer of 1x concentration was prepared by diluting 50 ml of the 10x buffer with 450 ml of distilled water.

Sample loading buffer

Tris-HCl 1.0 M (pH 6.8) 2.4 ml, 20% sodium dodecyl sulphate 3 ml, glycerol 3 ml, β-mercaptoethanol 1.6 ml and bromophenol blue 0.006 g were mixed. The mixture was filtered and stored in a sterilized bottle at 4^{o}C.

Staining solution

Coomassie brilliant blue G-250, 1.0 g was dissolved in 44 ml methanol and 12 ml glacial acetic acid was added and the mixture was made up to 100 ml with distilled water.

De-staining solution

This was a mixture of 437.5 ml distilled water, 25 ml methanol and 37.5 ml glacial acetic acid.

Gel fixative

Mixed 50 ml methanol, 50 ml distilled water and 50 µl formalin.

3.2.2. Collection and maintenance of insects:

Larvae of the mango leaf webber, *Orthaga exvinacea*, collected from their natural habitat, *i.e.*, mango trees from different areas of Calicut and

Malappuram districts (Kerala), were brought and transferred to plastic troughs kept in the insectary. They were reared on mango leaves. The troughs were covered with cotton cloths and kept in large wooden insect cages of 14′ ×14′ ×14′ with three side covered with aluminium gauze and the top with glass. There was a glass door for the cage on one side. The larvae were protected from ants by water barriers. The plastic troughs were cleaned on alternate days. The insect culture was maintained at a temperature of about 27±2°C and relative humidity of 70-80 %.

3.2.3. Identification of different stages of *O. exvinacea*

Sixth instar larvae

Larval skin was wrinkled and two longitudinal lines were seen on either side of the larvae and length was about 19 to 22 mm.

Seventh instar larvae

Larval skin was dark brown in colour and they had a length of about 24 to 30 mm.

Pre-pupal stage

Pre-pupae were collected from the cocoon soon after its formation.

3.2.4. Collection of hemolymph from larvae

Hemolymph collected into calibrated capillary tubes were pooled separately from 3-4 day old 6th instar larvae,7th instar larvae and prepupae (5 to 10 nos.) by puncturing the proleg, to get sufficient volume of the hemolymph. Samples were transferred in to Eppendorf tubes containing 3 µl of insect ringer, kept on ice and were stored at 4^{o}C until use.

3.2.5. Collection of fat body

The larvae and prepupae were dissected mid-dorsally in cold insect saline. The digestive systems were removed with a forceps. The floating fat body were transferred to a Petri dish containing insect ringer. It was then transferred in to an Eppendorf tube containing 200 µl 4% SDS and from this 100 mg fat body samples were taken and homogenized with 1.0 ml phosphate buffer and were kept at 4^{o}C until use.

3.2.6. Collection of silk glands from larvae and prepupae

The larvae and prepupae were dissected mid-dorsally in cold insect saline. The digestive systems were removed with a forceps. Silk glands were taken from the larval and prepupal stage and transferred to a Petri dish containing insect ringer. It was then transferred in to an Eppendorf tube containing 200 µl 4% SDS and from this 100 mg samples were taken and homogenized with 1.0 ml phosphate buffer and were kept at 4^{o}C until use.

3.2.7. Collection of ovary from adults

Adult insects were dissected mid-dorsally in cold insect saline. The ovaries were transferred to a Petri dish containing insect ringer. The tissue was then transferred in to an Eppendorf tube containing 200 µl 4% SDS and from this 100 mg samples were taken and homogenized with 1.0 ml phosphate buffer and were kept at 4^{o}C until use.

3.2.8. Extraction of proteins from tissues

Tissue (fat body, silk glands and ovary) homogenates were thawed and the homogenates were subjected to centrifugation at 12000 rpm at 4^{o} C for 10 min. Supernatants thus obtained were collected for quantification and profiling of protein. Samples for protein profiling were stored at 20^{o}C.

3.2.9. Quantification of proteins from fat body, silk glands, ovary and hemolymph:

Quantitative analyses of proteins from fat body, silk glands, ovary and hemolymph of *Orthaga exvinacea* were done by using modified Lowry *et al*. *(*1951) method using BSA as the protein standard. Different concentrations of standard BSA solution were taken and made up to 400 µl with 1 % SDS. To this 2X Lowry concentrate 400 µl (alkaline copper reagent, 1% SDS and 0.1 N NaOH mixed in 3:1:1 ratio) was added and incubated at room temperature for 10 min. This was followed by the addition of 200 µl of 0.2 N

Folin'sreagent and was kept at room temperature for 30 min incubation. The absorbance was measured at 750 nm in UV-vis spectrophotometer. The amount of proteins/mg/ml of tissue was calculated from a standard graph, which was plotted as absorbance against concentration.

3.2.10. Statistical analysis

The data obtained were subjected to unpaired 't' test using GraphPad software.

3.2.11. Profiling of protein using SDS-PAGE

Poly acrylamide gel electrophoresis has been carried out using the method described by Laemmli (1970). From a stock solution of poly acrylamide (containing 30 g acrylamide and 0.8 g bisacrylamide), resolving gel of 10% and stacking gel of 4% were prepared for the separation of proteins present in the samples as follows.

Setting and casting of gel

Glass plates were cleaned using mild detergents, washed thoroughly with distilled water and dried. The spacers were fixed in between the glass plates in respective places. The bottom and two sides of glass plates were then sealed using petroleum jelly. The levels for filling stacking and resolving gels were marked using marker pen on the glass plate

Preparation of poly acrylamide gel

The resolving gel (10%) and stacking gel (4%) were prepared for the separation of proteins present in the sample as follows:

Resolving gel (10%)

Acrylamide solution (30%), 1.66 ml was pipetted out into a 25 ml beaker, 2 ml of 1.0 M Tris-HCl was added to it and mixed well. Then, 1.26 ml of water and 25 µl of 20% SDS were added, followed by 25 µl of 10% ammonium persulphate and 3 µl of TEMED. The mixture was stirred for proper mixing and transferred into gel casting unit without trapping any air bubbles between the glass plates. The unit was left undisturbed for 15 min for complete polymerization. After polymerization, the water came from the resolving gel was blotted using a blotting paper.

Stacking gel (4%)

Acrylamide (660 µl) and 1.0 M Tris-HCl (pH 6.8, 630 µl) were mixed. To this mixture 25 µl of 20% SDS, 25 µl of 10% ammonium persulphate and 5 µl of TEMED were added. The whole mixture was diluted to5 ml with distilled water. The gel was poured over the surface of polymerized separating gel. The teflon comb of the appropriate size was soon fixed above this stacking gel to make wells for loading the sample. The gel casting unit

was kept undisturbed for about 30 min. After polymerization, the teflon comb was removed and wells were cleaned with distilled water.

Running of the Gel

The teflon comb from the top and the spacer from the bottom of glass plate were removed after the polymerization of gel. The polymerized gel was transferred into the running unit and the glass plate fixed in the unit. Levelled the unit using a sprit level. The whole set up was maintained at a temperature of 15-16^{o} C.The upper and lower chambers of the running unit were filled with chamber buffer. After loading the samples and standard protein marker, the electrodes were connected to power supply unit and 220 v were supplied. When the dye reached the bottom of the gel, power supply was turned off. The glass plates containing polymerized gel were taken out. One plate was gently lifted from the other. The gel was transferred from the other glass plate into a Petri dish containing gel fixative (methanol, formalin and distilled water mixture). A small cut was made on the right bottom of the gel to indicate the direction and sequence of samples. The gel was kept in gel fixative overnight. Next day, it was transferred to the stain solution, Coomassie Brilliant Blue G-250 taken in another Petri dish and kept for about 4-6 h. This was followed by multiple washes with de-staining solution (glacial acetic acid, methanol and water mixture) to remove excess stain. The gel was stored in the same solution. The protein bands developed were

observed under illumination and photographed using gel documentation system (Bio-rad).

Scanning of gel

Bio-rad gel scanner was used for taking the photograph of gel. The software Quantity One was used for determining the molecular weight of protein bands. The molecular weight was also estimated manually by comparing with molecular weight markers.

3.3. Ecdysteroid titre measurement

3.3.1. Extraction of ecdysteroid

Ecdysteroids were extracted as detailed by Haegele and Wang (2004). Separately hemolymph (35 µl), collected from the 6th and 7th instar larvae and prepupae were mixed with 1.0 ml 70% methanol. The samples were then heated to 60^{o}C for 10 min, cooled to room temperature and centrifuged at 10000 g for 10 min. Supernatants were collected and the pellets re-extracted twice with 70% methanol. Combined supernatants were dried under reduced pressure and the dried residues partitioned between 300 µl 70% methanol and 300 µl hexane to remove apolar lipids (Dinan and Rees, 1981). The hexane phases were discarded and the lower methanolic phases were desiccated, re-dissolved in 500 µl each of 70% methanol and taken for FTIR and HPLC analyses.

Since clear hemolymph was not accessible from the pupae, the whole pupae were used for the extraction of ecdysteroids. Pupae (5 nos.) were wiped clean with tissue paper and were homogenized in 1.0 ml 70% methanol and extraction of ecdysteroid was done by using the same procedure used for the larval hemolymph. This procedure was also followed for the extraction of ecdysteroids from the whole larvae. For the extraction of ecdysteroids whole larvae (3 nos. each) of the 4th, 5th, 6th, 7th instars were taken.

3.3.2. Analysis of ecdysteroids using HPLC

Aliquots (100 µl) of the extracts prepared as described above were diluted with 100 µl each of 70% methanol. The samples were filtered using a filtration unit (Millipore, USA) with a filter of 0.45 µm pore size. Samples (20 µl) drawn from the diluted extracts were further mixed with 60 µl each of 70% methanol. Used 20 µl each of this diluted samples for injection into the HPLC instrument (Shimadzu, LC 20AD, LC 20AD, SPD 20A) using a Hamilton micro-syringe. High performance liquid chromatographic (HPLC) separations of all the samples and standard were carried out with a reverse phase column (C18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. All the solvents were filtered in Millipore filter of pore size 0.45 µm. The eluents were monitored at 242 nm

using a UV–visible detector. The chromatograms were exported to Microsoft Word file.

The standard ecdysteroid (20E) was a gift from Central Silk Board, Madivala, Bangalore, India. Samples of the standard were run on HPLC for purity check. The single large peak with retention time of 3.4 min confirmed 100% purity. Known concentration of the standard (20 µl equivalent to 10.6 µg) was injected in to the HPLC instrument maintained in the same condition as for the samples. Similarity of retention time of any materials (appearing as peak) of the extracts of *O. exvinacea* with that of standard ecdysone was confirmed by overlying this profile with that obtained for standard ecdysone. HPLC run was repeated for all the samples and the peak area calculated were almost same for all the runs.

3.3.3. Determination of the functional groups present in the ecdysteroids using Fourier Transform Infrared Spectroscopy (FTIR).

Fourier Transform Infrared Spectroscopy (FTIR) is used for analysing chemical bonds present in organic compounds. When infrared light from the radiation source of spectrometer passes through the interferometer in to a sample, the sample absorbs lights of some frequencies, while others are transmitted. The transmitted light is recorded with the help of a detector. The absorption of the light depends on the nature of chemical bond in the sample. The plot of percentage of transmittance against frequency gives the infrared

spectrum of the sample. The nature of a compound is identified by comparing the spectrum with those for known standards.

The secondary structural characterisation of the ecdysteroid samples were done by using JASCO FTIR-4100 instrument (USA) by placing a drop of ecdysteroid sample with KBr. The frequency of absorption was in the region of 4000 to 400 cm^{-1} at room temperature. Infrared spectra were recorded from 32 scans per samples with resolution of 4 cm^{-1}.

3.3.4. Sample preparation for FTIR

Samples (10 µl each) for FTIR analysis were taken from the extracts prepared from larvae and pupae of *O. exvinacea* as described earlier. The secondary structural characterisation of the ecdysteroid samples were done by using JASCO FTIR-4100 instrument (USA). To obtain the infrared spectrum, the samples were placed in the sample holder between a pair of KBr plates, referred to as salt plates. When the plates were squeezed gently, a thin liquid film formed between the plates. The pair of plates was then inserted into the holder that fits into the spectrometer. The frequency of absorption selected was in the region of 4000 to 400 cm^{-1} at room temperature. Infrared spectra were recorded for all the samples, each from 32 scans per samples with resolution of 4 cm^{-1}. The same procedure was employed for analysing the standard ecdysteroid.

3.4. Juvenile hormone titre measurement

3.4.1. Juvenile hormone extraction

Hemolymph samples from 6th and 7th instar larvae of *O. exvinacea* (20 µl each) were mixed with methanol/isooctane (1:1 v/v) and were kept at room temperature for 30 min. Then samples were subjected to centrifugation at 8500 rpm for 30 min. The upper octane phase was transferred in to a new vial using micro pipette and the remaining methanol phase was vortexed and centrifuged at 10000 rpm for 30 min and then the upper phase combined with the initial isooctane phase. Then methoprene (660 µl) was added to it as an internal standard. The resulting mixture was concentrated to dryness under a stream of N_2. Dried pellets were dissolved in 20 µl of methanol and subjected to LC-Q-TOF-MS.

3.4.2. LC-Q-TOF-MS

LC-Q- TOF -MS system provides accurate mass analyses for a variety of analytical applications including profiling, identification, characterization, and quantification of both small and large molecules.

Juvenile hormone extract 20 µl each prepared as described above (Section 3.4.1) and the standard JH-III was injected into LC-Q-TOF-MS instrument with the following specifications.

Acquity H class (Waters) Ultra Performance Liquid Chromatography

with BEH C18 column (50 mm × 2.1 mm× 1.7 μm) were used. Water+0.1% formic acid and acetonitrile were used as mobile phase at a flow rate of 0.4 mL/min. Total run time was 10 min. Xevo G2 (Waters) Quadrapole – Time-of-Flight (Q-TOF) mass spectrometer were used. Both positive and negative mode of ionization were used. In positive mode electrospray capillary voltage: 3.50 kV, sample cone: 30 V, extraction cone: 1 V, source temperature was 135°C, desolvation temperature was 350°C, gas flow (nitrogen gas)cone Gas: 50 (L/H), desolvation gas: 900 (L/H). In negative mode of electron spray, capillary voltage was 2.50 kV, sample cone was 30 V, extraction cone was 1.0 V. Source temperature was 135°C, desolvation temperature 350°C. gas flow (nitrogen gas), cone gas: 50 (L/H), desolvation gas:900 (L/H).

Concentration of hormone present in the samples were calculated from the chromatogram using the equation:

$$\frac{\text{Concentration of standard}}{\text{Area of standard}} = \frac{\text{concentration of sample}}{\text{Area of sample}}$$

CHAPTER IV
RESULTS

4.1. Proteins in the fat body, hemolymph, silk glands and ovary of *O. exvinacea* during development

4.1.1. Quantitative changes of proteins present in various tissues during development

Colorimetric analyses of total proteins present in the fat body, hemolymph, silk glands and ovary of *O. exvinacea* showed that there is significant change in the protein concentration during development. The concentration of proteins present in the fat body of 6th instar larvae was 19.4±1.2 µg of protein per mg of tissue. The value increased to 22.60±1.5 µg/mg in the 7th instar larvae. A remarkable increase was seen in the concentration of protein in the prepupal fat body and it was 47.30±2.0 µg/mg (Fig. 1). Concentration of protein present in the pupal fat body was 123.60±4.6 µg/mg and this value was very high compared to all other stages of development. The concentration of protein present in the adult fat body showed a value (43.7±3.9 µg/mg) which was less than that of prepupal fat body protein concentration. This change in protein concentration in the different developmental stages (with in groups) were found to be statistically significant (P 0.001) (Table. 1).

Analysis of the concentration of proteins present in hemolymph of 6th instar larvae showed a value 35.75±6.1 µg/ml of hemolymph. It increased to 52±7.4 µg/ml in the hemolymph of 7th instar larvae. There was a further slight increase in the amount of protein in the prepupal stage to 58.25±6.0 µg/ml of hemolymph sample (Fig. 2, Table. 2).

The concentration of proteins present in silk gland of 6th instar larvae showed a value 6.31±0.98 µg/mg. It increased to 7.08±1.12 µg/mg in the silk gland of 7th instar larvae. There was a dramatic increase in the amount of protein in the prepupal stage to 21.98±2.41 µg/mg of silk gland sample (Fig. 3, Table 3).

The changes in concentrations of proteins present in the ovary of adults were also studied. The concentration of proteins present in the ovary of 3-day old adult was 37±3.0 µg/mg and was the highest compared to the concentration of protein present in the ovary of 1-day old adult and 2-day old adult. Concentration of protein in the ovary 1-day old adult was 30±2.4 µg/mg and that of 2-day old adult was 35±1.9 µg/mg (Fig. 4, Table 4). These changes were found to be statistically significant (P 0.001).

4.1.2. Protein maps of *O. exvinacea*

The molecular weight of proteins present in the bands obtained for various samples prepared from fat body, hemolymph, silk glands and ovary as described earlier (Section 3.2.8), run in sodium dodecyl sulphate poly acryl

amide gel were derived by plotting the relative mobility of protein bands with respect to antilog of standard marker run in the same gel and confirmed by Quantity One programme in a gel documentation system (Bio Rad).

The results showed that the proteins present in the 6th instar larval fat body samples appeared as two bands (Fig. 5, lane 1) with molecular weights 70.49 kDa and 74.34 kDa, which were narrow and clear. Proteins of the 7th instar larval fat body showed 6 bands and the molecular weights ranged from 36kDa to 74.34 kDa (Fig. 5, lane 2). All the bands were narrow and proteins with molecular weights 70 and 74 kDa showed clear and well defined bands. Proteins present in the prepupal fat body showed 3 bands with molecular weights 35.25, 62.5 and 68.3 kDa, (Fig. 5, lane 3). Pupal fat body showed seven clear bands with molecular weights 36.5, 38.25, 41.36, 42.74, 59.35, 62.18 and 65.44 kDa (Fig. 5, lane 5). Adult fat body showed four protein bands with molecular weights 37, 45, 50 and 59 kDa (Fig. 5, lane 4).

In the case of hemolymph, the concentration of protein was higher in the 7th instar larval sample compared to the 6th instar larvae. The number of protein bands was also more in the 7th instar larval hemolymph sample compared to the number of protein bands present in the 6th instar larval hemolymph sample. Seventh instar larval hemolymph sample showed six protein bands with molecular weights ranging from 24 to 73 kDa (Fig. 6, lane 2). The number of protein bands present in the 6th instar larval and prepupal

hemolymph samples were 3 and 6 respectively. The molecular weight of the 6th instar larval hemolymph proteins were 60.57, 72.50 and 100.83 kDa (Fig. 6, lane 1). The molecular weight of proteins in the prepupal hemolymph sample (Fig. 6, lane 3) were having molecular weights 40, 44, 70, 74, 84 and 105 kDa.

Protein profiling of silk glands showed variation in the quality of proteins during development. Silk gland from 6th instar larvae showed 13 bands most of the intense bands of which were in the low molecular weight region (Fig. 7, lane 1). Silk gland of 7th instar larvae showed 10 bands, in which bands in the molecular region 48, 54, 77 and 80 kDa showed high intensity (Fig. 7, lane 2). In the prepupal stage, number of protein bands were less compared to 6th and 7th instar larval stage and bands with molecular weight 32, 38, 46 and 77 kDa showed high intensity. (Fig. 7, lane 3).

As far as the proteins in the ovary was concerned, the concentration of protein was higher in the 3-day old adults compared to 1-day and 2-day old insects. Samples from 1-day old ovary showed five protein bands with molecular weights 40.34, 45.79, 70.30, 74.95 and 80 kDa (Fig. 8, lane 1). Even though the concentration of protein is higher in the 2-day and 3-day ovary samples compared to 1-day old ovary, the number of protein bands were less in the 2-day and 3-day old ovary samples. Two day old ovary sample showed three bands with molecular weights 70, 74 and 80 kDa (Fig. 8,

lane 2). In the case of 3-day old ovary four bands with molecular weights 45, 70, 74, 80 kDa were observed (Fig. 8, lane 3). Proteins with molecular weight 80, 74 and 70 kDa were found to be common in 1-day, 2-day and 3-day ovary samples. A protein band with molecular weight 45 kDa was present in the 1-day and 3-day old ovary samples.

The analysis of qualitative and quantitative changes in the proteins in various tissues of the insect, such as hemolymph, fat body, ovaries and the silk glands gives the general pattern of translocation of proteins during development. Fat body, being the site of storage of proteins showed a higher level of protein concentration, concentration of proteins present in the fat body showed an increasing pattern during the development of insect from larvae to adult. But pupal fat body showed a higher value compared to adult fat body, this increased concentration of protein in pupal fat body may be due to the sequestration of both the hemolymph and silk gland proteins in to the fat body during pupal development. Concentration of proteins is higher in hemolymph compared to fat body and silk gland. In the case of ovary, protein concentration was found to be increasing from day one to day three. The quantitative analysis of proteins on different tissues of the insect showed an increasing pattern during development.

4.2. Analyses of ecdysteroids

4.2.1. Qualitative analysis of ecdysteroids

Ecdysteroids extracted from whole larvae (4th, 5th, 6th and 7th instar), hemolymph of 6th and 7th instar larva, prepupae and whole pupae of *O. exvinacea* and ecdysteroid standard (20 µl equivalent to 10.6 µg) were separated on HPLC. An obvious peak and some small peaks were found in all samples. The main peak had a retention time of 3.2 min which was similar to that of standard 20E (Fig. 9). This shows that the main ecdysteroid present in the extract derived from the hemolymph of 6th and 7th larval instars and prepupae, whole larvae and pupae of *O. exvinacea* is 20-ecdysteroid.

4.2.2. Quantitative analysis of ecdysteroids

Extracts prepared from the whole bodies of larvae, pupae, hemolymph, fat body etc. were subjected to HPLC separation. Concentrations of ecdysteroid present in the samples were calculated by using the area per cent of the peaks obtained from the sample run, comparing with that of a known quantity of standard ecdysteroid.

$$\text{Concentration of the sample} = \text{Concentration of the standard} \times \frac{\text{Area of sample}}{\text{Area of standard}}$$

1.Whole body of 4th instar larvae

The ecdysteroid sample extracted from the whole body of 4th instar larvae of *O.exvinacea* was subjected to HPLC analysis. It showed a major absorption peak with an area percentage of 54.543. The peak showed similarity with the peak obtained for standard ecdysteroid. The titre of ecdysteroid present in the sample was calculated to be 1.72 ng/ larva (Fig. 10).

2. Whole body of 5th instar larvae

The HPLC chromatogram of ecdysteroid sample obtained from the whole body of 5th instar larvae of *O. exvinacea* showed a major absorption peak at retention time of 3.289. The peak area of the ecdysteroid present in the sample was 69.365%. Showed 99% similarity in retention time with the standard ecdysteroid. The titre of ecdysteroid present in the sample was calculated as 201.3 ng/larva (Fig. 11).

3. Whole body of 6th instar larvae

The ecdysteroid present in the whole body extract of 6th instar larvae of *O. exvinacea* showed a major peak of ecdysteroid at a retention time of 3.282 which was near to the retention time observed for the peak obtained for the standard ecdysteroid. Area of the peak was 61.816% of the total for the

sample. The titre of ecdysteroid present in the sample was found to be 224.7 ng/larva (Fig. 12).

4. Whole body of the 7th instar larvae

The HPLC separation of ecdysteroid sample present in the whole body of 7th instar larvae of *O. exvinacea* showed only one major peak in the chromatogram with a retention time of 3.270. Area percentage of the peak was 67.398%. Peak showed 99% similarity in retention time with that of standard ecdysteroid. The titre of ecdysteroid present in the sample was derived to be 433.8 ng/larva (Fig. 13).

5. Hemolymph of 6th instar larvae

The ecdysteroid sample extracted from the 6th instar larval hemolymph was subjected to HPLC for the quantitative and qualitative analysis of ecdysteroids present in it. It showed a major peak at retention time of 3.298 which was similar to that of standard and area percentage of the peak was 35.167%. The titre of ecdysteroid present in the sample was thus found to be 700 ng/10 µl (Fig. 14).

6. Hemolymph of 7th instar larvae

The ecdysteroid sample obtained from the hemolymph of 7th instar larvae of *O. exvinacea* showed one major absorption peak from HPLC analysis and it was at retention time of 3.296. Similarity in retention time of

ecdysteroid sample with that of standard ecdysteroid was 99%. The titre of ecdysteroid present in the 7th instar larval hemolymph was found to be 600 ng/10 µl (Fig. 15).

7. Hemolymph of prepupae

The ecdysteroid sample obtained from the hemolymph of prepupae of *O. exvinacea* showed one major absorption peak in HPLC analysis and it was at retention time of 3.311. Similarity in retention time of ecdysteroid sample with that of standard ecdysteroid was 99% and area percentage of the peak was 98.735%. The titre of ecdysteroid in the prepupal larval hemolymph was 1800 ng/10 µl (Fig. 16).

8.One day old whole pupae

The ecdysteroid sample extracted from one day old pupae was subjected to HPLC analysis for checking the concentration of the ecdysteroid. The HPLC chromatogram showed only one major peak with a retention time of 3.272. Peak area represented 45.386% of the total area of peaks. This peak also showed similarity in retention time with ecdysteroid standard. The titre of ecdysteroid present in the 1-day old pupal extract was 8.62 ng/pupa (Fig. 17).

9. Two day old whole pupae

The HPL chromatogram obtained for the ecdysteroid sample extracted from the 2-day old pupa of *O. exvinacea* showed similarity in retention time with the standard ecdysteroid. It showed a major peak at retention time 3.224 and the area of the peak was 66.5%. of the total. The titre of ecdysteroid present in the 2 day old pupa was 13 ng/pupa (Fig. 18).

10. Three day old whole pupae

A major peak with retention time 3.209 was observed for ecdysteroid sample extracted from 3-day old pupa of *O. exvinacea* and the area percentage of the sample was 79.865%. The titre of ecdysteroid present in the 3 day old pupa was 644 ng/pupa (Fig. 19).

11. Four day old whole pupae

Major peak obtained for the ecdysteroid sample extracted from 4-day old pupa of *O. exvinacea* showed a retention time of 3.220 and area percentage of the peak was 77.902%. The titre of ecdysteroid present in the 4 day old pupa was 1230 ng/pupa (Fig. 20).

The titre of ecdysteroid in the hemolymph of 6th instar larvae was a higher titre value 700 ng/10 µl whereas it was slightly lower in the 7th instar larval hemolymph (600 ng/10 µl). But the hemolymph of prepupal larvae showed a higher value (1800 ng/10 µl) than 6th and 7th larval hemolymph

(Table 5, Fig. 21). The ecdysteroid in the extract of whole body of the 4th instar larvae showed a titre value 1.72 ng/ larva which increased considerably in the 5th, 6th and 7th larval instars. The whole body ecdysteroid in the 5th instar larvae showed a value of 201.3 ng/larvae and the quantity further increased in the sixth and the seventh instar larvae to 224.7 ng/larva and 433.8 ng/larva respectively (Table 6, Fig. 22). The prepupae showed a higher titre value for ecdysteroid (1800 ng/pupa) compared to ecdysteroid titre of all other samples. The titre of ecdysteroid in the pupae showed a higher value (1230 ng/pupae) for the four day old pupae compared to the 1st, 2nd and 3rd day old pupae. The titre of ecdysteroid for 1 day old pupae was 8.62 ng/pupa and for 2 day old pupae it was 13 ng/pupa. The titre of ecdysteroid was higher (644 ng/pupa) in 3-day old pupae compared to 1-day and 2-day old pupae (Table 7, Fig. 23).

4.3. Determination of the functional groups present in the ecdysteroid using FTIR

4.3.1. Ecdysteroid standard

FTIR analysis of ecdysteroid standard showed three major peaks for three functional groups present in the ecdysteroid structure (Fig. 24). The presence of a number of hydroxyl groups (OH) on most ecdysteroids exhibit strong absorption in the infrared (IR) spectrum in the region of 3340 cm^{-1} – 3500 cm^{-1}. The standard showed absorption peaks of OH at 3452.92 cm^{-1} and

carbonyl (C=O) at 1637.27 cm^{-1}. Normally C=O shows strong absorption in the range of 1640 cm^{-1} – 1670 cm^{-1}. Here, the small decrease shown in frequency is due to the presence of adjacent C=C bond in the structure of ecdysteroid. A strong absorption peak at 1407.78 cm^{-1} was also observed. This is due to the presence of C=C in the structure.

4.3.2. Extracted samples

1. Whole body of the 4th, 5th, 6th and 7th instar larvae

The FTIR spectrum of the ecdysteroid extracted from the 4th instar of larvae showed mainly three prominent peaks. A strong absorption peak was observed at 3455.33 cm^{-1} for OH functional group present in the ecdysteroid sample. Prominent absorption peak observed at 1635.34 cm^{-1} is due to the presence of C=O and the third peak is for C=C at 1384.16 cm^{-1} (Fig. 25).

The ecdysteroids extracted from the 5th instar larvae also showed three major peaks, at 3488.59 cm^{-1} for OH group, at 1635.82 cm^{-1} for C=O and the third peak at 1453.09 cm^{-1} for C=C (Fig. 26). Similarly the ecdysteroids extracted from the 6th instar larvae of the mango leaf webber showed three major absorption peak, one at 3424.96 cm^{-1}, second at 1635.34 cm^{-1} and the third at 1450.21 cm^{-1} for OH , C=O and C=C respectively (Fig. 27).

The spectrum of ecdysteroid present in the 7th instar larvae likewise, showed three major absorption peaks. Carbonyl absorption peak showed a

shift from 1635.34 to 1628.59 cm^{-1} and OH absorption peak shifted from 3424.96 cm^{-1} to 3266.44 cm^{-1} and C=C at 1444.44 cm^{-1} compared to that of ecdysteroid from the sixth instar larval whole body (Fig. 28).

2. Hemolymph of 6th instar larvae

The FTIR spectrum of ecdysteroids extracted from the hemolymph of 6th instar larvae of *O. exvinacea* showed three main peaks with similarities in the finger print region of 20 ecdysteroid. The prominent absorption peak observed at 1634.86 cm^{-1} is due to the presence of C=O. A broad peak appeared in the 3445.69 cm^{-1} region due to the OH functional group and the third peak was for C=C which appeared at 1384.16 cm^{-1} (Fig. 29).

3. Hemolymph of 7th instar larvae

The spectrum of ecdysteroids present in the 7th instar larval hemolymph also showed three major absorption peaks similar to those obtained in the case of 6th instar larvae. Carbonyl absorption peak appeared at 1634.86 cm^{-1} and the OH absorption peak was at 3434.61 cm^{-1}. The peak corresponding to C=C appeared at 1454.55 cm^{-1} (Fig. 30).

4. Hemolymph of prepupae

The FTIR spectrum of ecdysteroid present in the hemolymph of prepupae of *O. exvinacea* showed three major peaks. Carbonyl absorption

peak appeared at 1633.41 cm^{-1} and the OH absorption peak was at 3448.58 cm^{-1}. The peak corresponding to C=C appeared at 1459.84 cm^{-1} (Fig. 31).

5. Pupae of *O. exvinacea*

The FTIR spectrum of the main eluting peaks for the ecdysteroids extracted from whole pupae during the different days of its development also showed three major absorption peaks for C=O, OH and C=C. The ecdysteroid extract obtained from the pupae, during the 1st day of its pupal stage showed a medium range absorption peak for C=O at 1636.30 cm^{-1} and a broad peak at 3423.99 cm^{-1} for OH group and the third peak at 1434.77 cm^{-1} corresponding to C=C functional group (Fig. 32). Spectra for ecdysteroid sample obtained from the pupae during the 2nd day of its development showed a broad absorption peak at 3423.45 cm^{-1} for OH functional group, a medium range absorption peak for C=O at 1632.45 cm^{-1} and a narrow peak for C=C at 1403.52 cm^{-1} (Fig. 33). Ecdysteroid from the 3rd day of pupal development also showed similarities in the absorption peaks present in the FTIR spectra with that of other ecdysteroid samples, a narrow range C=C absorption peak at 1404.52 cm^{-1} , at 1634.38 cm^{-1} for C=C and a broad absorption peak for OH at 3444.24 cm^{-1} (Fig. 34). The FTIR spectra obtained for the ecdysteroid sample from the 4th day of the pupae showed C=O absorption peak at 1635.34 cm^{-1},for C=C group absorption peak was at

1053.91 cm^{-1} and OH functional group showed a peak of absorption at 3465.46 cm^{-1} (Fig. 35).

Some small peaks were also seen, which are due to the presence of CH_3 groups in the structure of ecdysteroid and aromatic compounds present in the solvent.

Ecdysteroid titre in the whole larvae, hemolymph and pupae of the insect during development showed an increasing pattern. The titre was higher in prepupal hemolymph compared to all other samples. The FTIR spectrum of all the ecdysteroid samples showed three major absorption peaks corresponding to C=C, C=O and OH functional groups, Suggesting/confirming its structure in this insect as 20 ecdysone.

4.4. Juvenile hormone titre measurement using LC-Q-TOF-MS

Juvenile hormones (JHs) are acyclic sesquiterpenoids found in insects. They play a major role in the regulation of metamorphosis, reproduction and development in insects. In some instances they are also involved in the regulation of polyphenisms including color, morphs and castes. Juvenile hormone extract prepared from hemolymph samples from 6th and 7th instar larvae of *O. exvinacea* were subjected to LC-Q-TOF-MS analysis. Juvenile hormone extract 20 µl each prepared as described in the materials and methods (section.3.4.1) and 20 µl of standard JH-III with 2.5 µg concentration were injected into LC-Q-TOF-MS instrument.

Concentration of hormone present in the samples were calculated from the chromatogram using the equation.

$$\frac{\text{Concentration of standard}}{\text{Area of standard}} = \frac{\text{concentration of sample}}{\text{Area of sample}}$$

In ESI-MS JH-III standard give m/z 267 for $[M+H]^+$ (Fig. 40). By visually inspecting the mass spectra (Fig. 41) obtained for the juvenile hormone sample extracted from the 6th instar larval hemolymph of *O. exvinacea* 15 ions differing from the background with *m/z* 45, 89, 133,178, 200, 222, 240, 267, 309, 377, 417, 464, 502, 535 and 588.

The juvenile hormone extracted from the 7th instar larval hemolymph of *O. exvinacea* showed 14 ions differing from the background. Mass of ions were *m/z* 45,89, 117, 133, 178, 200, 222, 245, 267, 332, 385, 471, 536, 588 (Fig. 42).

The total ion chromatogram (TIC) showed retention time on x axis and relative abundance of ions on y axis. The TIC of JH-III standard (Fig. 36) showed a prominent peak at retention time of 6.14 with an area of 267094. The samples, JH extracted from the hemolymph of 6th and 7th instar larvae showed absorption peak with similar retention time, also had similarity in retention time of the standard. The TIC of juvenile hormone extracted from the hemolymph of 6th larval instar showed an absorption peak at retention time 6.15 and the area of peak was 822, the peak was broad in nature (Fig.

37). Similarly, an absorption peak with retention time of 6.16 with an area of 491 was observed in the TIC of juvenile hormone extracted from the hemolymph of 7th instar larvae (Fig. 38).

Concentration of juvenile hormone present in the samples were calculated using the above mentioned equation (Section 4.4). The concentration of JH present in the exact of 6th instar larval hemolymph was 3.8 ng/10µl and which is found to be higher than the concentration of JH present in the 7th instar larval hemolymph which was 2.3 ng/10µl (Table 8, Fig. 39).

The juvenile hormone titre in this insect showed an usual pattern as expected.

CHAPTER V
DISCUSSION

Qualitative and quantitative changes in proteins in different tissues of the lepidopteran pest of mango, the mango leaf webber, *Orthaga exvinacea*, and the role of insect growth regulating hormones, juvenile hormone and ecdysone, in the regulation of the movement of different proteins have been studied in different developmental stages of the insect. It has been revealed that the different proteins present in the tissues such as the fat body, hemolymph, ovary and silk glands showed fluctuation of various levels during development. Proteins serve as one of the major structural and functional molecules meeting demands in the changing internal milieu during growth and development in animals. Storage, mobilisation and transport of proteins are influenced by hormones secreted from brain or such other glands in response to signals arising from external as well as internal milieu. In insects, fat body serves as the main site of storage of proteins which has been considered equivalent to the vertebrate liver and adipose tissue combined. Besides proteins, lipids in the form of triacylglyerols and carbohydrates in the form of glycogen are also stored in the fat body. In both hemimetabolous and holometabolous insects, voracious feeding and accumulation of the above reserves takes place during the early stages of postembryonic development. Hemolymph act both as a pool of proteins and several other macromolecules

including the blood sugar, trehalose, as well as a medium of transport of these macromolecules from and to different organs during changing demands. The usual targets for the mobilised metabolites are flight and leg muscles, ovary and silk glands (in the case of silk spinning insects).

In the present investigation, it has been shown that the fat body protein accumulation increases as the larva grows and during the last larval instar and the prepupal stage, the quantity shows a considerable and sharp increase. Maximum accumulation is achieved towards the time of pupation. However, during metamorphosis involving complete reorganisation of the different larval structures into adult structures and ultimate emergence of the adult, the stored materials almost get depleted. My results clearly agree to this phenomena. This pattern of protein accumulation and utilization cycle had been demonstrated in several insects. Qualitative and quantitative changes of protein in different tissues during development of sericigenous and non-sericigenous insects have been studied, and it reveals that different proteins affect various life stages of insects such as moulting, metamorphosis, diapause, reproduction etc. An increase in concentration of protein was observed in the fat body and hemolymph from early larval stadia in *Cydia pomenella,* Colorado potato beetle and *A. mylitta*to shortly before pupation (Brown, 1980; Koopmanschap*et al*., 1992; Kumar *et al*., 2008). Damara *et al.* (2010) and Wang and Jiang (2010) reported the hemolymph protein sequestration by the fat body in *Corcyra cephalonica* and *Manduca sexta*

respectively. According to Kumar *et al*. (2011), comparatively less concentration of protein is found in feeding and mature stage of 4th instar larvae than the 3rd and 5th instar of *A. mylitta* due to the requirement of protein for the development of silk gland, hemolymph protein concentration was high in the feeding larvae compared to mature larvae in 3rd instar. . Proteins are capable of forming complexes with the divalent metals in the hemolymph of insects, their presence and role in different physiological conditions of insect hemolymph was reported (Florkin and Jeuniaux, 1974; Mullin, 1985; Choudary*et al*., 2004; Kumar *et al*., 2008; An and Kanost, 2010; Damara and Gupta, 2010). The effect of AKH and CC-extract on the protein synthesis by the fat body of *Iphita limbata* was studied in vitro and found that both syn AKH-1 and CC-extract inhibit protein synthesis by the fat body. Same result was observed for the male and female insects. The changes were also observed for protein profiling of hemolymph samples. During starvation concentration of the protein showed a decrease in both the hemolymph and fat body of the *Iphita limbata* corresponding results were obtained for protein profiling, the number of bands get decreased (Rasheed and Gokuldas, 2002). In *A. mylitta* fat body protein concentration was found to increase from 3rd to 5th instar larvae and the concentration of protein is higher in pupal fat body (Kumar *et al*., 2011). It was also seen that a higher concentration of protein was found in the hemolymph and midgut of 3rd and 5th instar feeding larvae and in the 4th instar mature larvae.

The hemolymph protein levels also showed corresponding increase during development from 6th instar to prepupal stage. However, it should be noted that the increase was gradual and very small. The average level of protein in the hemolymph of 6th instar larvae appeared higher (expressed as µg/ml) compared to the level proteins in the fat body expressed as µg/mg). The rate of increase was slow in the hemolymph, whereas the rate of increase in the case of fat body protein,especially during the prepupal-pupal development.

Changes in protein concentration in the fat body and hemolymph of *Bombyx mori* was studied in 5th instar larvae and was found to be high in hemolymph (6584±384 mg/100ml) compared to fat body (246.4±26.1 mg/g) (Surendranath *et al*., 1997).

According to the report of Murthy (2015), in multivoltine, bivoltine and crossbreed of silkworms, there occurs a change in hemolymph protein concentration during development. On day one of the 5th instar larval development, hemolymph showed protein concentrations of 13.18±0.51, 20.10±0.51 and 16.82±0.86 mg/g respectively for multivoltine, bivoltine and crossbreed. All the strains showed increase in protein concentration from day one to day 7 of the larval development (Murthy, 2015). The concentration of protein present in the hemolymph of muga silkworm *Antheraea assamensis* grown on two host plants were studied and result showed that there occurs an

increase in protein concentration from 3rd to 5th instar larvae (Talukdar, 2015). An increase in concentration of protein in the hemolymph of *M. americanum* was observed by Loughton and West (1965), concentration remained between 2-3% during the first five larval instars and thereafter a rapid increase in the concentration of protein was observed in the 6th larval instar (5-6%) and this trend continued up to first half of the pupal stage, but gradually decreased in the later half of the pupal stage. Studies on Eri silkworm showed an increase in concentration from mid instar of 5th instar larva to ripening and observed a significant difference between the protein concentration in the middle period and ripening period, this increase in concentration of protein is related with larval growth, particularly at metamorphosis, an extensive synthesis of protein take place and leads to the conclusion that protein synthesis is active during spinning.(Goswami, 1990., Priyanki and Jogen, 2017).

In the present study, a pattern similar to the above reports was observed. Protein concentration showed a gradual increase from 6th instar larvae to prepupal stage. The studies showed that increase in concentration of protein considered to be related with the larval growth because during growth particularly in metamorphosis an extensive synthesis of protein is known to be take place.

Silk secreting glands are unique in insects, mites and spiders. Silk is produced by modified labial glands (salivary glands) or by glandular cells scattered in different parts of the body in insects such as the skin and Malpighian tubules and by specialised glands of various types in the case spiders. Silk in these arthropods serve different functions in them. In insects, silk threads are used for egg attachment, dispersal and nesting, for the construction of protective galleries etc. Major groups of insects that produce silks are Bombycidae, Saturniidae, Lasiocampidae, Pyralidae etc. In most of these cases, silk is produced only during pupation whereas in the other cases, webs of various shapes and structure are used throughout their entire life span. The elaborate cocoon, which is the source of the silk yarn in the silk industry, is continuously secreted and extruded from the labium in the form of fine threads. The entire cocoon is made up of a single continuous thread which may have a length of 400-600 meters. The silk in the case of *Orthaga exvinacea* is secreted by the larvae throughout its larval stages, serve to hold the leaves of mango together to form a loose cage-like structure using the silken web, while the larvae eat the entire leaves from inside, leaving the veins of the leaves. During the later part of the larval stage, the prepupa prepares a loose cocoon, inside which pupation take place. The silk material used for this loose cocoon is very much less than the same cocoon found in the case of *Bombyx*. This probably explains the very slow and gradual increase in the amount of protein in the silk gland during development from

the 6th instar larva to the prepupal stage and the smaller quantity of total protein in the prepupal stage.

During development from 6th, through 7th to prepupal stage, there was an increase in the quantity of protein, but this increase was very small and gradual. This is due to the reason that in *O. exvinacea*, there is no formation of an elaborate cocoon as found in mulberry and non-mulberry silkworms which requires large quantities of silk fibres and hence large amount of proteins. In *Orthaga*, the cocoon is only a very loose bag and the silk fibres are not continuous and compact. The higher quantity of protein in the prepupal salivary gland is to meet the requirement during cocoon spinning in the prepupal stage which is a non-feeding stage, and need to be drawn from the storage in the silk gland.

In the case of fat body, concentration of protein increases from 6th instar to pupal stage, after that a decrease in concentration occurred. During 6th instar of larval stage the concentration of protein was less compared to all other stages in all tissues examined this may be due to the back forth movement of proteins from fat body to hemolymph –hemolymph to silk gland and silk glands to fat body. All the samples of different tissues of 6th instar larva showed bands for proteins in the molecular weight region 60-74 kDa.

Seventh instar larvae had a higher concentration of protein in hemolymph compared to fat body and silk gland. Proteins in the molecular

weight region 54-59 kDa was found in all tissues of 7th instar larvae, proteins in the molecular weight region 43-48 kDa was found to be common in hemolymph and silk gland sample and proteins in the region of 36-38 kDa was common in hemolymph and fat body sample.

During the prepupal stage of the insect protein concentration was higher in the hemolymph sample and low concentration of protein was observed for the silk gland sample. Fat body and silk gland sample showed proteins bands in the molecular weight region 70-77 kDa and 40-46 kDa. Proteins in the low molecular weight region 35- 38 kDa was observed for hemolymph and silk gland samples.

Protein profile of pupal and adult fat body showed several bands in the low molecular weight region 36-65 kDa.

The concentration of protein present in the silk glands were studied in *Bombyx mori* (CSR_2XCSR_4) from day one to day 12 of the 5th instar larval development by Reddy *et al*. (2015) and showed a variation in all the days of development. It showed an increasing pattern during day 1 to day 3, a decrease in concentration of proteins observed on day 4. Thereafter a gradual increase was observed till day 9. From day 10 to day 11 of the larval development it showed a decrease in protein concentration. Again an increase was observed on day 12 compared to day 11. Concentration of protein was

found to be increasing gradually and reaches the maximum during the ripening period of *Bombyx mori* larvae for the preparation of cocoons.

Observations from the present study agree with the above reports. Protein concentration in the silk glands increases from 6th instar to prepupal stage. The high protein content in the prepupal stage may be associated with the cocoon formation for the development of pupa.

In the case of ovary, the amount of protein in the newly hatched adult was considerably high compared to the silk glands. It is to be presumed that the ovary of 1-day old moth is already ready to receive the fertilized egg, in order that the oocyte is supplied with proteins. This amount of protein gradually increases during the second and third day. The protein profile of all ovary sample showed bands in the molecular weight region 70-74 kDa. One day old ovary sample showed more bands compared to 2-day and 3-day old ovary samples, a band in the molecular weight region 45 kDa was found to be common for these two samples.

Quantitative and qualitative analysis of proteins present in the fat body and ovary of *Meliponini quadrifasciata anthidioides* showed a high value of protein concentration for ovary compared to fat body of newly-emerged workers. In nurse workers concentration of protein was high in the ovary extract compared to fat body extract, but in the case of forager workers an increased concentration of protein was observed in fat body compared to

ovary. The protein profile of fat body extract of newly emerged workers lacks bands in the high molecular weight region compared to that of ovary. In the case of nurse workers bands were observed in the high molecular weight region. A similarity can be observed between the molecular masses of protein bands present in the fat body and ovaries in each stage and little variation among the same tissues in the different stages of life cycle. (Paes de Oliveria and Landim, 2004).

In my study using HPLC and FTIR, it was found that the main ecdysteroid component present in the larvae and pupae of *O. exvinacea* is 20-ecdysteroid (20E). Occurrence of 20E as one of the major ecdysteroid was reported in *Schistocerca gregaria* (Tawfik *et al*., 1999). Along with 20E, α-ecdysone also was reported to be a major component. Other compounds included 26E, 2dE and 20, 26-hydroxy ecdysone. Ahmad *et al*. (2007) studied the titre of ecdysteroids in 5th instar larvae of *Bombyx mori*. The titre value was 99.83 µg/ml on 6th day of the larval development and it shows an increase until the 7th day up to 115.45 µg/ml. In the 5th larval stadium of *Bombyx mori* the concentration of ecdysteroids was found to be high on day 7 and it decreases after that until larvae stopped eating and started making cocoon. The minimum amount of ecdysteroid was observed on day 8, two days before larvae started making cocoon. The loss of ecdysteroid soon after eclosion may account for the initial titre decline (Handler, 1982; Schwartz *et al*., 1989). Bownes *et al*. (1984) reported a 5 pg of ecdysone equivalents per

mg live weight in females that were 3 to 4 days post-eclosion. During the first 3 days of post eclosion period in *D. melanogaster* an increase in ovarian ecdysteroid titre was reported.

In the present study it was found that ecdysteroid titre is high in 6th instar larval hemolymph compared to 7th instar larval hemolymph but, it increases in prepupal stage and again an increase was observed during the last day of pupal development. The titre of ecdysteroid in the hemolymph of 6th instar larvae showed a higher titre value of 700 ng/10 µl than that of 7th instar larval hemolymph (600 ng/10 µl). The ecdysteroid in the whole body of the 4th instar larvae showed a titre value 1.72 ng/larva which is less than the titre value of ecdysteroid present in the 5th, 6th and 7th larval instars. The whole body ecdysteroid in the 5th instar larval stadium showed a titre value of 201.3 ng/larva and the sixth and the seventh instar larval ecdysteroid showed titre value 224.7 ng/larva and 433.8 ng/ larva respectively. The titre of ecdysteroid in the pupae showed a higher value (1230 ng/pupa) for the four day old pupae compared to the 1st, 2ndand 3rd day old pupae. The titre of ecdysteroid for 1-day old pupae was 8.62 ng/pupa and for 2-day old pupae it was 13 ng/pupa. The titre of ecdysteroid was higher in 3-day old pupae (644 ng/pupa) compared to 1-day and 2-day old pupae. The four day old pupae showed a higher titre value for ecdysteroid (1230 ng/pupa) compared to ecdysteroid titre of all other samples.

The amounts of the hormone in the larval stages appear to be in the normal ranges reported in insects. The hormone titre maintained in the hemolymph meets the requirement for moulting during the larval stages. However, the pupal ecdysteroids show a higher range value than some of the reported values. In *Opogona sacchari*, for example, the titre of ecdysteroids in both male and female pupae reaches a peak of 80 ng/pupae which later dropped to 30 ng/ pupae. It was also seen that the content of ecdysteroids is higher in female pupae than in male pupae during development (Wang *et al*.,2006). Investigations on the sex difference in the titre of ecdysteroids was not attempted in our study. The high value for the pupal hormone titre obtained for this species of insect can only be explained in terms of the species difference.

Structural studies using FTIR showed 3 major absorption peaks, for OH, C=O, C=C functional groups present in the ecdysteroid structure. These functional groups are characteristic of ecdysteroids. Similar functional groups have been reported in some plants using FTIR. For example, in the Caryophyllaceae plant, *Lychnisflos-coculi,* FTIR have shown the presence of functional groups characteristic of insect ecdysteroids which were identified as phytoecdysteroids present in those plants (Louden *et al.,* 2001).

Juvenile hormones are a group of acyclic sesquiterpinoids that regulate many aspects of insect physiology. The changes in titre of juvenile hormone

during the development of the insect modulate metamorphosis in both hemimetabolous and holometabolous insects. Concentration of JH-III present in the hemolymph of 6th and 7th insatar larvae of *O.exvinacea* were determined using LC-Q-TOF-MS and it showed that the titre decreases from 6th instar to 7th instar.

In adult females the JH titre is associated with vitellogenesis and oocyte maturation in most species (Raikhel *et al*., 2005). Treiblmayr *et al*. (2006) determined the concentration of JH-III in hemolymph of *B. germanica* during the last two nymphal instars. The concentration of JH-III in the hemolymph of the fifth nymphal instar was low and the value was found between 5-20 ng/ml and 10 ng/ml was the most frequent value. A low titre value was observed on day 1 and day 6 and higher value was obtained for day 5 hemolymph sample. During the 6th nymphal instar a higher titre value were measured just after the molt (5 ng/ml). In the case of hemolymph from the adult female JH-III titre was found to be increasing during day 1 to day 5 of adult development (70 ng/ml). On day 6 of the development the titre value found to be decreasing and again an increase was observed in 7 day of development until the value reach 80 ng/ml. According to Elekonich *et al*. (2001) the JH titres in the worker honey bee was found to be increasing progressively through the first 15 or so days of the life cycle before the onset of foraging. Juvenile hormone I, II and III in the hemolymph of silkworm *B. mori* were quantified from the 3rd instar to early pupal stage. The titre of JH-

I in the hemolymph had peaks on day 0 of the 3rd and 5th instar immediately after ecdysis (1.5-2.0 ng/ml) and decreased to the lowest level before ecdysis. The titre of JH-II changed similarly to JH-I, but the titre value was lower at 0 day of 3rd and 4th instar, JH-III was not detect during the same period. During the beginning of 5th instar larval stadium JH could not be detected. On day 7 of 5th instar a slight increase in JH-III titre was observed and an apparent increase of JH-I was observed just after pupation (Furuta *et al*., 2013).

The results showed a different pattern of JH titre compared to the above reported cases. This difference in titre value may be species specific.

In my study during the 6th instar of larval development concentration of protein in the fat body was 19.4±1.2 µg/mg and in the case of hemolymph, the value was higher, i.e., 35.75±6.1 µg/ml. In silk gland it was 6.31±0.98 µg/mg. During this stage ecdysteroid concentration was 700 ng/10 µl and that of JH-III was 3.8 ng/10 µl. When larvae develops into 7th instar concentration of protein increases in all the tissues tested. In fat body, concentration of protein was 22.60 ±1.5 µg/mg, in the case of hemolymph sample protein concentration (52±7.4 µg/ml) was high compared to fat body sample. In silk gland, protein concentration increased to 7.08 ±1.12 µg/mg compared to 6th instar but the concentration of protein is less compared to fat body and hemolymph sample of the 7th instar. During this stage the

ecdysteroid concentration in the hemolymph was 600 ng/10 µl and JH-III titre value was 2.3 ng/10 µl. In prepupal stage again an increase in protein concentration was observed in tissues such as fat body, hemolymph and silk glands were observed and which was higher compared to that of the 6th and 7th larval instars.

In pupa concentration of protein was high in fat body compared to all other stages of the development. Ecdysteroid concentration was also high for pupal stage. This high concentration of protein in the pupal fat body may result from the sequestration of proteins from the hemolymph and silk glands into the fat body.

During adult stage the fat body protein concentration was found to be decreasing and this may be due to the utilization of protein for ovarian development in female moths.

Ecdysteroids as well as juvenile hormones, the hormones that regulate the overall development and metamorphosis, are collectively referred to as insect growth regulators (IGRs). Insects are unable to develop resistance to molecules that mimic their own hormones. Compounds such as methoprene, hydroprene, kinoprene, pyriproxifen, RH-5992, diflubenzuron are found to be effective against many dipteran, coleopteran, homopteran and lepidopteran insect pests.Ketokonazole, a synthetic imidazole derivative, is known to inhibit the ecdysone-20 monooxygenase and is very effective in inhibiting the

terminal hydroxylation steps of ecdysteroid biosynthesis in adult locusts and crickets (Jarios *et al.*, 1994; Lorenz *et al.*, 1995). The newest member, RH-2485 of the bisacylhydrazine class, are more effective than tebufenozide and act against a wide range of lepidopteran pests, with a high degree of safety with respect to non-target organisms. A high level of insecticidal activity of methoxyfenozide against a wide range of important caterpillar pests was reported by Carlson *et al*. (2001). A reduction in the egg laying was observed in *Cydia pomonella* treated with RH-5992 and RH-2485 (Knight, 2000; Knight *et al.*, 2001). All these non-steroidal ecdysone agonists manifest their effects through the interaction with the EcR/USP receptor complex. Designing analogues of the ecdysteroids will be challenging in the development of non-chemical insecticides, as these compounds are highly specific to insects, eliciting no reaction on other species of animals. Determining the dosage and formulation will be other areas in which investigations are to be extended. Attempts to control more pests including *O. exvinacea* are to be carried out in order to establish the effectiveness of the new compounds.

SUMMARY

Investigations were carried out to analyse the protein profiles and the titre of hormones involved in the regulation in protein metabolism namely the juvenile hormone and ecdysone in the mango leaf webber, *O. exvinacea* (Pyralidae:Lepidoptera), a seasonal and serious pest of mango trees. Different tissues such as the fat body, hemolymph, ovary and silk glands from different stages of development were subjected to the analyses.

It was found that there was a marked variation in concentration and profile of proteins from fat body, hemolymph, silk glands and ovary during various developmental stages of the insect. Proteins from the fat body, hemolymph, silk glands etc. were measured by Lowry's method. Qualitative studies were done by using SDS-PAGE, method described by Laemmli.

In the case of fat body, concentration of protein increases from 6th instar to pupal stage, after that a decrease in concentration occurred. The 6th instar of larval stage had the lowest level of protein concentration compared to all other stages in all tissues examined. All the 6th instar larval tissue samples showed bands for proteins in the molecular weight region 60-74 kDa.

Seventh instar larvae had a higher concentration of protein in hemolymph compared to fat body and silk gland. Proteins in the molecular weight region 54-59 kDa was found in all tissues of 7th instar larvae, proteins

in the molecular weight region 43-48 kDa was found to be common in hemolymph and silk gland sample and proteins in the region of 36-38 kDa was common in hemolymph and fat body sample.

During the prepupal stage of the insect concentration of protein was higher in the hemolymph sample and low concentration of protein was observed for the silk gland sample. Fat body and silk gland sample showed proteins bands in the molecular weight region 70-77 kDa and 40-46 kDa. Proteins in the low molecular weight region 35- 38 kDa was observed for hemolymph and silk gland samples. Protein profile of pupal and adult fat body showed several bands in the low molecular weight region 36-65 kDa.

In the case of ovary, the amount of protein in the newly hatched adult was considerably high compared to the silk glands. It is to be presumed that the ovary of 1day old moth is already ready to receive the fertilized egg, in order that the oocyte is supplied with proteins. This amount of protein gradually increases during the second and third day. The protein profile of all ovary samples showed bands in the molecular weight region 70-74 kDa. One day old ovary sample showed more bands compared to 2-day and 3-day old ovary samples, a band in the molecular weight region 45 kDa was found to be common for these two samples.

Various biochemical and physiological processes in insects are regulated by the hormones secreted by endocrine glands. Major metamorphic

hormones in insects are juvenile hormone and ecdysteroids secreted by the corpora allata (CA) and prothoracic glands (PTG) respectively into the hemolymph and metabolic hormones are peptide hormones originating from neurosecretory cells in the nervous system. Ecdysteroids drive moulting and metamorphosis and juvenile hormone involved in the determination of nature and time of moult.

The titre of the growth hormones, ecdysteroid and juvenile hormone were determined during the development of the insect using HPLC and LC-Q-TOF-MS. 20-Ecdysone was the main ecdysone found in whole larvae, hemolymph and pupae of the experimental insect, *O.exvinacea*. The functional group determination of ecdysone was done by using FTIR. The presence of JH-III was confirmed in the hemolymph of 6th and 7th instar larvae of *O.exvinacea*

In the present investigation it was found that ecdysteroid titre is high in 6th instar larval hemolymph compared to 7th instar larval hemolymph but, it increases in prepupal stage and again an increase was observed during the last day of pupal development. In the case of JH the titre decreases during the development of the insect.

From the results of the present work we can correlate the concentration of proteins present in different tissues during development with the growth hormone titres. During the 6th instar of larval development concentration of

protein in the fat body was 19.4±1.2 µg/mg and in the case of hemolymph it was 35.75±6.1µg/ml. In silk glands the protein concentration was 6.31±0.98 µg/mg. During this stage ecdysteroid concentration was 700 ng/10 µl and that of JH-III was 3.8 ng/10µl. When larvae develops into 7th instar concentration of protein increases in all the tissues tested. In fat body concentration of protein was 22.60 ±1.5 µg/mg, in the case of hemolymph sample protein concentration (52±7.4 µg/ml) was high compared to fat body sample. In silk gland protein concentration increases 7.08 ±1.12 µg/mg compared to 6th instar but the concentration of protein is less compared to fat body and hemolymph sample of the 7th instar. During this stage the ecdysteroid concentration in the hemolymph was 600 ng/10 µl and JH-III titre value was 2.3 ng/10 µl. In prepupal stage again an increase in protein concentration was observed in tissues such as fat body, hemolymph and silk glands were observed and which was higher compared to that of the 6th and 7th larval instars.

In pupa concentration of protein was high in fat body compared to all other stages of the development. Ecdysteroid concentration was also high for pupal stage. This high concentration of protein in the pupal fat body may result from the sequestration of proteins from the hemolymph and silk glands into the fat body. During adult stage the fat body protein concentration found to be decreasing and this may be due to the utilization of protein for ovarian development in female moths.

It is thus concluded that in the insect *Orthaga exvinacea*, during development, the synthesis, storage and translocation of proteins between fat body, hemolymph, ovary and silk glands are strictly regulated by the presence of the hormones ecdysone and juvenile hormones. Early larval instar (6th) shows evidence for continuing synthesis of proteins which reaches a maximum at pupal stage. Mean while, protein also increases in the hemolymph and ovary. At this stage, the silk gland appears to have very little proteins since the silk requirement at this stage is minimum. Seventh instar protein level shows higher values at which time the level of ecdysone in the hemolymph was low (600 ng/10 µl) and juvenile hormone concentration was 2.3 ng/10 µl. This indicates that the insect is preparing for its next molt, building up protein levels. Ecdysone levels in the whole larvae tested in our experiments clearly indicate a parallel increase in proteins in the fat body of the later stages, viz., 7th instar whole larvae, whole prepupae and 3rd and 4th day adults. The results thus agree with the already reported increase in proteins in the developing *Drosophila* (Bownes, 1989) and *Aedes aegypti* (Hagedorn, 1985; Dittmann *et al*., 1989). A drop in the juvenile hormone titre as shown in figure 39 is also justified as the ecdysone is active as development progresses.

The fact that the hormones involved in the growth, development and metamorphosis of insects have triggered interest in developing mimics using

modern techniques and technologies opens up new avenues for future investigations to develop newer compounds and such derivatives may be used to tamper or derail the normal development and metamorphosis in insects. These methods could be directed to develop novel approaches in integrated pest management strategies.

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Table 1. Total protein present in the fat body of *Orthaga exvinacea* during different developmental stages

Different stages	Amount of Protein (µg/mg)
6th instar larva	19.4 ±1.2
7th instar larva	22.6 ±1.5
Pre pupa	47.3 ±2.0
Pupa	123.6 ±4.6
Adult	43.7 ±3.9

(Values are expressed as means ± SEMs, n= 7)

Figure 1. Pattern of changes in protein concentration present in the fat body of *Orthaga exvinacea* during development

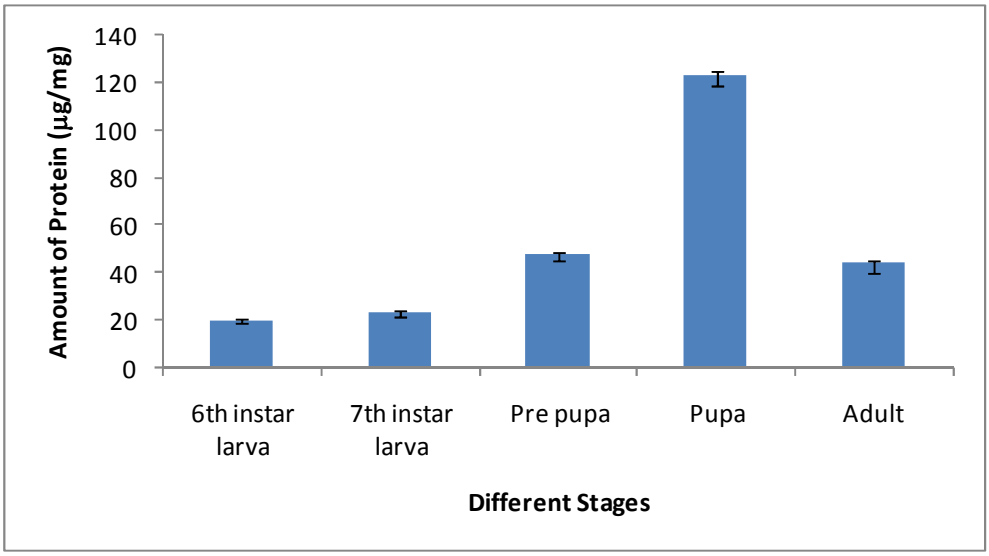


Table 2. Total protein present in the hemolymph of *Orthaga exvinacea* during different developmental stages

Different stages	Amount of protein (µg/ml)
6th instar larva	35.75 ±6.1
7th instar larva	52 ±7.4
Pre pupa	58.25 ±6.0

(Values are expressed as means ± SEMs, n= 7)

Figure 2. Pattern of changes in protein concentration present in the hemolymph of *Orthaga exvinacea* during development

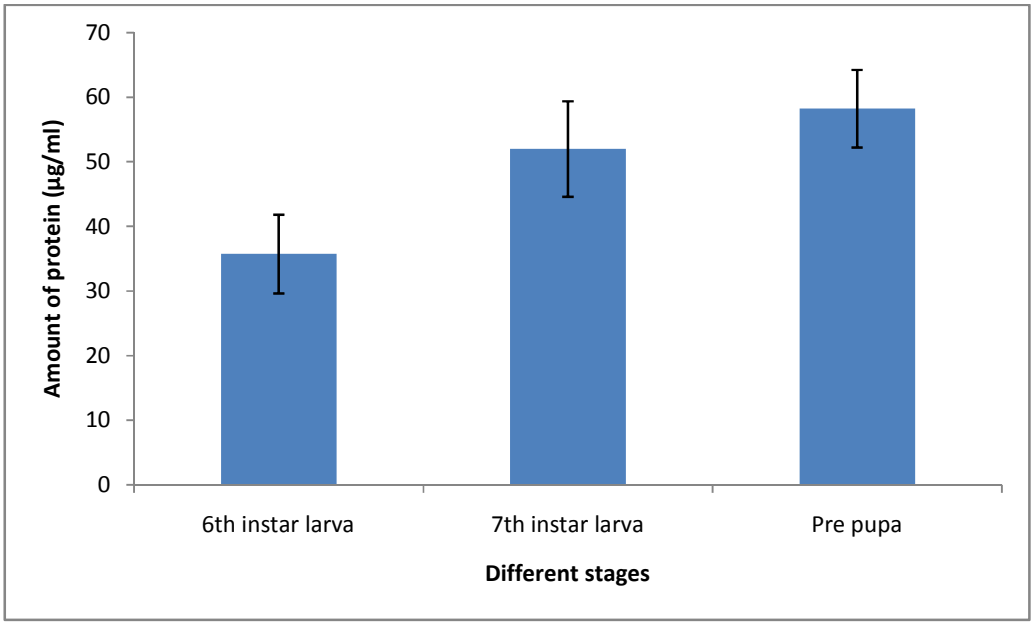


Table 3. Total protein present in the silk glands of *Orthaga exvinacea* during development

Different stages	Amount of protein (µg/mg)
6th instar larva	6.31±0.98
7th instar larva	7.08±1.12
Pre pupa	21.98 ±2.41

(Values are expressed as means ± SEMs, n= 7)

Figure 3. Pattern of changes in protein concentration present in the silk glands of *Orthaga exvinacea* during development

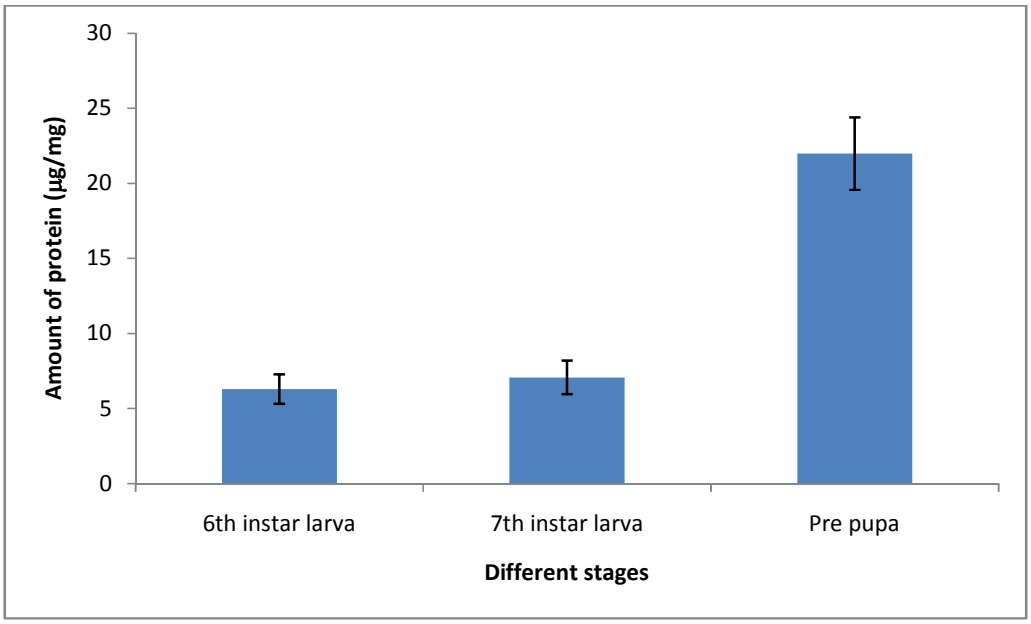


Table 4. Total protein present in the ovary of adult

Ovary		
Ist day (µg/mg)	**2nd day (µg/mg)**	**3rd day (µg/mg)**
30±2.4	35±1.9	37±3.0

(Values are expressed as means ± SEMs, n= 7)

Figure 4. Pattern of changes in protein concentration present in the ovary of adult of *Orthaga exvinacea* during development

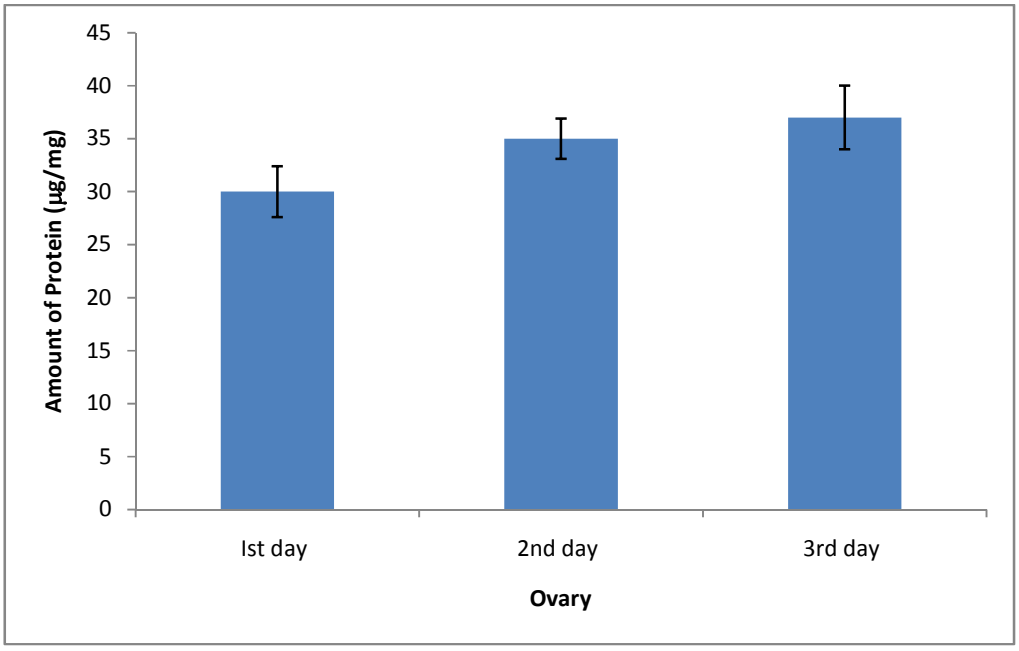
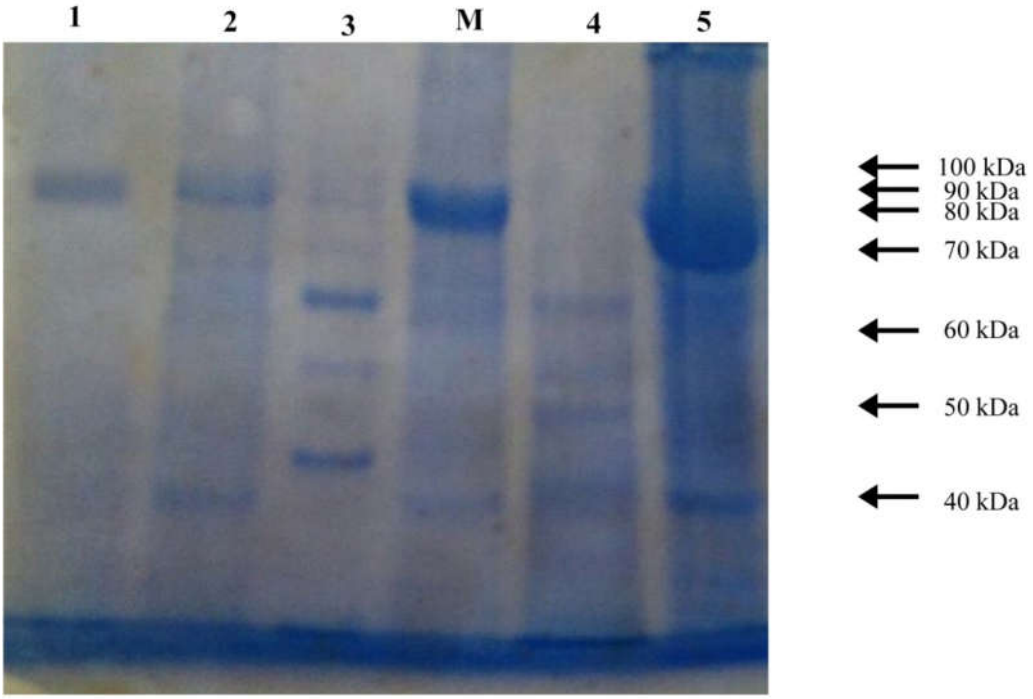
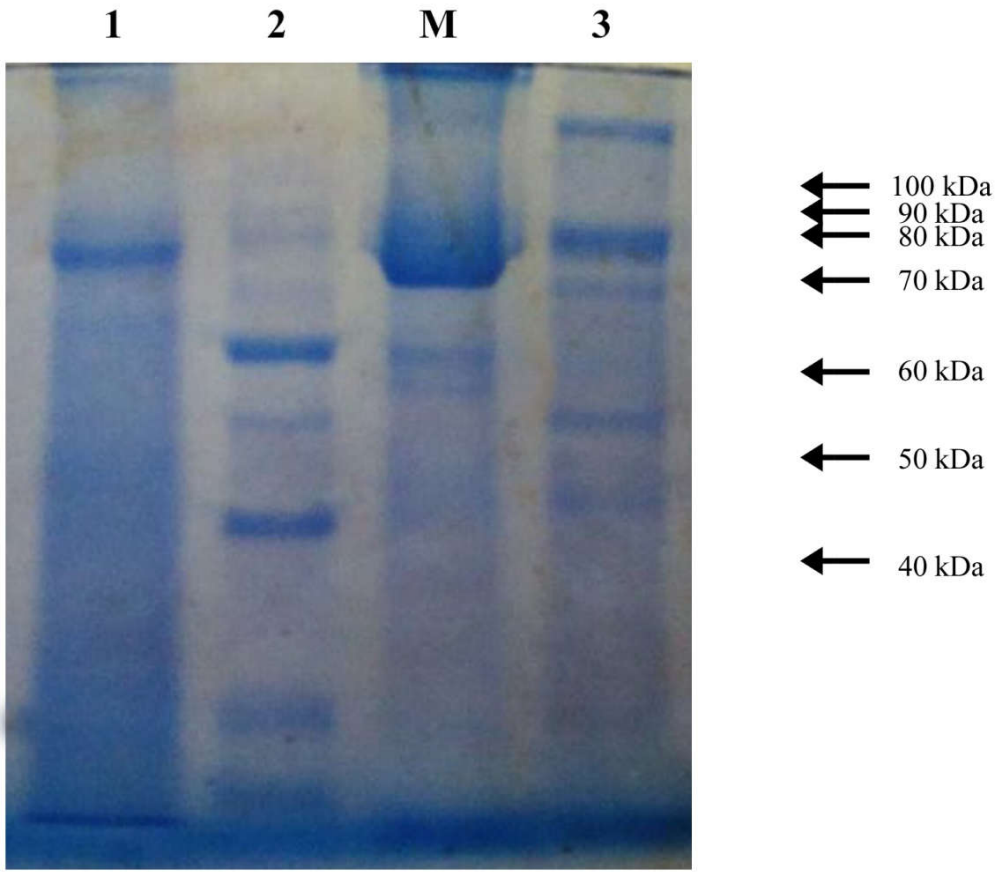


Figure 5. SDS-PAGE analysis of proteins from fat body of *Orthaga exvinacea* during different developmental stages



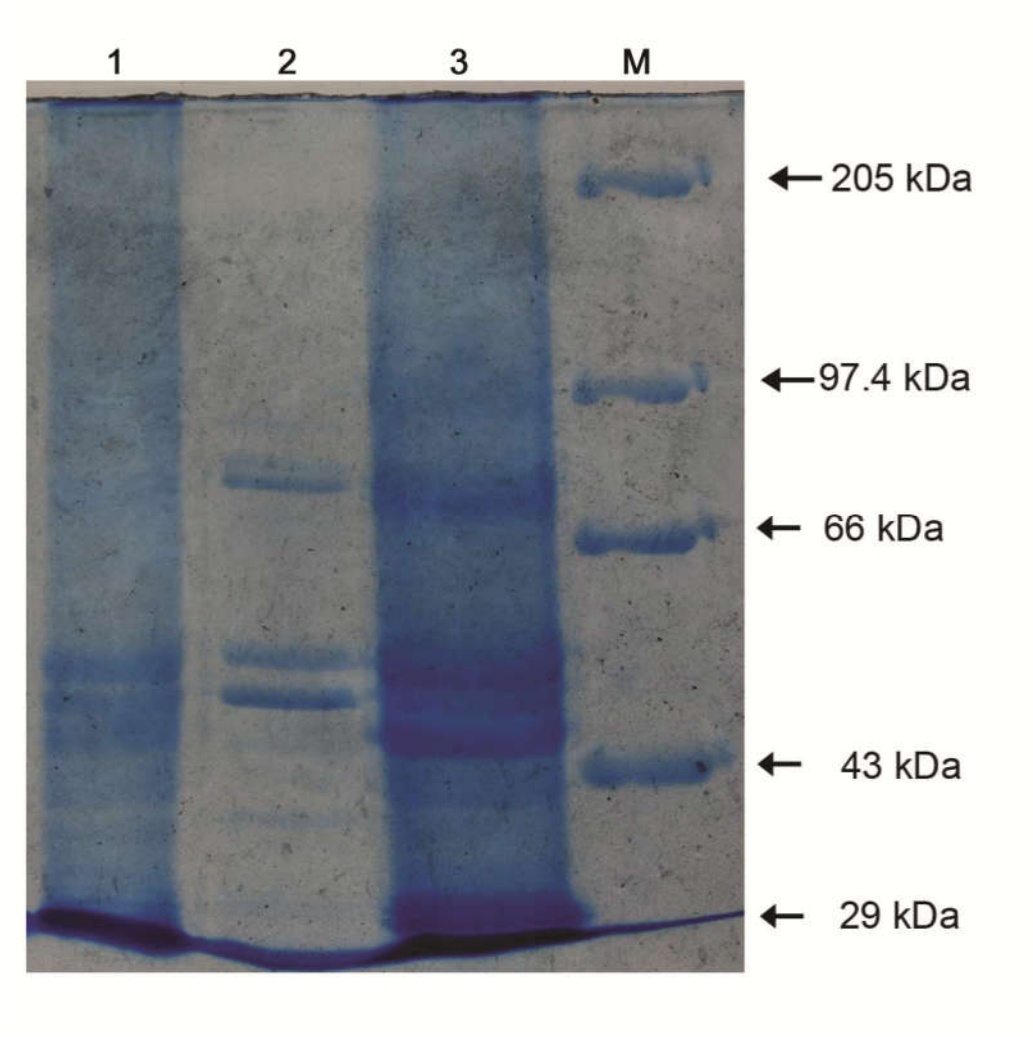
Lane 1. Fat body from sixth instar larvae, Lane 2. Fat body from seventh instar larvae, Lane 3 . Fat body from prepupal larvae, Lane M. Marker, Lane 4. Fat body from adult, Lane 5. Fat body from pupae

Figure 6. SDS-PAGE analysis of proteins from hemolymph of *Orthaga exvinacea* during different developmental stages



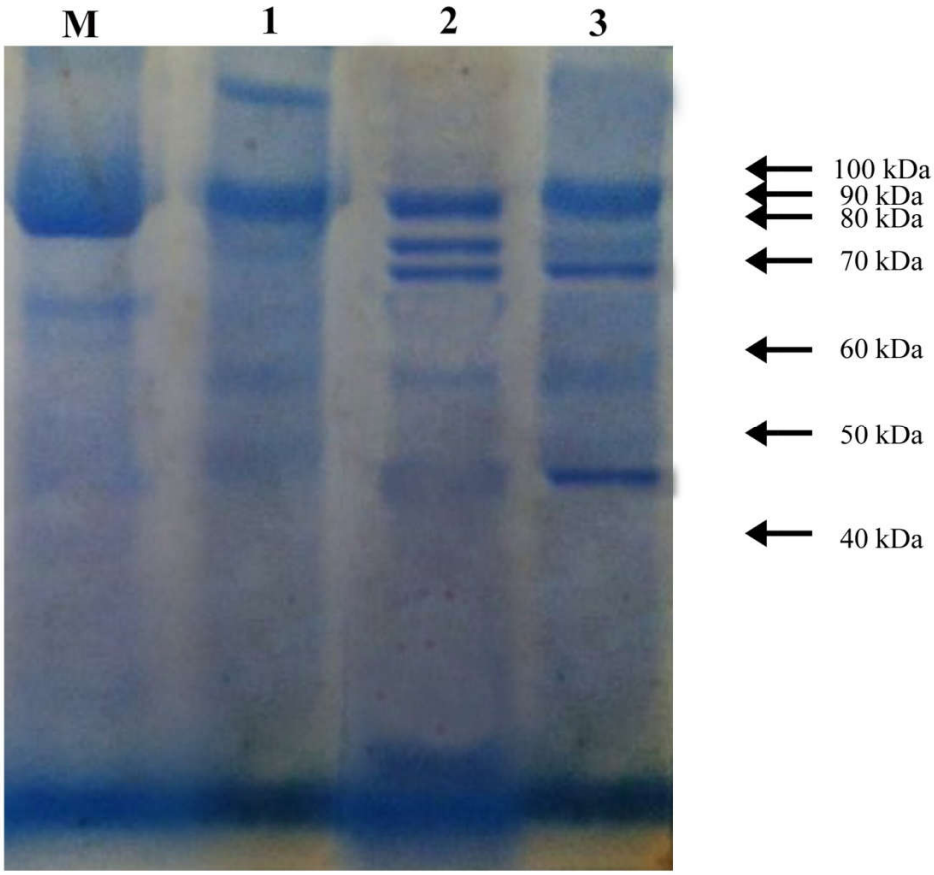
Lane 1.Hemolymph from sixth instar larvae, Lane 2. Hemolymph from seventh instar larvae, Lane M. Marker, Lane 3. Hemolymph from prepupal larvae

Figure 7. SDS-PAGE analysis of proteins from silk glands of *Orthaga exvinacea* during different developmental stages



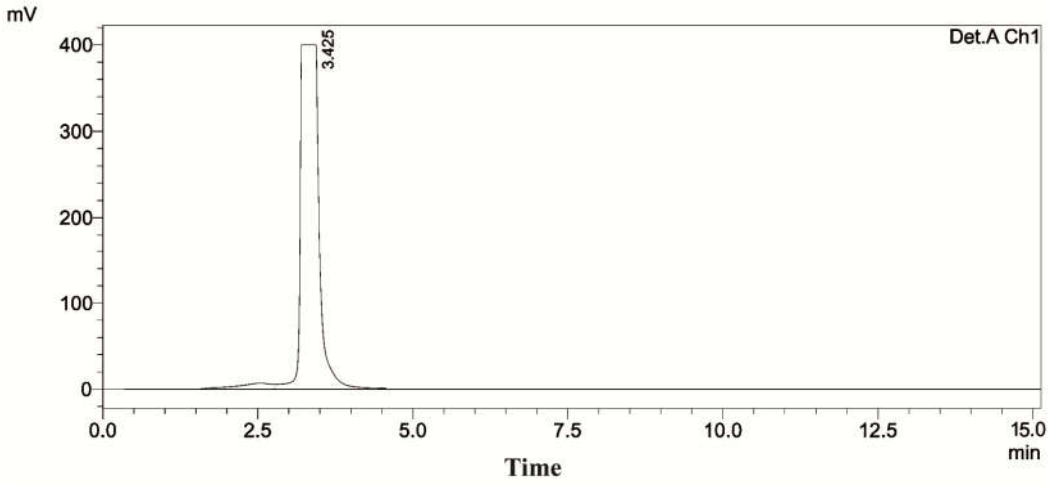
Lane 1.Silk glands from sixth instar larvae, Lane 2. Silk glands from seventh instar larvae, Lane 3. Silk glands from prepupal larvae, Lane M. Marker.

Figure 8. SDS-PAGE analysis of proteins from ovary of *Orthaga exvinacea* during different days of adult development



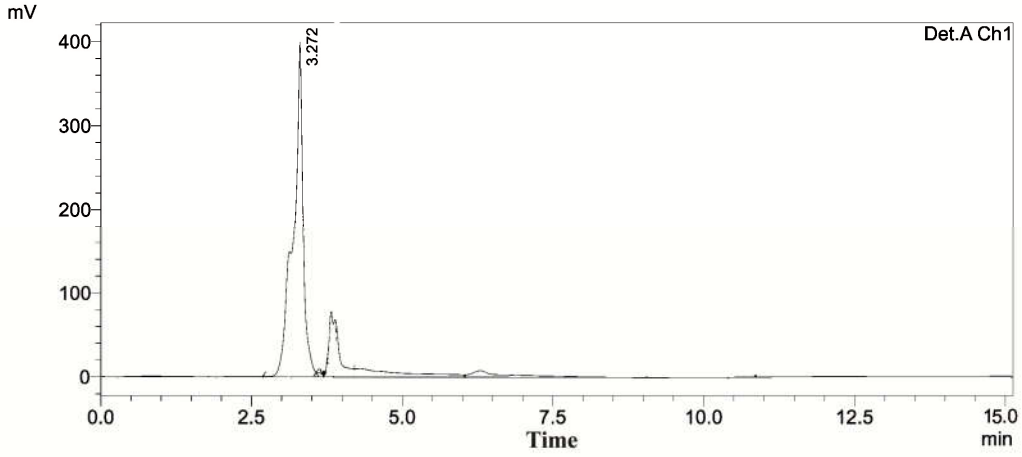
Lane M. Marker, Lane 1. Ovary from one day old adult, Lane 2. Ovary from two day old adult, Lane 3. Ovary from three day old adult

Figure 9. HPLC of the ecdysteroid standard



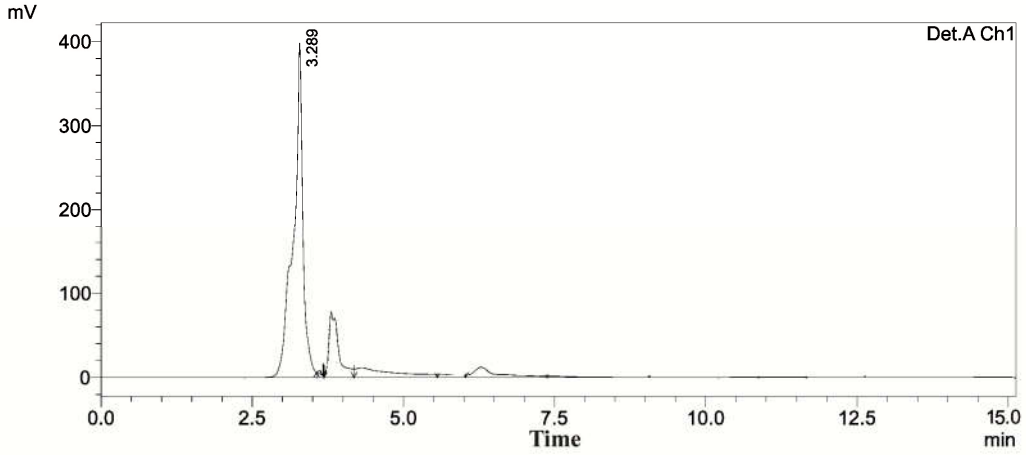
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 10. HPLC of ecdysteroids extracted from the whole body of the 4th instar larvae of *O. exvinacea*



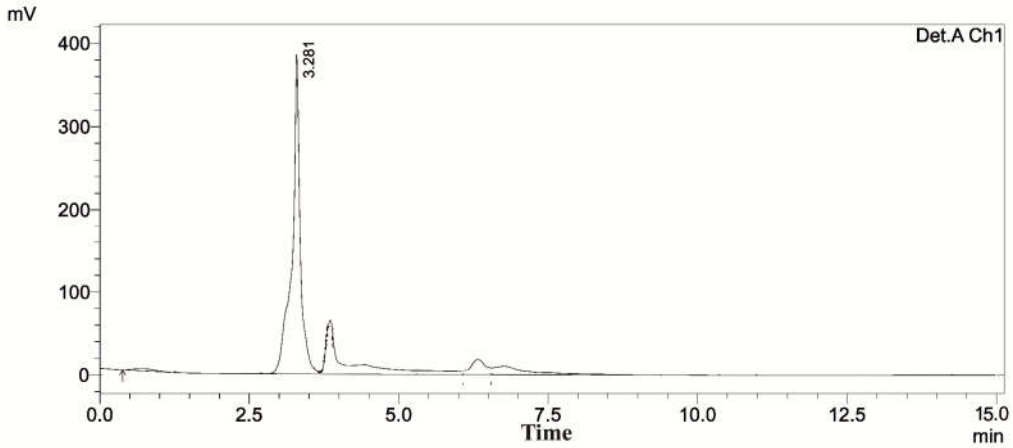
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 11. HPLC of ecdysteroids extracted from the whole body of the 5th instar larvae of *O. exvinacea*



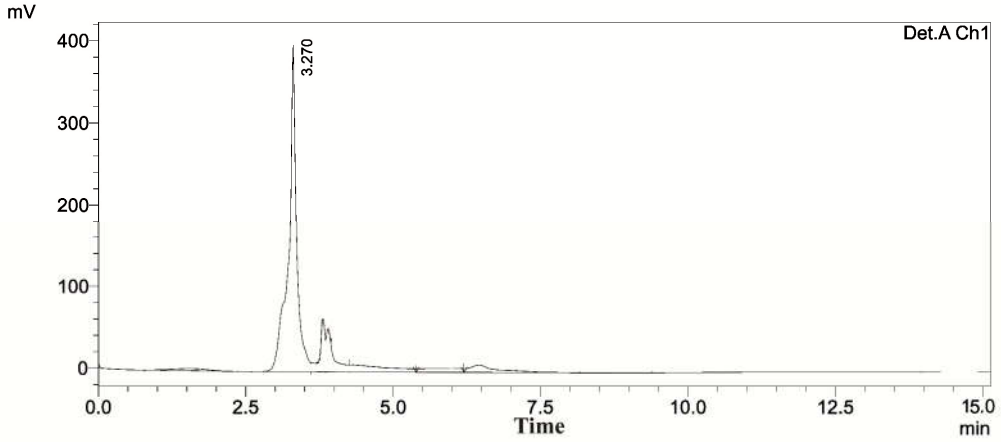
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 12. HPLC of ecdysteroids extracted from the whole body of the 6th instar larvae of *O. exvinacea*



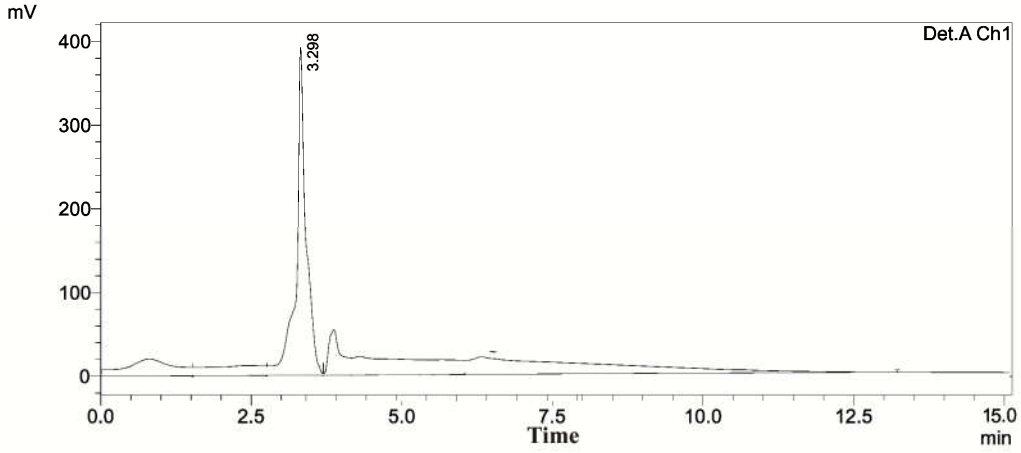
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 13. HPLC of ecdysteroids extracted from the whole body of the 7th instar larvae of *O. exvinacea*



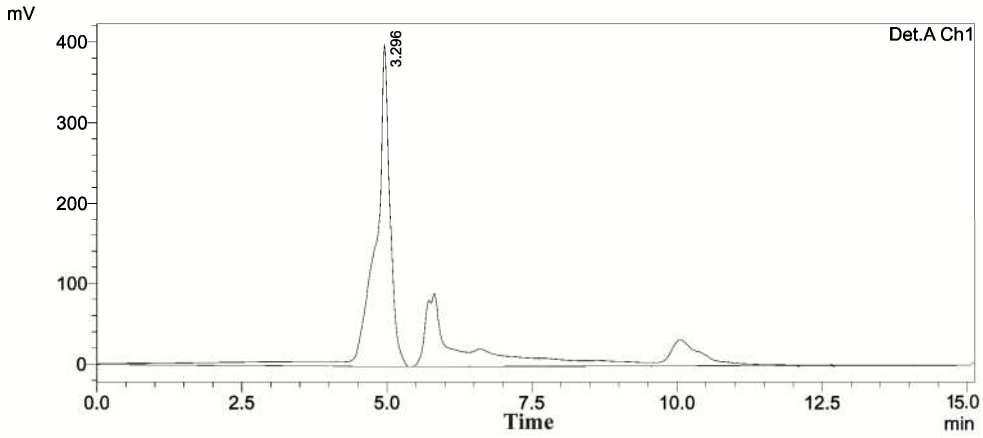
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 14. HPLC of ecdysteroids extracted from the hemolymph of the 6th instar larvae of *O. exvinacea*



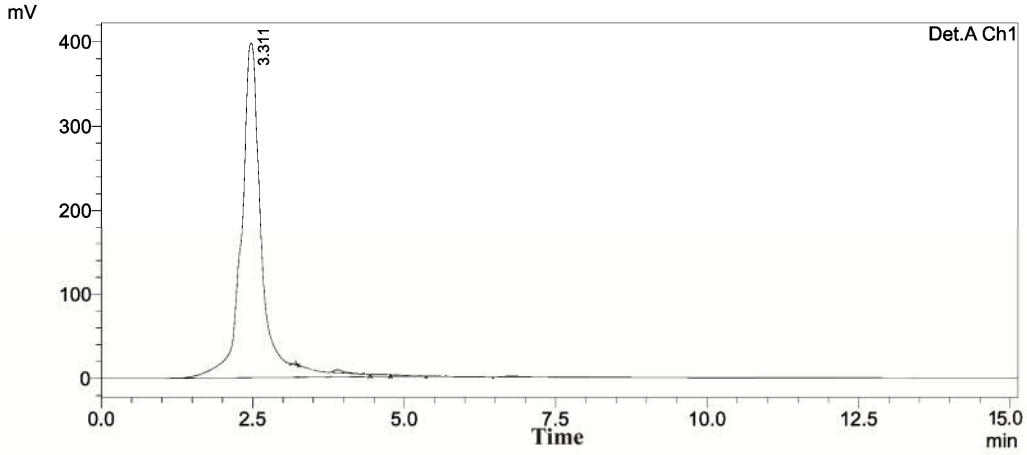
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 15. HPLC of ecdysteroids extracted from the hemolymph of the 7th instar larvae of *O. exvinacea*



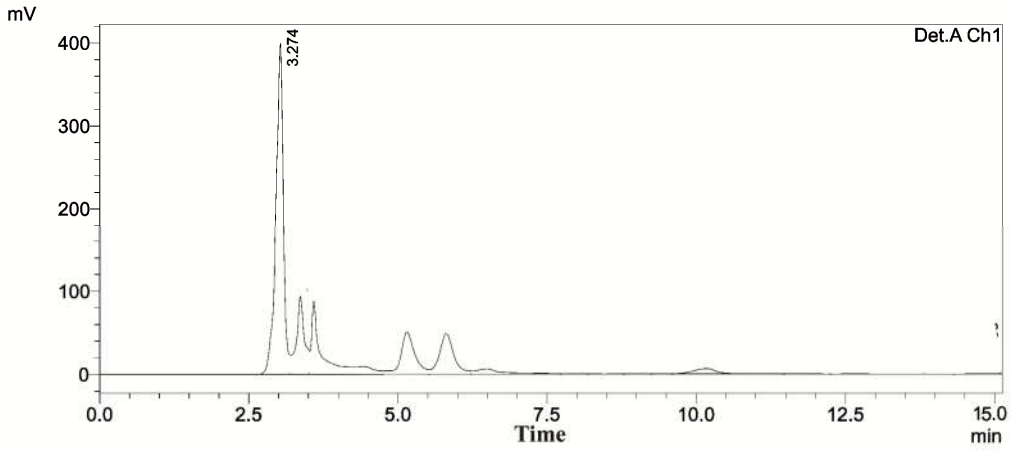
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 16. HPLC of ecdysteroids extracted from the hemolymph of the prepupae of *O. exvinacea*



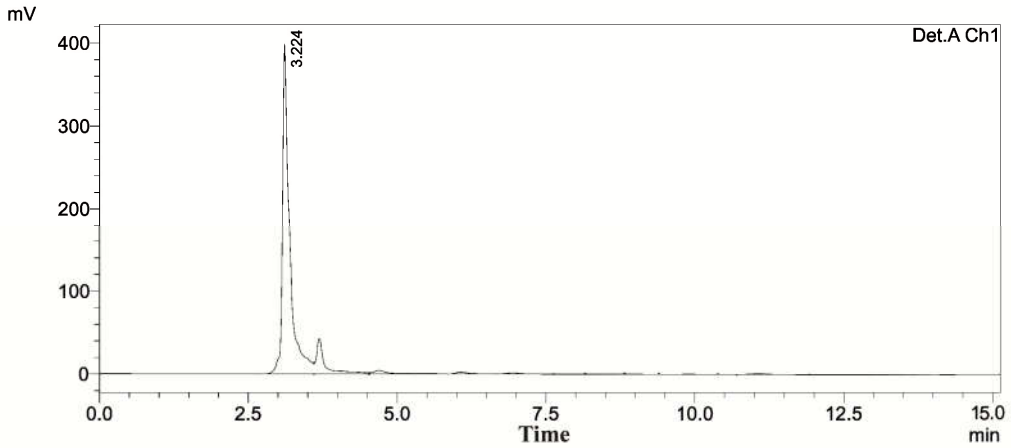
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 17. HPLC of ecdysteroids extracted from the 1-day old pupa *O. exvinacea*



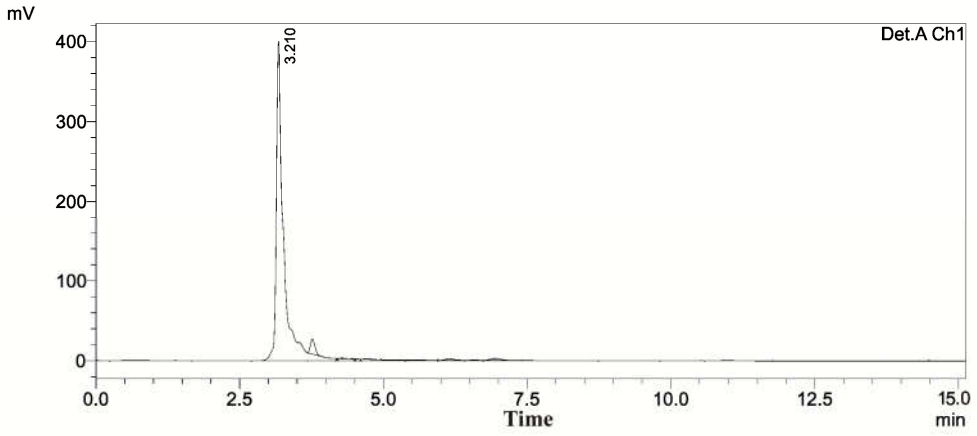
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 18. HPLC of ecdysteroids extracted from the 2-day old pupa *O. exvinacea*



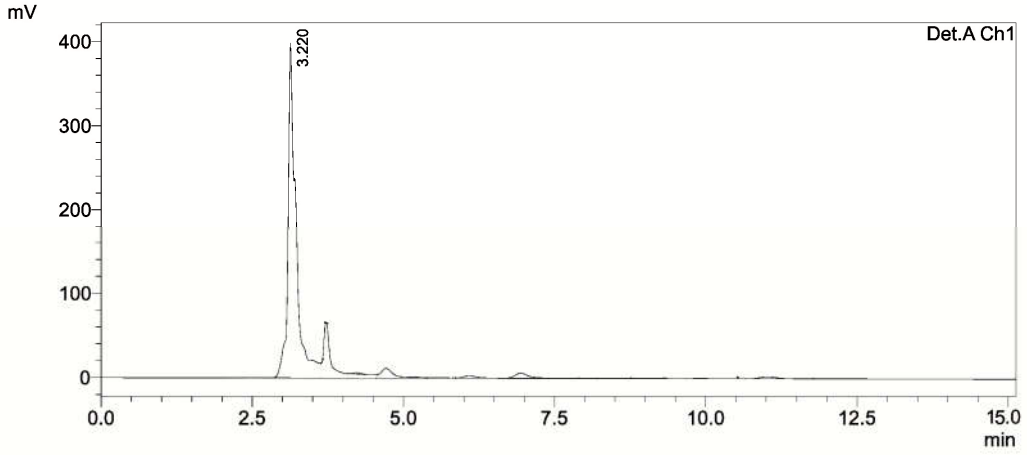
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 19. HPLC of ecdysteroids extracted from the 3-day old pupa *O. exvinacea*



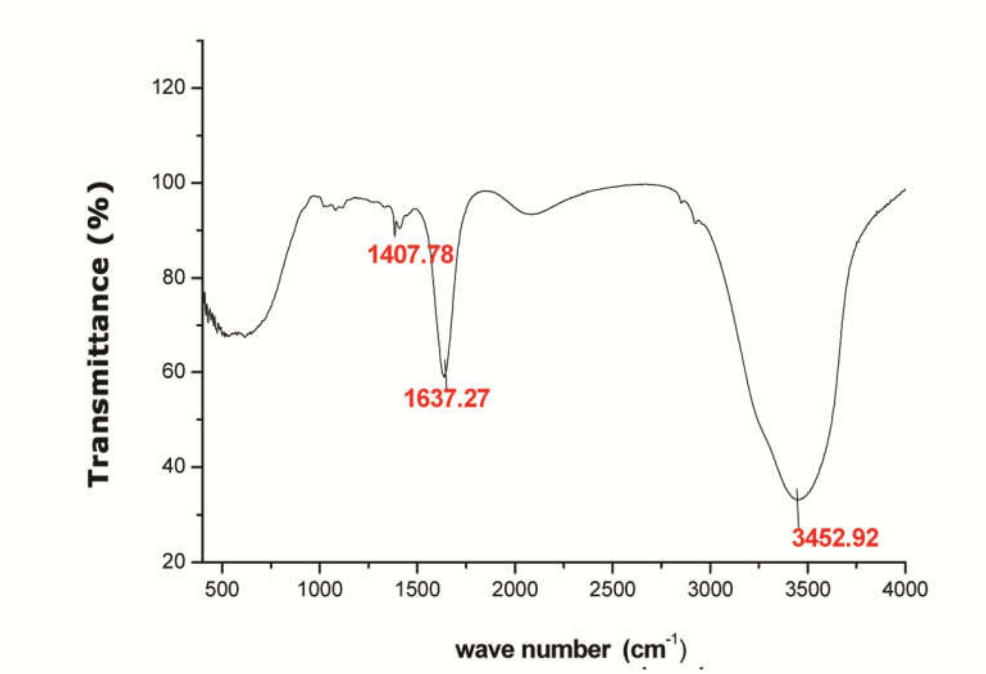
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 20. HPLC of ecdysteroids extracted from the 4-day old pupa *O. exvinacea*



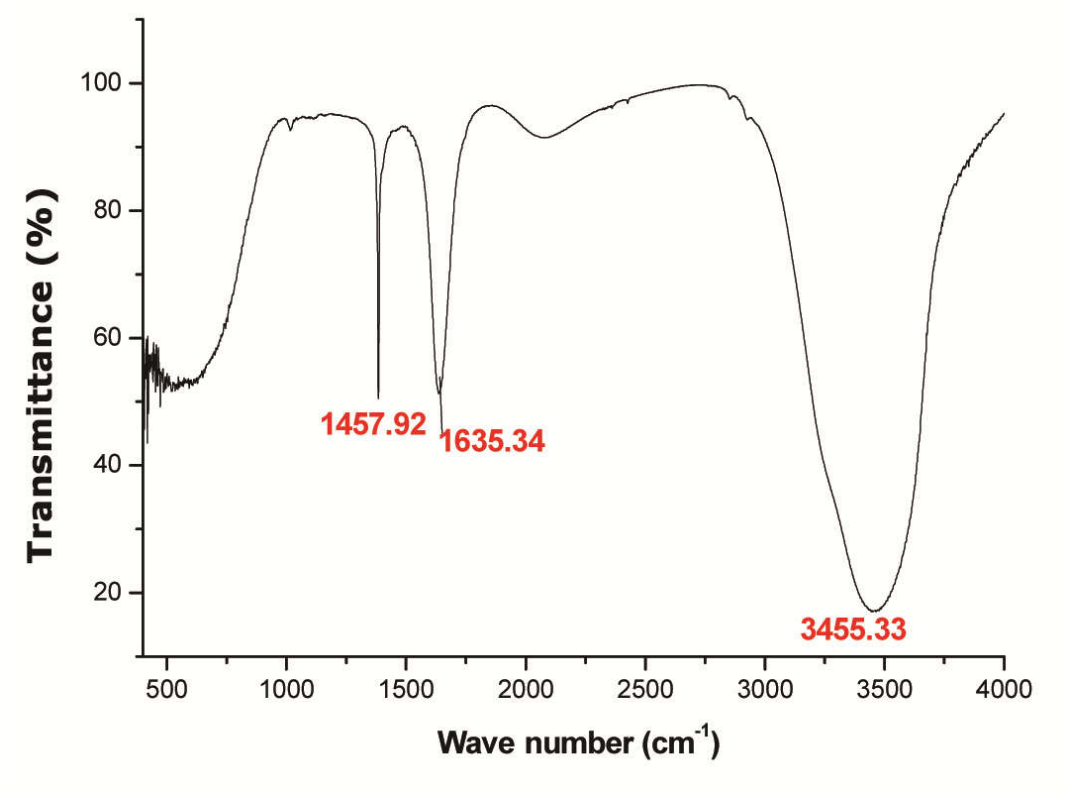
The analysis was carried out with a reverse phase column (C 18) of 250×4.6 mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 ml/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV-visible detector.

Figure 24. The FTIR spectrum of ecdysteroid standard



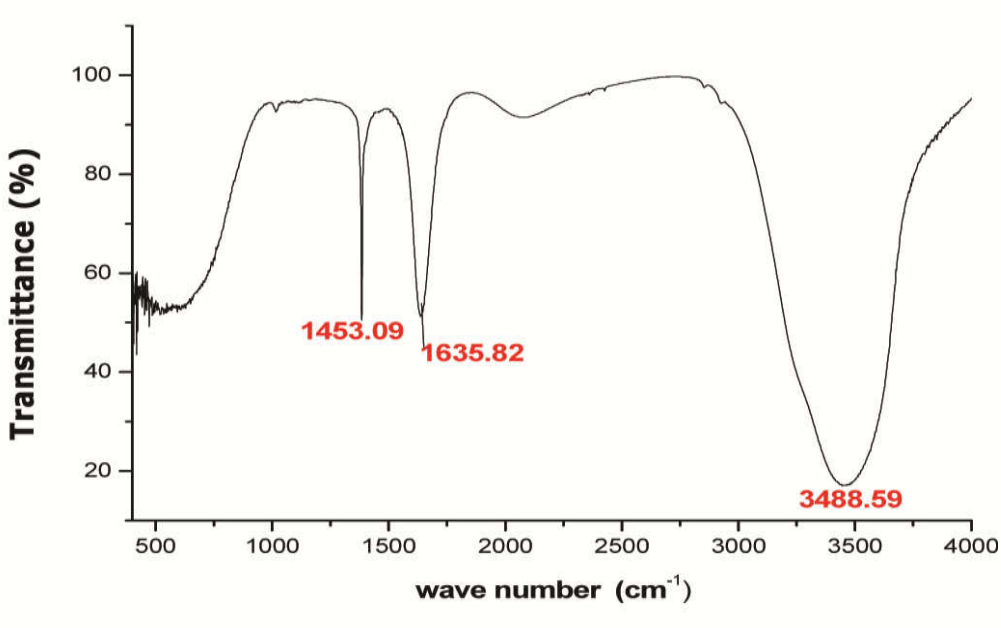
The FTIR spectrum of the ecdysteroid standard. The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 25. The FTIR spectrum of ecdysteroids extracted from the whole body of the 4th instar larvae of *O. exvinacea*



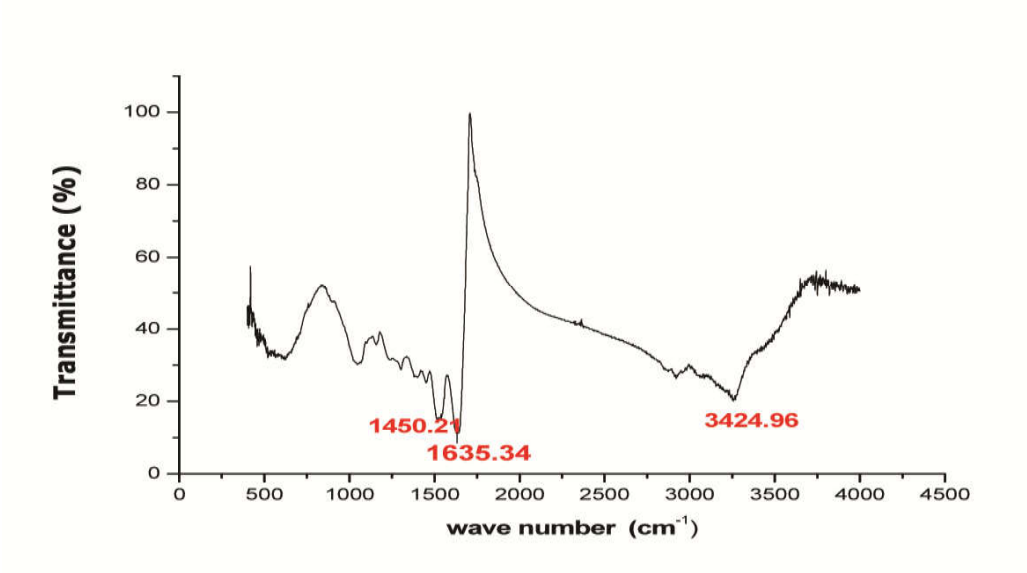
The FTIR spectrum of the ecdysteroid extracted from the hemolymph of the whole body of the 4th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 26. The FTIR spectrum of ecdysteroids extracted from the whole body of the 5th instar larvae of *O. exvinacea*



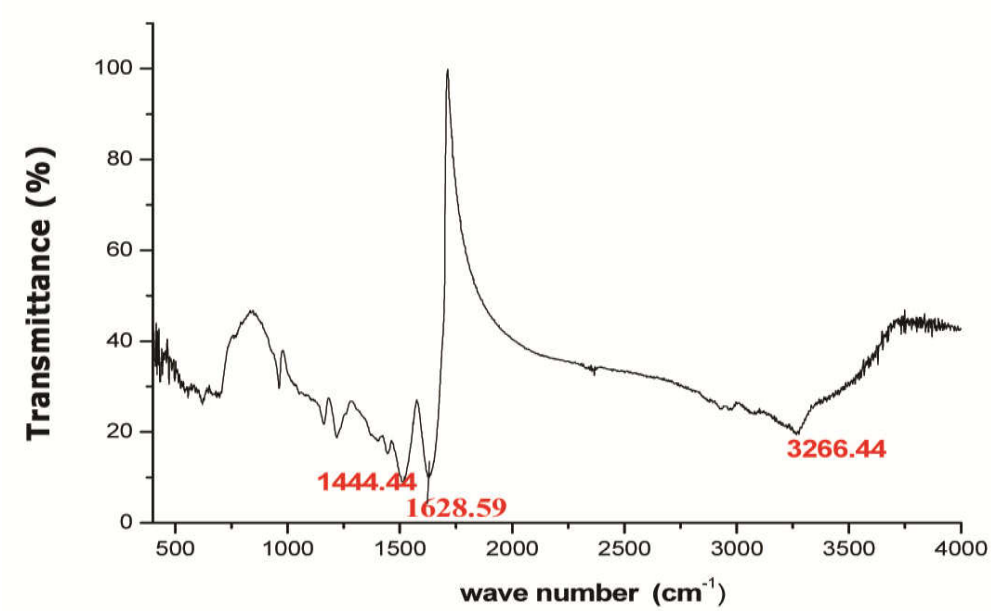
The FTIR spectrum of the ecdysteroid extracted from the hemolymph of the whole body of the 5th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 27. The FTIR spectrum of ecdysteroids extracted from the whole body of the 6th instar larvae of *O. exvinacea*



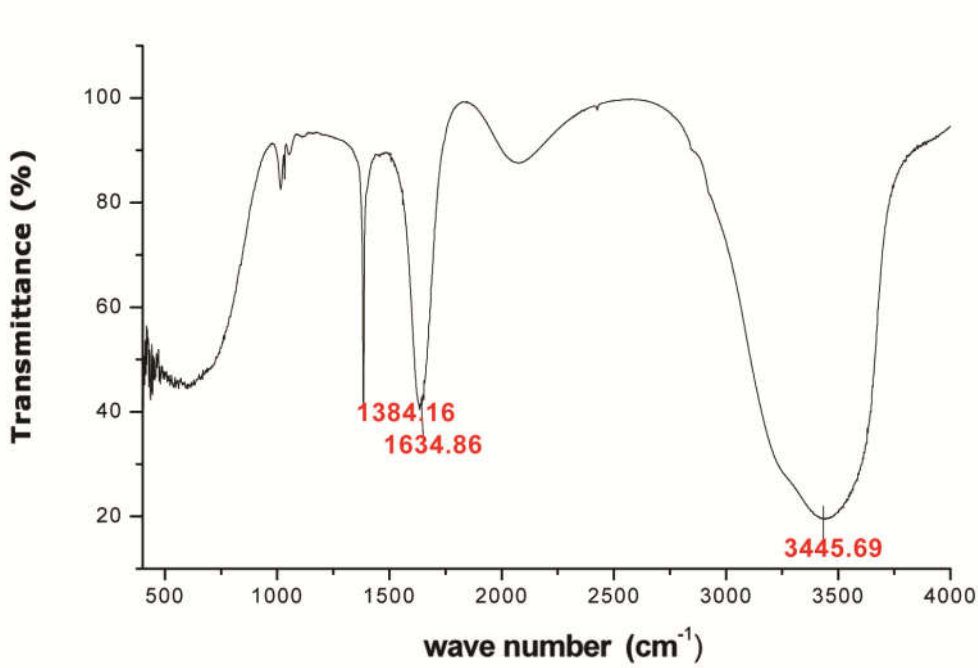
The FTIR spectrum of the ecdysteroid extracted from the whole body of the 6th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 28. The FTIR spectrum of ecdysteroids extracted from the whole body of the 7th instar larvae of *O. exvinacea*



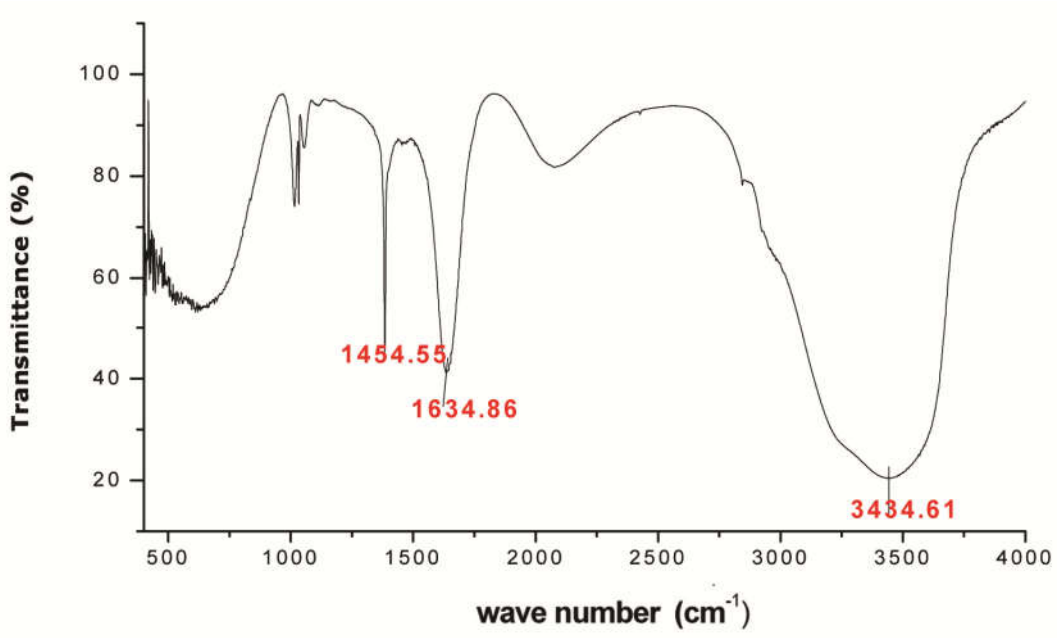
The FTIR spectrum of the ecdysteroid extracted from the whole body of the 7th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 29. The FTIR spectrum of ecdysteroids extracted from the hemolymph of the 6th instar larvae of *O. exvinacea*



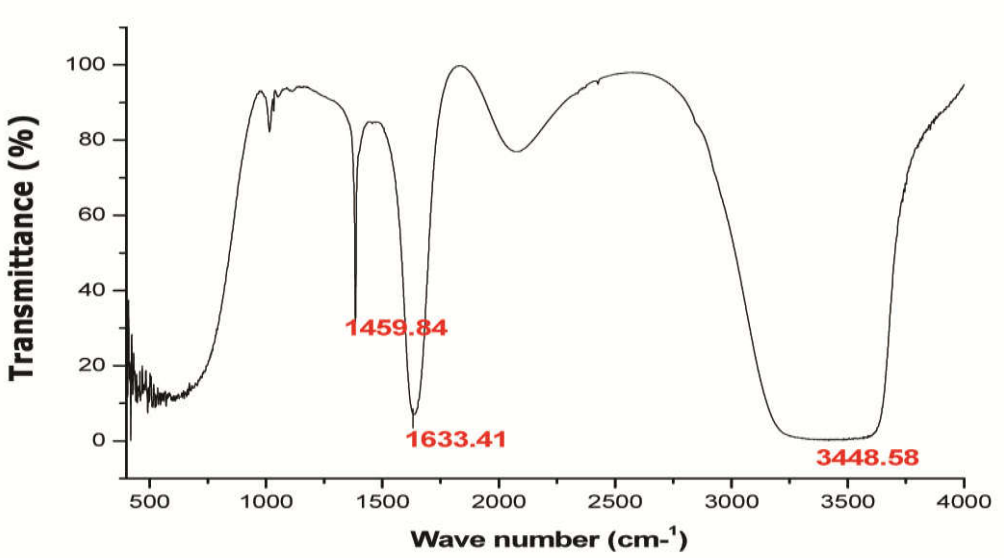
The FTIR spectrum of the ecdysteroid extracted from the hemolymph of the 6th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 30. The FTIR spectrum of ecdysteroids extracted from the hemolymph of the 7th instar larvae of *O. exvinacea*



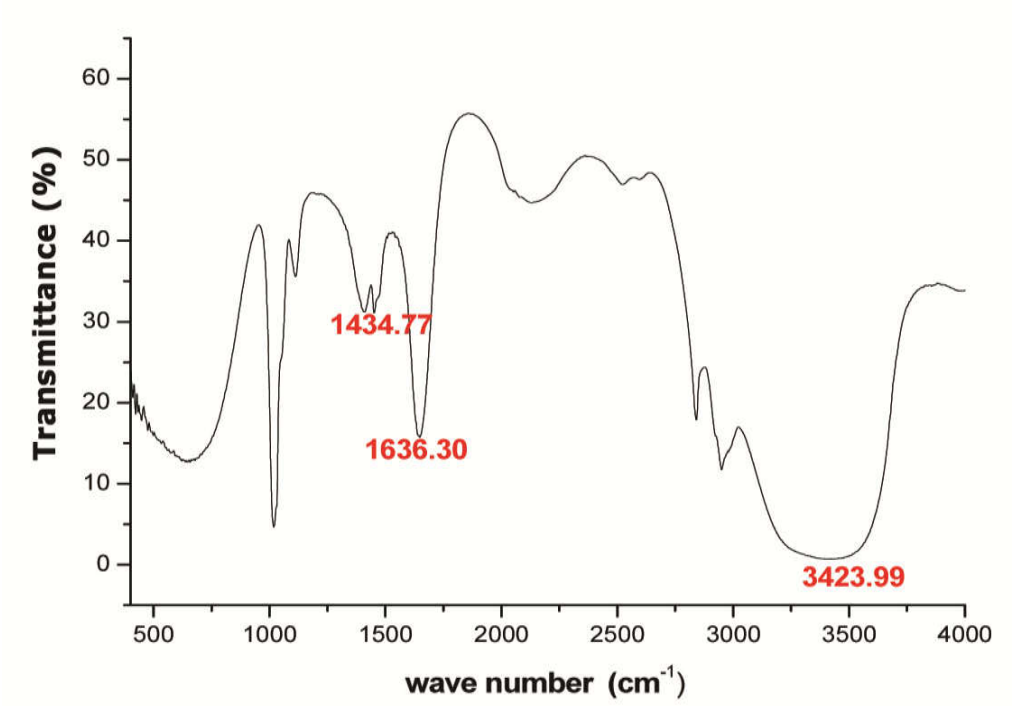
The FTIR spectrum of the ecdysteroid extracted from the hemolymph of the 7th instar larvae of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 31. The FTIR spectrum of ecdysteroids extracted from the prepupal hemolymph of *O. exvinacea*



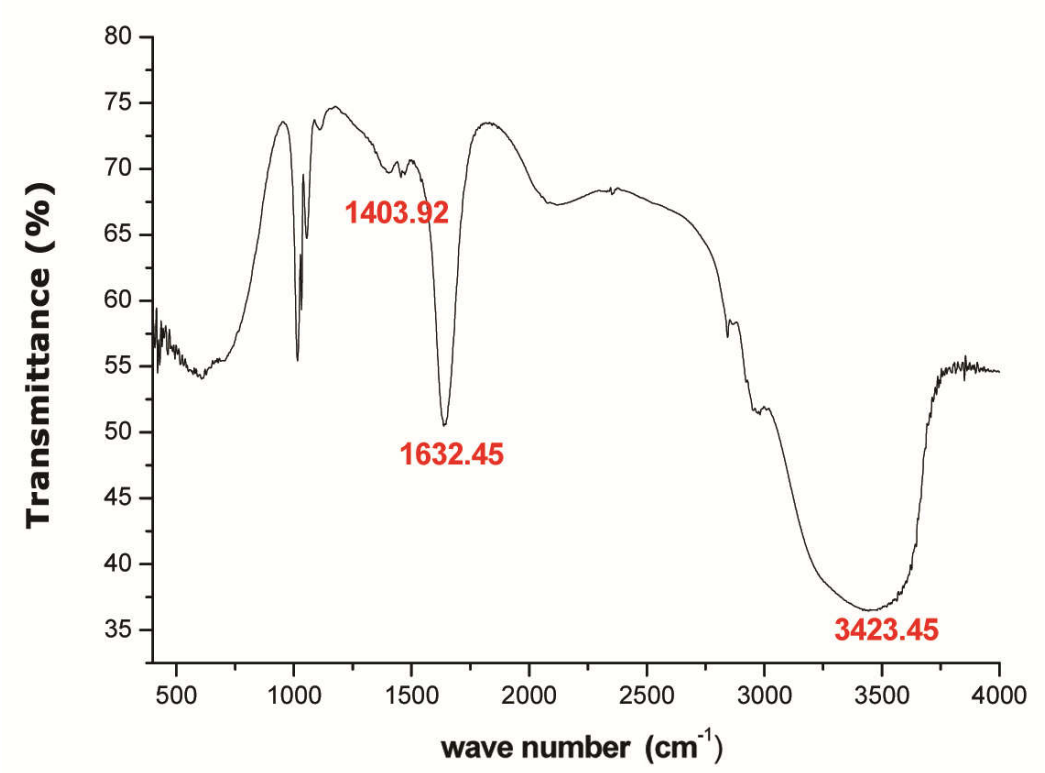
The FTIR spectrum of the ecdysteroid extracted from the prepupal hemolymph of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 32. The FTIR spectrum of ecdysteroids extracted from the 1-day old pupa of *O. exvinacea*



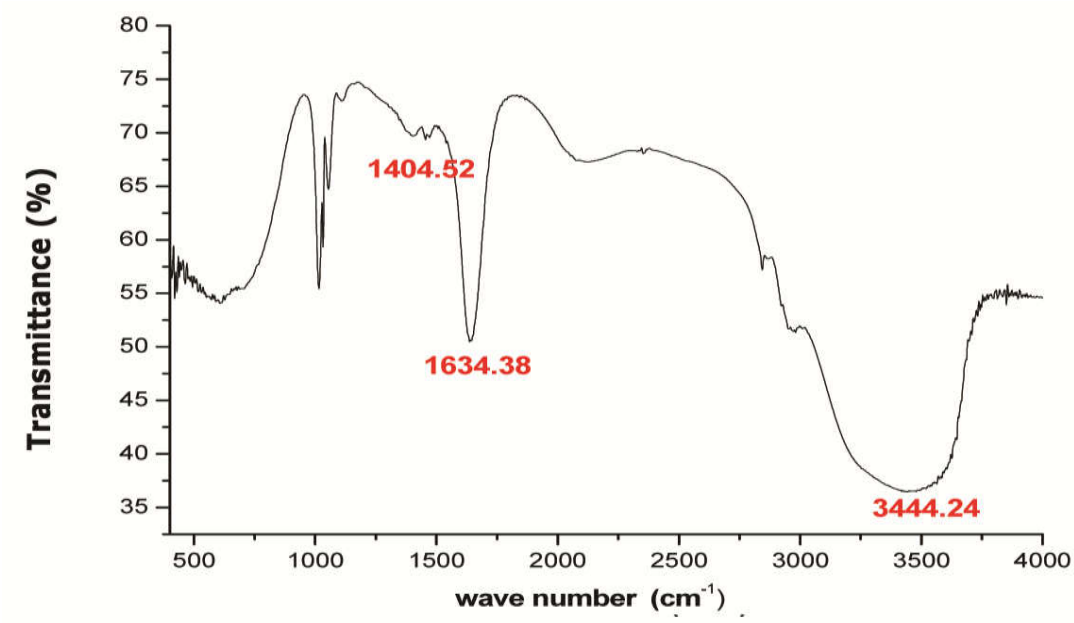
The FTIR spectrum of the ecdysteroid extracted from the 1-day old pupa of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 33. The FTIR spectrum of ecdysteroids extracted from the 2-day old pupa of *O. exvinacea*



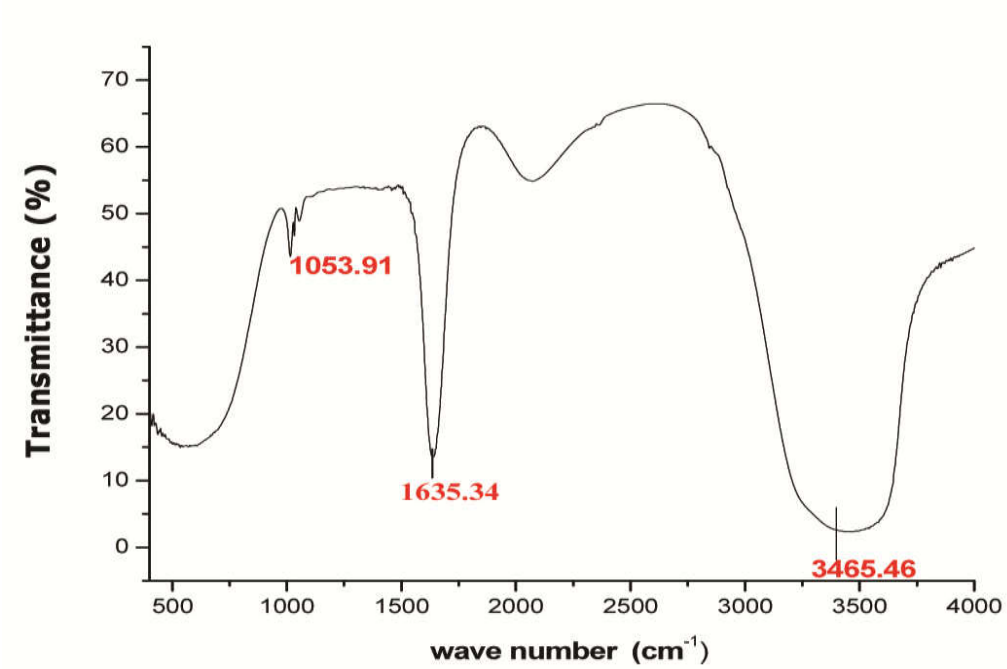
The FTIR spectrum of the ecdysteroid extracted from the 2-day old pupa of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 34. The FTIR spectrum of ecdysteroids extracted from the 3-day old pupa of *O. exvinacea*



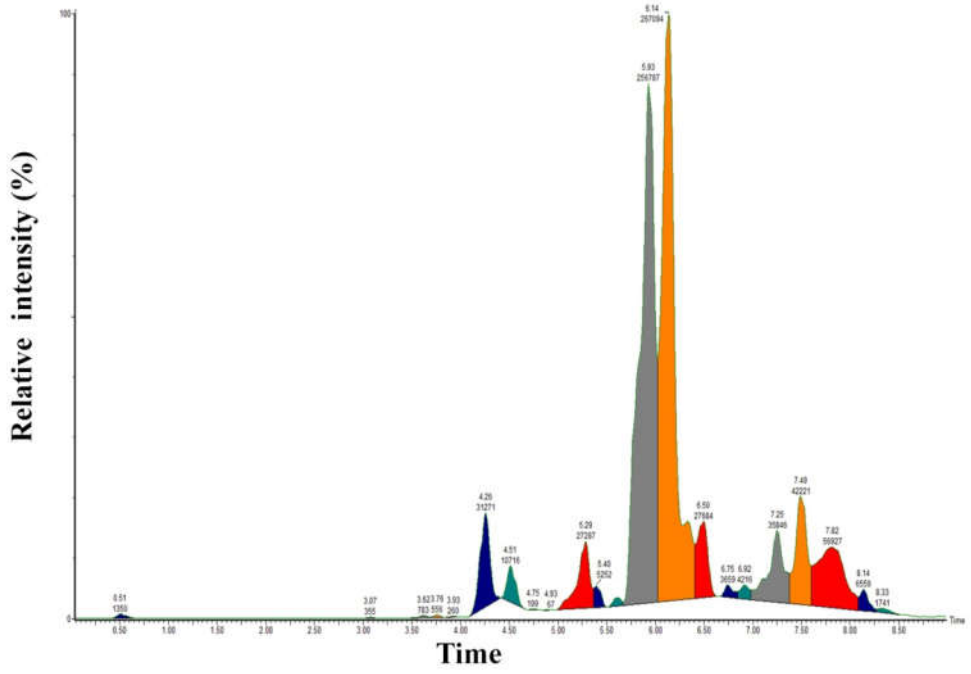
The FTIR spectrum of the ecdysteroid extracted from the 3-day old pupa of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 35. The FTIR spectrum of ecdysteroids extracted from the 4-day old pupa of *O. exvinacea*



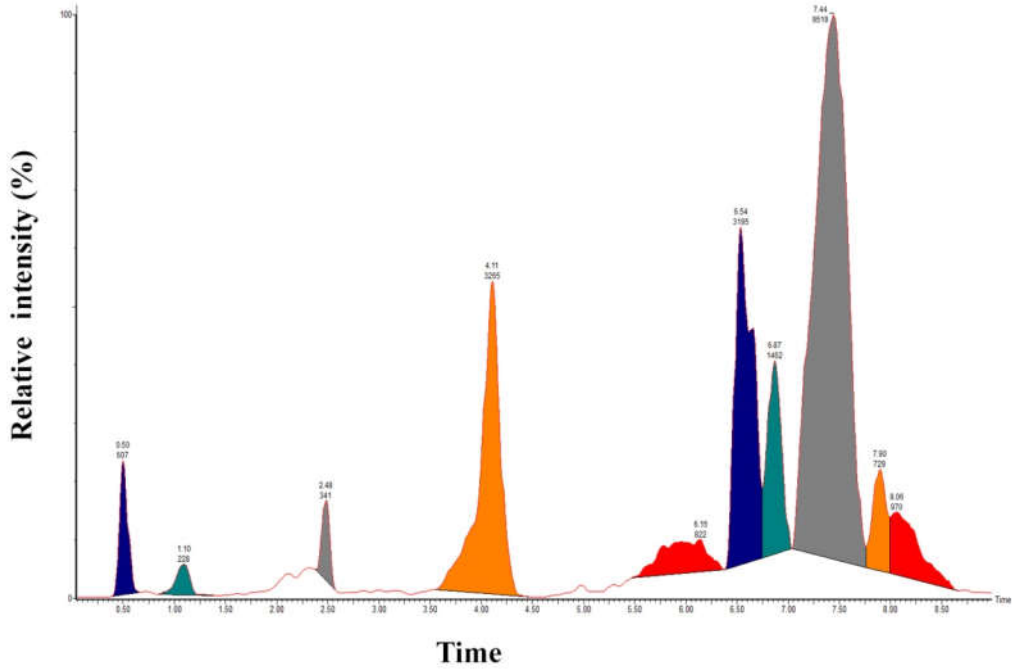
The FTIR spectrum of the ecdysteroid extracted from the 4-day old pupa of *O. exvinacea.* The secondary structural characterisation of the ecdysteroid sample was done by using JASCO FTIR-4100 instrument (USA). The frequency of absorption selected was in the region of 4000-400 cm^{-1} at room temperature. Infrared spectrum was recorded from 32 scans per sample with a resolution of 4 cm^{-1}. Three major absorption peaks were obtained viz., for OH, C=O and C=C functional groups.

Figure 36. TIC of JH-III standard



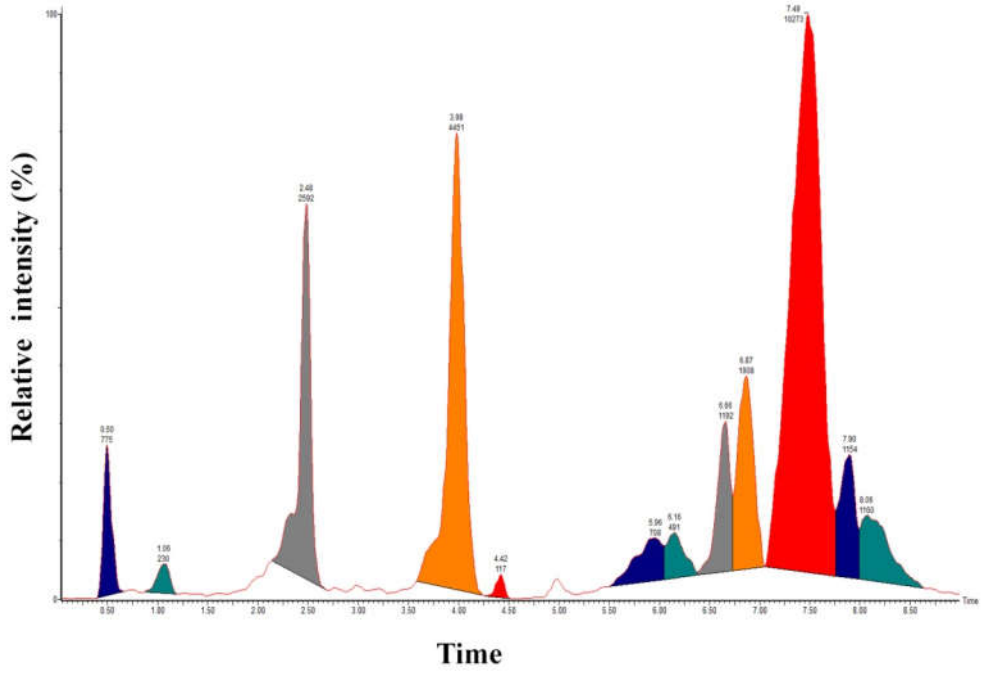
TIC of JH-III standard, 20 µl was injected into LC-Q-TOF-MS. Acquity H class (Waters) Ultra Performance Liquid Chromatography with BEH C18 column (50 mm × 2.1 mm× 1.7 µm) were used. Water+0.1% formic acid and acetonitrile were used as mobile phase at a flow rate of 0.4 mL/min. Total run time was 10 min. Xevo G2 (Waters) Quadrapole-Time-of-Flight (Q-TOF) mass spectrometer was used.

Figure 37. TIC of JH-III 6th instar larval hemolymph



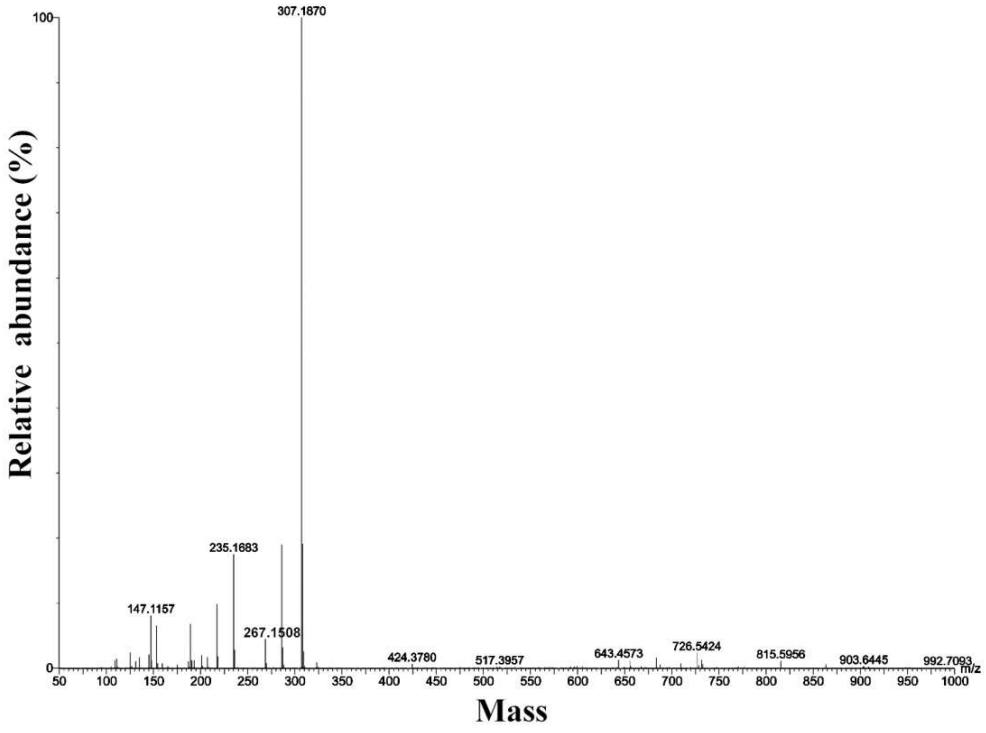
TIC of JH-III from 6th instar larval hemolymph, 20 µl of the sample was injected into LC-Q-TOF-MS. Acquity H class (Waters) Ultra Performance Liquid Chromatography with BEH C18 column (50 mm × 2.1 mm× 1.7 µm) were used. Water+0.1% formic acid and acetonitrile were used as mobile phase at a flow rate of 0.4 ml/min. Total run time was 10 min. Xevo G2 (Waters) Quadrapole-Time-of-Flight (Q-TOF) mass spectrometer were used.

Figure 38. TIC of JH-III 7th instar larval hemolymph



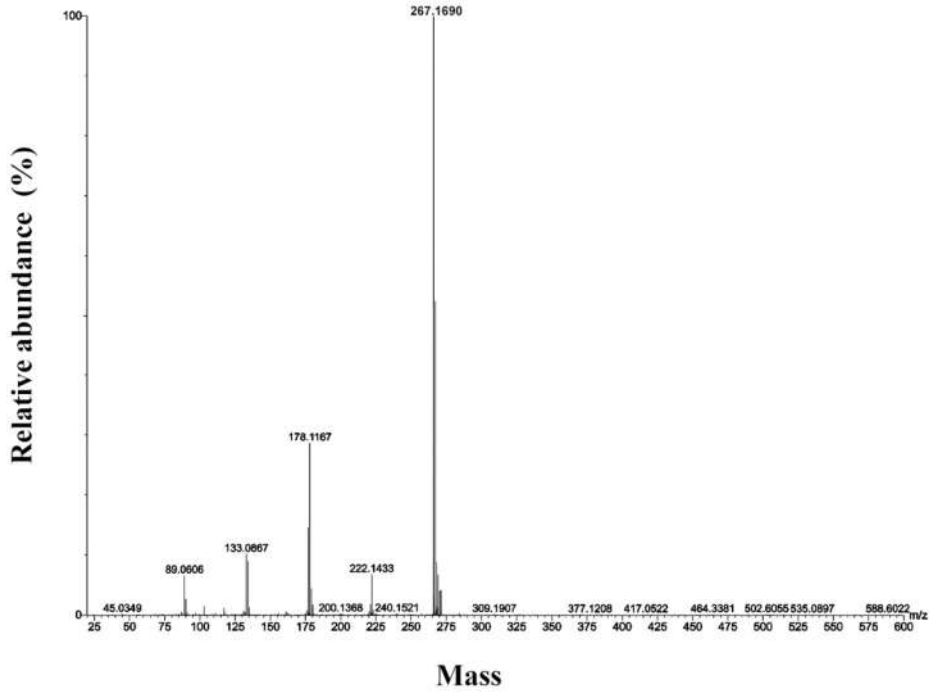
TIC of JH-III from 7th instar larval hemolymph, 20 µl of the sample was injected into LC-Q-TOF-MS. Acquity H class (Waters) Ultra Performance Liquid Chromatography with BEH C18 column (50 mm × 2.1 mm× 1.7 µm) were used. Water+0.1% formic acid and acetonitrile were used as mobile phase at a flow rate of 0.4 ml/min. Total run time was 10 min. Xevo G2 (Waters) Quadrapole-Time-of-Flight (Q-TOF) mass spectrometer were used.

Figure 40. Mass spectrum of JH-III standard



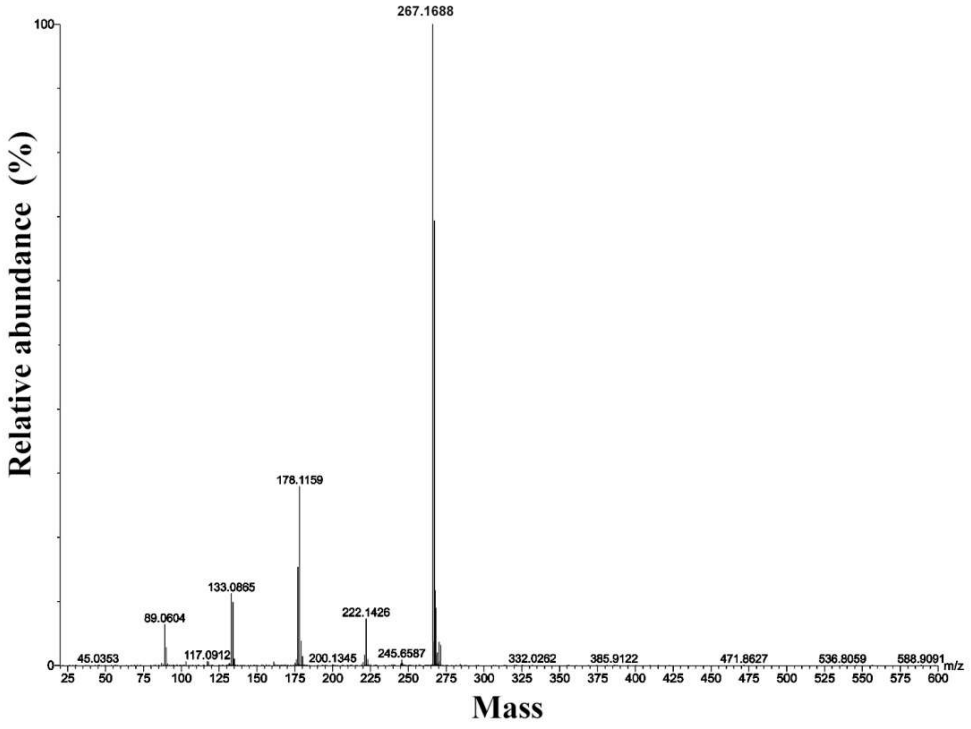
ESI-MS spectra of the JH-III peak in the standard

Figure 41. Mass spectrum of JH-III extracted from hemolymph of 6th instar larvae of *O. exvinacea*



ESI-MS spectra of the JH-III peak in the hemolymph of 6th instar larvae of *O. exvinacea*

Figure 42. Mass spectrum of JH III extracted from hemolymph of 7th instar larvae of *O. exvinacea*



ESI-MS spectra of the JH-III peak in the hemolymph of 7th instar larvae of *O. exvinacea*

Table 6. Titre of ecdysteroids in the whole larvae of *Orthaga exvinacea* during different developmental stages

Different stages	Concentration of hormone (ng/larva)
4th instar larva	1.72
5th instar larva	201.3
6th instar larva	224.7
7th instar larva	433.8

Figure 22. Pattern of changes in ecdysteroids concentration present in the whole larvae of *Orthaga exvinacea* during development

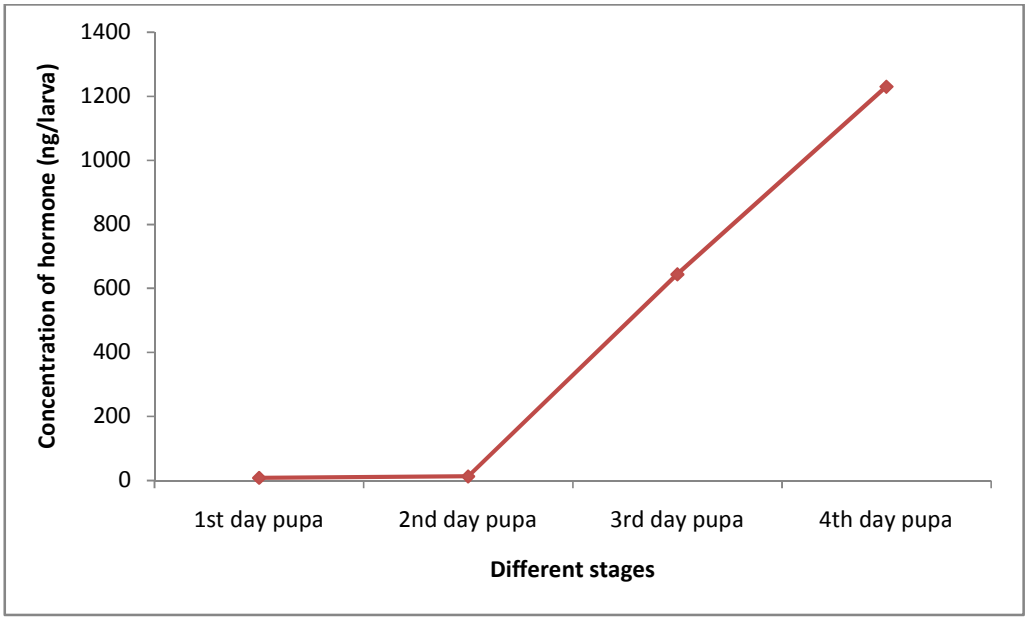


Table 7. Titre of ecdysteroids in the whole pupae of *Orthaga exvinacea* during different developmental stages

Different stages	Concentration of hormone (ng/pupa)
1st day pupa	8.62
2nd day pupa	13
3rd day pupa	644
4th day pupa	1230

Figure 23. Pattern of changes in ecdysteroids concentration present in the whole pupae of *Orthaga exvinacea* during development

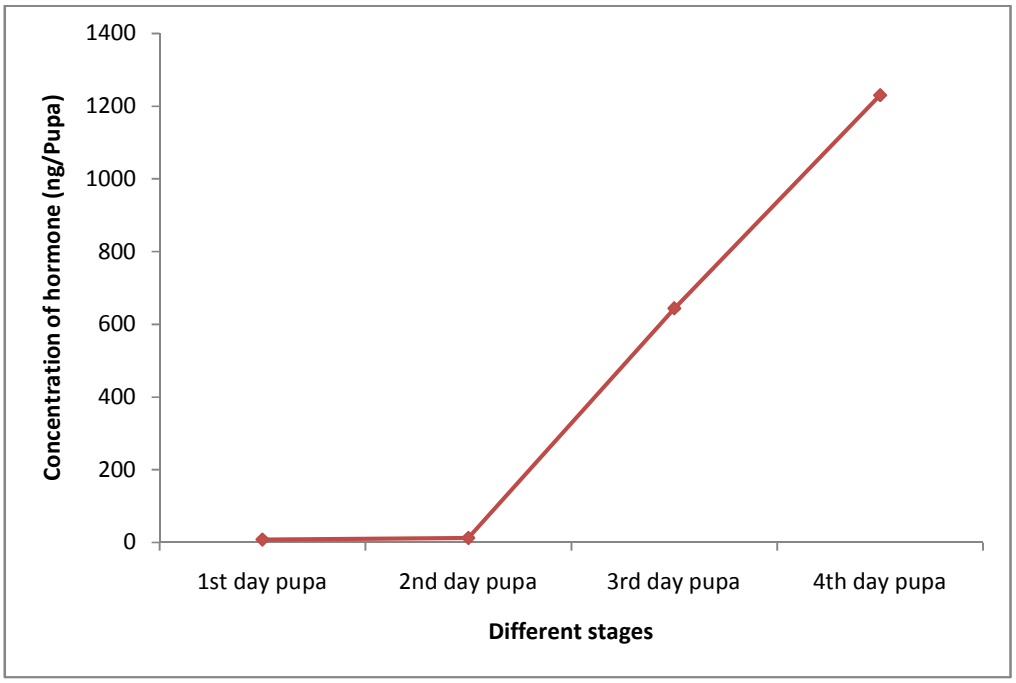


Table 5. Titre of ecdysteroids in the hemolymph of *Orthaga exvinacea* during different developmental stages

Different stages	Concentration of hormone (ng/10µl)
6th instar larva	700
7th instar larva	600
Prepupa	1800

Figure 21. Pattern of changes in ecdysteroids concentration present in the hemolymph of *Orthaga exvinacea* during development

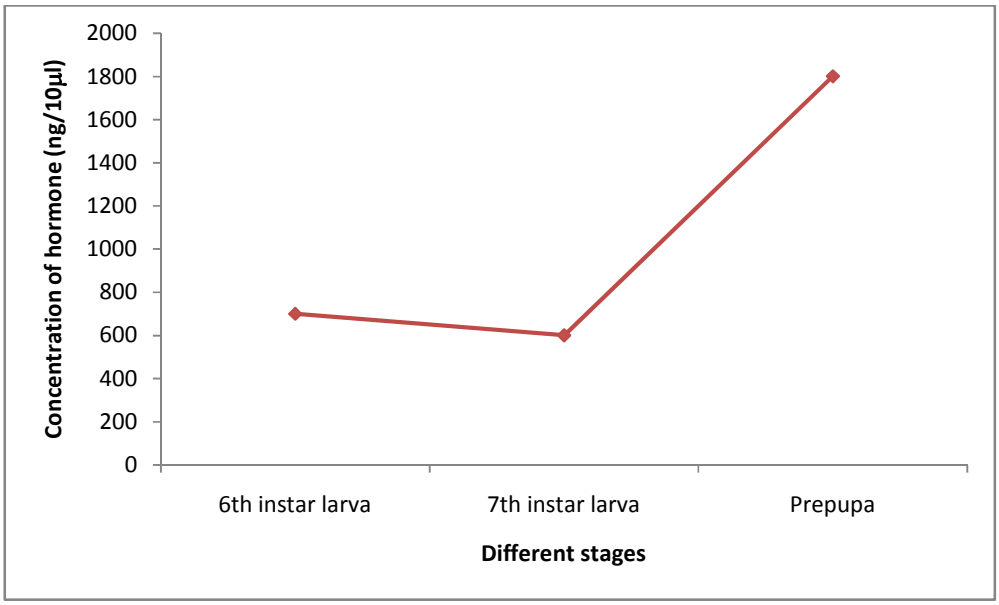


Table 8. Titre of juvenile hormone in the hemolymph of *Orthaga exvinacea* during different developmental stages

Different stages	Concentration of hormone (ng/10µl)
6th instar larva	3.8
7th instar larva	2.3

Figure 39. Pattern of changes in juvenile hormone concentration present in the hemolymph of *Orthaga exvinacea* during development