STUDIES ON THE EFFECT OF BOTANICALS ON MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF LAST INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)

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By

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CERTIFICATE

This is to certify that this thesis entitled "**STUDIES ON THE EFFECT OF BOTANICALS ON MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF LAST INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)**" is an authentic record of the work done by **Mrs. RANJINI K.D.,** for the Ph.D. course program of the University of Calicut from 2011 to 2017 under my guidance and supervision in fulfillment of requirements of degree of Doctor of Philosophy in Zoology under the faculty of Science of the University of Calicut. No part of this thesis has been presented before for the award of any other degree.

Malabar Christian College **Dr. Ranjini. K. R.**

DECLARATION

I, Ranjini. K.D., hereby declare that this thesis entitled "**STUDIES ON THE EFFECT OF BOTANICALS ON MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF LAST INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)**" is an authentic record of the work carried out by me under the supervision and guidance of **Dr. Ranjini. K. R**, Associate Professor, P G & Research Department of Zoology, Malabar Christian College, Calicut and that no part of this has been published previously or submitted for the award of any degree, diploma, title or recognition before.

Malabar Christian College **Ranjini. K.D**

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Dedicated to

My Beloved Family

CHAPTER I
GENERAL INTRODUCTION

1.1. Pest status of insects

Insects are remarkable organisms with unrivaled diversity and they outnumber all other animal categories. They are associated intimately with human society in positive and negative terms. Certain insect species greatly benefit us by providing with food directly or contributing to our food. Other species adversely affect human and domestic animal health and still others cause severe damage to our agriculture. They are the only invertebrates capable of flying and dominate the food chains and food webs in both volume and numbers with their ecological adaptability. Their ecologies vary incredibly. Their feeding specializations differ among taxa, including detritivores, herbivores, carnivores and as parasites. A tremendous proportion of all food crops are lost due to herbivorous insects and the plant diseases they transmit. Some insect species are vectors responsible for transmitting pathogens.

Insects acquire "Pest" status when their number increases beyond damage threshold level that leads to economic loss and cause problems for human welfare. For a variety of reasons, some species are resistant to contemporary insect management technologies and they have potential for agro ecological disasters. These potentials underscore the need for new pest control technologies, a point stressed decades ago by Djerassi *et al*. (1974).

1.2. Need for Pest Management

Even from the prehistoric days, man competes with insects for many of his food requirements. Pest problems and attempts to reduce pest populations are as old as agriculture. In order to eradicate the pest population, farmers

have used insecticides from time immemorial. Insects can be controlled by various methods- legislative, physical (direct killing using X-rays, heat and light), genetic (sterile male technique), ecological (removal, destruction and modification of materials that favour the existence of pest), biological (using predators, parasitoids, nematodes and microbial agents) and chemical means (using natural or synthetic chemicals). Genetically modified crops which have pest resistance have also been developed.

Discovery of DDT in 1939 marked the beginning of development and application of synthetic organic insecticides, which revolutionized insect control at that time. Later on, hundreds of compounds of differing insecticidal value have been discovered and many more new potential toxicants are also evaluated each year. Chemical control of insects have emerged fastly in the recent 100 years, starting with inorganic substances like arsenic, lime, sulfur and natural products such as pyrethrum, rotenone and nicotine. These are first generation pesticides. Second generation insecticides include organochlorines, organophosphates and carbamates which gave new approach to pest control. Most currently used pesticides are synthetic organics which have played an important role in the development of agriculture, but it cause a wide range of adverse effects. The chemical synthetic pesticides which are mainly neuro toxic agents, used for pest control are highly toxic, non-selective and persistent in nature (Carson, 1962). The widespread use of these pesticides have caused many deleterious effects such as environmental pollution, development of resistance, resurgence of pest population, destruction of beneficial insects, bio magnification of pesticide residues and health problems for human beings and other higher animals. This has led to search for an alternative ecofriendly, safer method of pest control using botanicals and other biopesticides and also for the emergence of new approach, Integrated Pest Management (IPM) which is a multidisciplinary ecological approach for

the management of pests. This includes biological, microbial and natural chemical pesticides and growth regulators.

1.3. Botanicals in pest management

Plant extracts are used as insecticides for about 4000 years ago. About 2400 plant species have been recorded as useful for pest control. Neem has emerged as most effective source of pesticide. In India, it was effective against 105 species of insects, 12 species of nematodes and 9 species of fungi (Singh and Kataria, 1991). About 417 species of insects which are key pests of agriculture are susceptible to neem all over the world (Schmutterer and Singh, 1995).

In the first half of 20th century only three insecticides i.e., nicotine from *Nicotiana tabacum,* rotenone from *Derris* sp. and pyrethrum from *Chrysanthemum cinerariaefolium* were commercialized and only pyrethrum is still being used and the other two gradually pushed out from the market by synthetic pesticides. Botanical pesticides have divergent activities like antifeedant and insect growth regulatory action. They also have some advantages over synthetic pesticides like no mammalian toxicity and health hazards, no environmental pollution and minimum risk of development of insect resistance and they are less expensive and easily available. The wide use of powerful synthetic pesticides which produce faster results have caused the neglect of botanical pesticides and failure of the systematic screening for biological activities of botanicals also led to the restricted use of biopesticides of plant origin for effective pest control.

Naturally occurring botanical pesticides have valuable potentials for development into wide use as one of several alternative pest management technologies. Plants produce a very wide range of novel chemical compounds and a small proportion of these plants have been used as effective pest

management tools for years. Many plants have evolved secondary metabolites such as phenolics, terpenoids, alkaloids and flavonoids which act in ecological roles to protect the plants from herbivory and they act as antifeedants, oviposition deterrents, attractants, repellents, growth inhibitors, juvenile hormone mimics, moulting hormones and antimoulting hormones.

Most of the botanicals used as insecticides acting on the insect's gut are fast acting, quickly inhibiting insect feeding and additional crop damage. Currently, there are numerous bio-pesticide plant extracts marketed as insecticides. Considerable attention has been focused on botanical pesticides because of their compatibility with the environment and efficacy in pest management. Their effects are restricted, in most cases, to target pests and closely related organisms. They are effective in very small quantities and they decompose quickly. Their use leads to residue-free food products and reduction in environmental degradation. Their renewability fit them well in a sustainable environment. When incorporated in integrated pest management programs botanical pesticides can decrease the use of chemical pesticides or, when used in combination with insecticides, potentially reduce overall application of synthetic chemicals. Farmers can easily take up the formulation of locally available plants and thus they can save money spent on costly synthetic chemical insecticides.

1.4. Botanicals in conventional use at present

Though a handful of botanicals are currently used in agriculture in the industrialized world and also there are few prospects for commercial development of new botanical products, several scientific studies documenting the bioactivity of plant derivatives to insect pests continues to expand. The major types of botanical products used for insect control includes pyrethrum, rotenone, neem and essential oils. A few other compounds such as ryania, nicotine and sabadilla are limited in use.

Neem and pyrethrin are well established commercial products. Some neem based insecticides were commercialized and are used in plant protection (Schmutterer, 1990). Azadirachtin is the most effective compound isolated from the seeds of Indian neem tree. Neem chemicals act as repellents, antifeedants, toxins and growth regulators. Neem products also affect fecundity of insects.

Pyrethrin is a potent insecticide and is used in developed countries as a natural insecticide. Most active insecticidal components of pyrethrum are pyrethrins I and II. These are neurotoxins responsible for rapid knockdown of pests on crop plants. Plant essential oils used as insecticides are produced commercially from several botanical sources. Most of these are produced from plants in the mint family (Lamiaceae).

Rotenone is produced in the roots or rhizomes of the tropical legumes *Derris*, *Lonchocarpus* and *Tephrosia*. Rotenone acts as a mitochondrial toxin, which interrupts the electron transport chain, lethally preventing cellular energy production in target insects (Hollingworth *et al.,* 1994).

Sabadilla is another botanical insecticide isolated from the seeds of the South American lilly *Schoenocaulon officinale*. The mode of action of these compounds is similar to that of pyrethrins.

Nicotine is an alkaloid obtained from the foliage of tobacco plants (*N. tabacum*) and related species, long known for insecticidal action. Nicotine and two closely related alkaloids, nornicotine and anabasine, are synaptic toxins that mimic the neurotransmitter, acetylcholine.

1.5. Biological significance of secondary metabolites in pest control

Plants are nature's "chemical factories" providing the richest source of chemicals on earth. Of the various defensive mechanisms developed,

chemicals elaborated by plants are the most important one. These chemicals are known as secondary plant substances and are produced as byproducts of major biochemical pathways. They include alkaloids, terpenoids, phenolics, flavonoids and many other compounds. Many of these chemicals have been successfully exploited by humanity for the control of arthropod pests (Swain, 1977). These chemicals deter feeding, disrupt development, provide barrier to attack, assist with wound healing, disrupt digestion and many are neurotoxic to herbivorous pests.

Most plants produce secondary metabolites that act in protection against herbivores and microbial pathogens based on their toxicity and repellency. Some are involved in defense against abiotic stress and act in animal-plant interactions (Rosenthal, 1991). These secondary metabolites are of three major groups, terpenes, phenolics and alkaloids. Terpenes are 5-C isopentanoid units with toxic and anti-feedant activity in many herbivores. Phenolics are derived primarily from the shikimic acid pathway, many of which have important roles in defense mechanisms. Nitrogen and Sulphur containing compounds (alkaloids) are synthesized from common amino acids (Van-Etten *et al.*, 2001). Plants apparently evolved metabolic pathways to produce these compounds in the co-evolution of plants and herbivores, indicating a genetic, heritable basis for production of these compounds (Agrawal *et al.*, 2012).

1.6. Control of leaf webbers using botanicals – Choice of Mango leaf webber

The insect order Lepidoptera includes moths and butterflies which feed on live plants. In many species, adult females can lay 200 to 600 eggs, or in others, it may reach 30,000 eggs in one day. The huge number of caterpillars hatching from these eggs is more than enough to cause damage to large quantities of crops. Hence, the management using effective control agents is

inevitable. Many botanicals have been reported for the control of these lepidopteran pests, especially the leaf webbers. Kodjo *et al.* (2011) reported that 10% oil emulsion of *Ricinus communis* products have strong larvicidal effect on *Plutella xylostella*, with 100% mortality recorded on 3rd instar larvae in both ingestion and contact toxicity tests. John and Jimmy (2015) reported that three concentrations (5%, 10% and 15%) of neem, tobacco extracts and garlic extracts at 15% led to 100% mortality of the amaranthus leaf webber, *Psara basalis*.

Moreover, *Hyptis suaveolens* and *Vitex negundo* used in the present scenario have been noticed to be biologically effective against many lepidopteran pests (Prakash *et al.*, 2008). Raja *et al.* (2005) reported significant ovicidal and antifeedant activity of the extracts of *H. suaveolens* against *Helicoverpa armigera*. Cyrille *et al.* (2011) reported the potential use of *H. suaveolens*, for the control of infestation by *Sesamia calamistis* on Maize. Pavunraj *et al.* (2013) confirmed the antifeedant and larvicidal activities of *H. suaveolens* leaves crude extracts and their fractions towards the control of four lepidopteran pests namely *H. armigera*, *Spodoptera litura*, *Earias vittella* and *Leucinodes orbonalis*. Similarly, *V. negundo* also reported to possess insecticidal and growth inhibitory action against *S. litura* (Deepthy *et al.*, 2010). Sahayaraj (2011) reported the efficacy of *V. negundo* in significantly reducing the population of *H. armigera* and *S. litura*. Arivoli and Tennyson (2013) reported maximum antifeedant activity against *S. litura* with the hexane extract of *V. negundo*. Similarly, several other plant based pesticides are larvicidal in nature (Ogendo *et al.*, 2008; Agboka *et al.*, 2009).

India is the largest mango producer, at about 53% of global mango production. Among mango pests, the most widely distributed insect pest is the mango leaf webber, *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae), responsible for restricted productivity. *O. exvinacea* is a mango leaf defoliator

responsible for crop reduction and economic loss. Earlier *O. exvinacea* was a minor pest and presently acquired major pest status. Heavily infested trees present a burnt look and are conspicuous with numerous webbed leaves and dry apical shoots leading to flower dropping and poor fruit formation; it prevents further flower formation and fruit setting (Rafeeq and Ranjini, 2011).

There are no reported works on using botanicals to manage the mango leaf webber. In this context, the effect of *Hyptis suaveolens* and *Vitex negundo* on the various biochemical parameters, histopathology and lethal toxicity were studied. Changes in the total protein content, free amino acid content and the inhibition and induction of metabolic enzymes in different tissues of the botanical-treated insect were measured as potential impacts of plant extracts on the pest. Although most studies focus on qualitative data to examine the pathology associated with toxicants, quantitative data are needed for hypothesis testing.

Histopathological studies can be used as effective biomonitoring tools in toxicity studies since they provide early signs of pathological conditions (Meyers and Hendricks, 1985). Moreover, histopathological changes are useful biomarkers to study the effect of exposure to environmental toxicants, because they can reveal prior alterations in physiological or biochemical functions in an organism (Hinton *et al.*, 1992). Generally, exposure to toxic elements may cause major structural damages and histological changes. These visible changes can be due to underlying metabolic changes. Histopathological characteristics of specific organs express pathological condition and represent time-integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization (Chavin, 1973). Histological changes can be considered as more sensitive indicators of pathology than growth or reproductive parameters and

provide a better evaluation of the organism than a single biochemical parameter (Segner and Braunbeck, 1988).

Many botanical extracts studied have shown remarkable larvicidal activity against insect pests. Lethal dose (LD_{50}) is a common measure of toxicity that causes death in 50 percent of the test population. When the LD_{50} value is small, the tested doses are considered to be highly toxic for the exposed organism. Toxicity can be evaluated by counting the number of animals dead or alive after exposure to different doses at fixed time and suitable intervals (Finney, 1971). Because test doses of insecticide must enter the insect body through the cuticle, study of contact toxicity through topical application is necessary before field application. As toxicity studies are necessary to obtain lethal doses, biocidal properties of the two plant extracts were also carried out.

Generally, plant secondary metabolites are of vital importance in contributing towards the biopesticidal properties of plants. Phytochemical evaluation of the plants for their secondary metabolites includes both qualitative and quantitative analytical techniques. For the proper understanding of the type of compounds present in the active fraction of the botanicals employed in the present study, High Performance Thin Layer Chromatography (HPTLC) was performed. Furthermore, identification of the volatile components present in the active fractions of both plants were carried out with Gas Chromatographic- Mass Spectrometric (GC-MS) analysis.

1.7. Relevance of the study

Cultivation of mango is severely affected by the mango leaf webber and the economic losses drive farmers to make repetitive sprays of synthetic chemicals to improve yield. As almost all fruits are raw food material, human directly consume the insecticides within mangoes, leading to increased health

risks. There is only few management tools available for this pest, including pruning of infested branches and burning them completely, proper orchard maintenance, augmentation of predators and insecticide treatments (carbaryl at 0.1 % or quinalphos at 0.05 %) after infestation. Hence, alternate eco-friendly insect pest management approaches are urgently required. The present research work is designed to look into this need. Botanicals prepared from two plant species *V. negundo* and *H. suaveolens* are analyzed for their pesticidal efficacy against *O. exvinacea*. Botanical preparations from these plants are promising management tools.

1.8. Objectives

- To separate the constituents of leaf extracts and to evaluate the toxicity of major fractions by estimating the mortality rate.
- To identify the phytochemical constituents of toxic fractions using HPTLC and GC-MS analysis.
- To study the effect of botanicals on the histomorphological and ultrastructural changes in the midgut tissue using light microscopy and transmission electron microscopy.
- To evaluate the effect of leaf extracts on the protein concentration of midgut tissue, fat body and haemolymph by spectrophotometry.
- To study the electrophoretic protein profiling of midgut tissue, fat body and haemolymph with polyacrylamide gel electrophoresis.
- To evaluate the effect of leaf extracts on the total free amino acid concentration of midgut tissue, fat body and haemolymph using visible spectrophotometer.

- To study the effect of leaf extracts on the activity of catalase, acid phosphatase, alkaline phosphatase, aspartate amino transferase and alanine amino transferase in midgut tissue, fat body and haemolymph by spectrophotometry.

CHAPTER II
REVIEW OF LITERATURE

2.1. Introduction

Heretofore the use of pesticides to combat various pest problems is imperishable in nature. But years after, the widespread and continuous use of pesticides resulted in the emergence of resistant varieties of pests which indirectly provoked an increase in the concentration of powerful chemical ingredients. The uncontrolled usage of synthetic pesticides in improving agriculture have resulted in complex problems due to the side effects they produced on many beneficial and non-target animals along with contamination of the environment. According to USEPA (2011) more than 2.5 million tons of pesticides are used in agricultural crop protection for every year and the global damage caused by synthetic insecticides reached more than $100 billion annually.

Many researchers noticed various pesticidal issues such as hazards to human health and non-target organisms (Sighamony *et al.*, 1986), environmental pollution (Wright *et al.*, 1993) and development of resistant strains (White, 1995). Ghosh (1991) noted that most of the chemical pesticides are hazardous to humans and to the whole environment leaving residues and spreading toxic effects. Later on, the extermination of pest with the over-dependence and non-judicious use of synthetic insecticides has resulted in the buildup of many deleterious effects especially the 3 R's *viz*, resurgence, resistance and residue aspects (Mahapatro and Gupta, 1998) in addition to the health hazards.

Literature studies shows many investigations on the negative effects of synthetic insecticides and concluded that these pesticides are non-selective in

action but there are pesticides with selective action which are found to be expensive, so the inexpensive, nonselective pesticides are primarily used in developing countries (Chowanski *et al.*, 2014). Besides this, the indiscriminate application caused biodiversity threat for natural enemies, pest outbreak, residual accumulation in the food and also imbalance of ecosystem (Krishnamurthy, 1999; Praveen *et al.*, 2001). Jeyasankar and Jesudasan (2005) reported that the arbitrary use of organic pesticides instigate pest problems and other precarious effects on the environment endangering the sustainability of ecosystems. All these issues sow the seeds of world-wide interest to fabricate novel methods of pest control and the re-assessment and deployment of traditional botanical pest control agents (Heyde *et al.*, 1983).

This chapter deals with literature on the mango pest, *Orthaga exvinacea* and its management strategies, assessment of botanicals for their control, drawbacks of synthetic insecticides, importance of botanical insecticides, currently using botanicals and their mode of action, toxic effects of botanicals, effect of botanicals on biochemical parameters, histopathology and also the effect on enzyme activity.

2.2. Mango pest- *Orthaga exvinacea* Hampson

India's major fruit crop, the mango tree is infested by various insect pests. Rajkumar *et al.* (2013) reported that among the insect pests infesting mango tree, mango leaf webbers are found to be a major limiting factor in mango production regions of agro-climatic zones in India and recently gained the status of a serious pest in mango orchards (Singh *et al.*, 2006). The leaf webbers *Orthaga exvinacea* and *Orthaga euadrusalis* are considered as major pests of mango and cashew (Verghese, 1998). Mishra (2001) reported that *O. exvinacea* affects crop yield by damaging shoots and inflorescence from August to December. A heavily infested tree shows many clusters of webbed and dried leaves, presenting it a conspicuous burnt up appearance (Rafeeq and

Ranjini, 2011). Timely management of this insect pest is crucial to save the mango crop for the coming years. Densely planted orchards have higher infestation rates than the normal spaced and canopy managed orchards.

Reddy *et al.* (2001) reported that some of the mango cultivars found to be resistant to the pest attack. The large size of mango tree is an important limiting factor for management of mango leaf webber and also the mango orchard provides a safe micro-ecosystem for breeding of the pest in active period and for a safe living in the same orchard during off-season (Shukla *et al.*, 2001).

2.3. Management strategies

Cultural Control: Inspection of mango orchards once a month for webbed leaves and shoots and systematical pruning; removal of infested clusters of leaves and shoots and destruction of infested leaves along with the larvae.

Host-Plant Resistance: Degree of infestation varies with different commercial varieties of mango. It was earlier noted that young trees were more susceptible than old trees for *Orthaga* infestation (Cherian and Ananthanarayanan, 1943), whereas Srivastava *et al.* (1982) reported severe pest attack even in very old trees.

Biological Control: Many studies reported potent natural enemies against the larvae of *Orthaga* sp. (Srivastava and Tandon, 1980). The egg parasitoids such as *Trichogramma chilonis* or *T. pretiosum* may be effective for their control.

Chemical control: Spray of lambda cyhalothrin 5 EC (2 ml/ lit of water) or quinalphos 25 EC (1.5 ml/ lit of water) manages the pest. The most effective treatments for the management of mango leaf webber were Diflubenzuron

(0.01 %), Trizophos (0.06 %), Chlorpyriphos (0.04 %) and Indoxacarb (0.01 %) (ICAR, 2014).

2.4. *Hyptis suaveolens* Poit.

Ghosh *et al.* (2012) noticed that the extraction of active biochemical compounds from plants depends mainly upon the polarity of the solvents used. Generally polar solvents will always extract polar molecules and non-polar solvents extract non-polar molecules. Many solvent systems ranging from hexane/ petroleum ether (the most non polar) to that of water (the most polar) can be used for extraction purpose. Usually nonpolar solvents extract essential oils and polar solvents separate high molecular weight components such as proteins, glycans etc. The moderately polar solvents, chloroform and acetone mainly extracts steroids, alkaloids etc. Thus, potency of extracted plant compounds depends on different solvent types used which reveals difference in the chemo-profile of the plant species.

Many researchers studied and reported various bioactive constituents of plants such as alkaloids, tannins, saponins, terpenoids, steroids, glycosides, flavonoids and phenolic compounds both qualitatively and quantitatively (Florence *et al.*, 2012, 2014). There are several reports on qualitative analysis by means of HPTLC and GC-MS for the identification of phytochemical constituents present in *H. suaveolens* (Moreira *et al.*, 2010; Uzama *et al.*, 2013; Joseph and Jeeva, 2016).

Cowan (1999) noticed that different solvents possess various degrees of solubility for different phytochemicals. Pachkore and Dhale (2011) carried out the phytochemical analysis of *H. suaveolens* and revealed the presence of volatile oil, starch, proteins, tannins, saponins, fats, alkaloids and glycosides in leaves and the absence of saponins in stem and root of the plant. Agarwal and Varma (2013) revealed the presence of alkaloids, carbohydrates, reducing

sugars, flavonoids, glycosides, tannins, phenolic compounds, proteins, aminoacids, terpenoids and steroids with the methanolic extract of *H. suaveolens*. Joseph and Jeeva (2016) carried out phytochemical extraction of *H. suaveolens* leaves with aqueous, petroleum ether, chloroform, ethanol and acetone and the extracts were evaluated qualitatively. In all five extracts alkaloids, quinones, steroids, coumarins, proteins, flavonoids and terpenoids were present. Tannins were present in all extracts except petroleum ether extract. Aqueous, petroleum ether and acetone extracts showed the presence of saponins.

Many researchers worked on the phytochemical aspects of *H. suaveolens* and identified several compounds (Moreira *et al*., 2010; Syamasundar *et al*., 2012; Azeez *et al*., 2014). The presence of various bioactive principles recommends the use of *H. suaveolens* as an effective pest control measure.

H. suaveolens is a potent herb with very strong aromatic property due to the presence of certain essential oils. These essential oils constitute lipid-like secondary metabolites with insecticidal properties. Many of these components are lost usually during drying especially if exposed to sunlight. It is also recognized that *Hyptis* sp. releases secondary metabolites with diverse biological activities including potent specific activity against insects (Aguirre *et al*., 2004).

Many researchers studied the insecticidal properties of *Hyptis* sp. (Facey *et al*., 2005; Othira *et al*., 2009). Raja *et al*. (2005) conducted toxicity works with ethyl acetate leaf extract of *H. suaveolens* and reported that it possess insecticidal activity against *S. litura* and *H. armigera*. Essential oil of *H. suaveolens* exhibited toxicity against *P. xylostella* larvae (Kéïta *et al*., 2006). Gbehounou (2007) reported that *Hyptis* extracts can be used for the effective control of cow pea borer, *Maruca testulalis*. Anandan *et al*. (2010)

reported that crude extracts of *H. suaveolens* and *Melochia chorcorifolia* exhibit insecticidal activity against *S. litura*. Moreover, Pavunraj *et al.* (2014) evaluated the larvicidal efficacy of *H. suaveolens* crude extract against *H. armigera, S. litura, E. vittella* and *L. orbonalis.*

The essential oil constituents of *H. suaveolens* was found to be effective against several stored product insect pests such as *Callosobruchus chinensis*, *Sitophilus oryzae* (Park *et al.*, 2003), *Sitophilus zeamais* and *Tribolium castaneum* (Wang *et al.*, 2009). Piozzi *et al.* (2009) reviewed the literature and found that *Hyptis* sp. are known to produce a wide range of terpenoids, flavonoids and pyrones. Similarly, Musa *et al.* (2009) also noticed the importance of this plant for managing *Trogoderma granarium.* In addition, it was also noticed that *H. suaveolens* possess marked toxicity and repellency against adults of *S. zeamais* (Conti *et al.*, 2010). Furthermore, ethanolic extract of *H. suaveolens* found to be effective against *C. maculatus* (Kolawole *et al.*, 2011). Olotuah (2013) observed that methanolic extract of *H. suaveolens* exhibited insecticidal activity against *S. oryzae*, *S. zeamais* and *C. maculatus.*

Various parts of the plant exhibited feeding deterrency (Simmond and Blaney, 1992) and also insect repellency (Oparaeke *et al.*, 2002). Many workers proved that the fumes of dried leaves of the plant can be used as an insectifuge to repel mosquitoes and also for the control of stored grain pests (Alok *et al.*, 2010; Hemen *et al.*, 2013).

The plant has been reported to be rich in plant chemicals. Methanolic fraction of whole plant of *H. suaveolens* constitutes the presence of alkaloids, flavones, flavonols, terpenoids, tannins, aldehydes and ketones through phytochemical screening (Agarwal and Varma, 2013).

2.5. *Vitex negundo* Linn.

Many researchers carried out phytochemical analysis for the identification of chemical constituents by employing qualitative methods such as HPTLC and GC-MS techniques and reported several bioactive compounds from various parts of *V. negundo* (Zaware and Nirmala, 2010; Sridhar *et al.*, 2015; Thakur and Pandey, 2016).

The studies on *V. negundo* revealed the presence of volatile oil, triterpenes, diterpenes, sesquiterpenes, lignan, flavonoids, flavonoid glycosides, iridoid glycosides and stilbene derivative. Maurya *et al.* (2007) carried out isolation of flavonoid glycosides from the ethanolic extract of the leaves of *V. negundo*. Sahayaraj and Ravi (2008) conducted extraction of *V. negundo* leaves with the solvents, chloroform, benzene and water and observed the presence of triterpenoids, steroids, flavonoids, saponins, alkaloids, tannins and phenolic compounds. Singh *et al.* (2010) proved the presence of volatile oil which contains ten volatile components. Meena *et al.* (2010) identified compounds like negundoside, agnuside, and vitegnoside of the methanolic extract of *V. negundo* and the bark extract of the same plant had p-hydroxybenzoic acid and β-sitosterol. Jeyaseelan *et al.* (2011) conducted experiments for extracting *V. negundo* leaf, fruit and flower with various solvents dichloromethane, ethyl acetate, ethanol, methanol and aqueous extracts and found components like alkaloids, flavonoids, tannins, saponins, glycosides and terpenoids. The stem and leaf extract of *V. negundo* also showed the presence of tannin, glycoside, volatile oils, resins, flavonoids and saponins (Nirmalkumar, 2014).

Since phytophagous insects and other herbivores prefer leaves as their main food, plants deposit much of their defensive secondary metabolic compounds especially in the leaves than in any other parts of their body as a

defensive mechanism (Mazid *et al*., 2011). Hence, it is generally stated that the leaf extracts were found to exhibit higher toxicity and mortality.

There are many reports on the efficacy of *V. negundo* extracts towards lepidopteran pests. Das (1995) noticed that *V. negundo* can be used for the management of potato tuber moth, *Phthorimaea operculella*. Dayrit *et al.* (1995) observed the potential of topical application of *V. negundo* volatile oils in the mortality of third instar larvae of *S. litura*. Singh *et al.* (1996) reported that *V. negundo* leaves when mixed with maize showed insecticidal activities against *S. zeamais*. The insecticidal property of *V. negundo* extract against *S. litura* was also reported by Sahayaraj (1998). High mortality was recorded for *Achea janata, P. xylostella* and *S. litura* larvae with the treatment of extracts of *V. negundo, Clerodendrum inerme, Lantana camara* and *Eupatorium odoratum* (Kulkarni, 2002). Khetagoudar and Kandagal (2012) carried out toxicity studies and reported that *V. negundo* was very effective for the management of *S. litura* larva. Arivoli and Tennyson (2013) analysed the potential of *V. negundo* in causing ovicidal activity towards *S. litura*.

V. negundo leaf extract was also found to be effective against many stored product pests. Mannan *et al.* (1993) described the insecticidal potential of *V. negundo* methanolic leaf extract against *T. castaneum*. Raja *et al.* (2000) recorded the effect of solvent residues of *V. negundo* and *Cassia fistula* on *C. maculatus*. Paneru and Shivakoti (2001) also observed the insecticidal activity against *C. maculatus* caused by *V. negundo*. Tandon (2005) reported that oils of *V. negundo* leaf possessed repellency against stored product pests. Chowdhury *et al.* (2009) reported that the leaves of *V. negundo* exhibited insecticidal activities against *T. castaneum*.

Many researchers noticed the larvicidal potential of *V. negundo* against various species of mosquitoes (Yankanchi *et al*., 2014; Nayak and Rajani, 2014; Hemavathy and Anitha, 2016). Nevertheless, a lot of works in the

scientific literature studies support the insecticidal properties of *V. negundo* extracts.

2.6. Synthetic insecticides

Synthetic pesticides are of different classes which consist of organochlorines, organophosphates, carbamates and pyrethroids. Organochlorines which include DDT, toxaphene, dieldrin, aldrin etc. affect reproductive, nervous, endocrine and immune system of susceptible organisms. Organophosphates such as diazinon, glyphosate, malathion etc act on central nervous system. Carbamates like carbofuran, aldicarb, carbaryl etc also affect central nervous system. Examples for pyrethroids include fenpropanthrin, deltamethrin, cypermethrin etc were found to be highly toxic but their exact site of action is poorly understood. When animals and humans are exposed to these synthetic chemicals, it can cause either acute or chronic effects in them, mainly in the physiological systems, specific on reproductive, endocrine or central nervous systems.

Though synthetic insectides cause immediate pest control and intensified crop yield, they are disadvantageous in many respects. Many of the scientific literature studies reveal its drawbacks. Carriger *et al.* (2006) stated that generally an estimation of only 0.1% of the pesticides applied reaches the target organisms and the left bulk quantity contaminates the surrounding environment. The indeterminate, repeated and excessive use of non-biodegradable pesticides leads to pollution of water, air and soil ecosystem and have also entered into the food chain and bioaccumulated in higher trophic levels causing many acute and chronic illnesses in human beings (Mostafalou and Abdollahi, 2012). The pollution of water reservoirs with pesticides from fields occur generally through runoff or drainage induced by rain or irrigation (Larson *et al.*, 2010). Similarly air also may get polluted with pesticides in various modes like spray drift, volatilization from the

treated surfaces and aerial application of pesticides. Frequent and intensive pesticide applications tend to disperse the target pests to adapt into the novel environmental conditions and also results in the development of resistance in many targeted pest species (Cothran *et al.*, 2013). Dhaliwal *et al.* (2006) reported the resistance developed in many insect species towards different insecticide groups as 291 species against cyclodiene, for DDT (263 species), organophosphates (260 species), carbamates (85 species), pyrethroids (48 species), fumigants (12 species) and other (40 species). Thus the intensive use of pesticides has led to the development of resistance in many targeted pest species (Tabashnik *et al.*, 2009). The indiscriminate use of pesticides and the resultant insecticide resistance led to pest resurgence (Dhaliwal *et al.*, 2006).

Along with the destruction of natural enemies (Fabellar and Heinrichs, 1986) population of soil invertebrates also drastically disturbed. Pelosi *et al.* (2013) reported that the excessive pesticide use caused destruction of soil structure with negative effects on earthworms and several links in the food web. Moreover, pesticides were responsible for declining predator population and loss of insect pollinators and thereby caused indirect loss to crops (Fishel, 2011).

The very high toxic effects persisting in nature and capacity to enter into the food chain are the various risk factors of pesticides in causing bad effects on human health through direct contact or with food especially fruits and vegetables, contaminated water or polluted air. Pesticides possess the capacity to change the levels of antioxidant enzymes such as superoxide dismutase, glutathione reductase and catalase due to induction of oxidative stress (Sharma *et al.*, 2014) which in turn results in disruption of glucose homeostasis and several health problems such as Parkinson disease (Mostafalou and Abdollahi, 2012). A variety of circumstances which bring about pesticide exposure include direct ingestion of pesticide granules and

treated seeds, treatment of crops, direct exposure to sprays, contamination of water or feeding on contaminated prey and baits which cause birds mortality (Guerrero *et al*., 2012).

The recognition of the unintended effects of insecticides initiated the search for new techniques of insect control to be established. Alternative methods of pest control involves IPM, which enables a combination of different control tactics such as cultural control, physical and mechanical control, use of resistant genotype and also the use of biorational pesticide could reduce the number and amount of pesticide applications. IPM is designed with the intention to reduce indiscriminate pesticide use and also to replace hazardous chemicals with safe plant chemistries. Gill *et al*. (2013) reported that cultural control methods are farmer's most important tool for preventing crop losses due to its eco-friendly nature and minimal costs. Other potential management options include physical control, host plant resistance, biocontrol and the use of biopesticides etc. Biopesticides/biorational pesticides are pesticides of natural origin that have limited or no adverse effects on the environment or beneficial organisms and are rapidly gaining popularity. Biopesticides include microbial pesticides, plant pesticides and biochemical pesticides which are derived from micro-organisms and other natural sources.

However a good pesticide should be always effective against pests, should not cause health hazards to humans and non-target organisms and should not be persistent or should ultimately dissociate into harmless compounds in environment. To minimize the pesticide related tribulations, advanced approaches such as biotechnology and nanotechnology might be useful in developing resistant genotype or pesticides with fewer adverse effects. Furthermore, we can reduce deleterious impact of pesticides on our environment by educating and encouraging farmers to adopt the innovative

IPM strategies for pest control including the introduction of inevitable plant allelochemicals for effective pest management.

2.7. Botanical insecticides

Plant products and secondary metabolites of plant origin have been found to be in use by human beings very early. Although many alternate substitutes have been suggested by many authors, plant based pesticides have been emerged as more viable method than any other biorational techniques, in terms of availability and adaptability. It was already reported that the use of plant and plant-derived products to control pests in the developing world is well known and also prior to the discovery of synthetic pesticides, plant or plant-based products were the only pest-managing agents available to farmers around the world (Owen, 2004). Senthil-Nathan and Kalaivani (2005) also stated that botanical pesticides are highly effective, safe and ecologically acceptable.

Rahuman *et al*. (2008) noted some earlier used botanical insecticides in many countries such as chrysanthemum, pyrethrum, derris, quassia, nicotine, hellebore, anabasine, azadirachtin, d-limonene, camphor and turpentine etc. These traditional botanical pest control agents have got much attention of chemists and biologists because of their structural complexity, potency and selectivity.

2.8. Botanicals currently in use and their mode of action

The botanical insecticides which are available commercially consist of pyrethrin/pyrethrum, rotenone, sabadilla, ryania, nicotine, neem and citrus oil extracts.

Pyrethrin/pyrethrum: Pyrethrin, a fast acting insecticide derived from the seeds or flowers of *C. cinerariaefolium* is one of the oldest

household insecticides still available and presents an immediate “knockdown” effect on insects after application. The flower extraction yields an orange-colored liquid that contains the active principles (Casida and Quistad, 1995), which include three esters of chrysanthemic acid and three esters of pyrethric acid. Among these, pyrethrins I and II are the most abundant with more pesticidal activity. Pyrethrin possesses low mammalian toxicity, very short residual activity, degrading rapidly and acts both as a contact and stomach poison. The mode of action of pyrethrin is disruption of sodium and potassium ion-exchange process in insect nerves and interruption of the normal transmission of nerve impulses. Pyrethrin is active against a wide range of insects and mites, including flies, fleas, aphids, thrips, mosquitoes, whiteflies, leaf hoppers, caterpillars, mealybugs, beetles, lice and spider mites.

Rotenone: Rotenone is an isoflavonoid obtained from the roots or rhizomes of the tropical legumes *Derris*, *Lonchocarpus* and *Tephrosia*. *Lonchocarpus* grown in Venezuela and Peru provides the main source of rotenone used at present. Organic solvent extraction of the root yields resins containing major constituents of rotenone and deguelin (Fang and Casida, 1998). It is effective only if ingested and so regarded as a stomach poison. It acts as a mitochondrial poison, which prevents energy production by blocking electron transport chain (Hollingworth *et al.*, 1994). Rotenone can be applied against a number of insect and mite pests, including leaf-feeding beetles, caterpillars, thrips, lice, mosquitoes, aphids, spider mites, ticks, fleas, flies and fire ants.

Sabadilla: Sabadilla is an alkaloid similar to that of pyrethrins obtained from the seeds of the South American lilly *S. officinale*. Its pure form, cevadine-type alkaloids as such are extremely toxic to mammals, where as its commercial preparations have less than 1 % active ingredients to ensure

safety. Isman (2006) reported that sabadilla is used primarily on citrus crops and avocado by organic growers. In insects, it affects nerve cell membrane action and cause loss of nerve function leading to paralysis and death.

Ryania: Ryania is an alkaloid rich compound derived from the roots and woody stems of the Caribbean shrub, *Ryania speciosa* found to be declining in use. It acts as a slow-acting stomach poison with very low mammalian toxicity. The powdered wood contains an alkaloid, ryanodine which interferes with calcium release from muscle tissue (National Research Council, 2000). Ryania works best on caterpillars of codling moth, cabbage worm, corn earworm and also has activity on a wide range of insects and mites, including citrus thrips, beetles, lace bugs, whiteflies, squash bug and aphids.

Nicotine: Nicotine, an alkaloid obtained from the foliage of *N. tabacum* and two closely related alkaloids, nornicotine and anabasine, are synaptic poisons that mimic the neurotransmitter acetylcholine. Regnault-Roger and Philogène (2008) reported that nicotine causes poisoning symptoms similar to that with organophosphate and carbamate insecticides. Nicotine has seen declining in use due to its extreme toxicity to mammals and its rapid dermal absorption in humans. Nicotine is very fast-acting and acts as a nerve toxin in both insects and mammals. Nicotine can be applied against soft-bodied insects, mites, aphids, thrips, leaf hoppers and spider mites.

Neem: The Indian neem tree yields two types of botanical pesticides from its seeds (Schmutterer, 1990, 2002). Neem products (Azadirachtin) and neem oil are found to be effective against soft-bodied insects and mites and are also useful against phytopathogens. The disulfides present in the oil increase bioactivity of this material (Dimetry, 2012). Neem has varied mode of action such as feeding deterrent, insect-growth regulator, repellent and sterilant and as oviposition inhibitor. These remarkable properties of

azadirachtin provoked search for natural pesticides in the most closely related tree, *M. azedarach*, which has a number of triterpenoids, the meliacarpins, with some similarity to the azadirachtin and also have insect growth regulating bioactivities (Kraus, 2002). Neem is effective against many insects, including aphids, whiteflies, mealybugs, thrips, Japanese beetle and caterpillars.

Citrus oil extracts: Orange and other citrus fruit peels possess active compounds, limonene and linalool which are acting as contact poisons. They can influence the spontaneous activity of the sensory nerves, causing overstimulation of motor nerves leading to convulsions and paralysis. Both limonene and linalool are used on lice, ticks, aphids, mites, fire ants, house flies, paper wasps and crickets.

In recent years many researchers are motivated to promote research on pest control methods at low environmental cost with the increasing demand of consumers searching for healthier products with minimal environmental impact (Souza *et al.*, 2008). Since biopesticides possess many advantages over synthetics along with low environmental pollution and low toxicity to humans, botanicals are generally regarded as perfect alternatives to synthetic pesticides (Elumalai *et al.*, 2010). Some of the advantages of botanical insecticides are discussed below.

- Rapid degradation under environmental conditions like sunlight, humidity and rainfall and are less persistent in nature, thereby reducing deleterious impact on beneficial and nontarget organisms.
- Botanical insecticides are fast acting, kill insects rapidly or may deter insects from feeding soon after pesticide application.
- Most of the botanicals are having low mammalian toxicity

- Botanical insecticides are highly selective in their action and considered generally less harmful to beneficial insects and mites than synthetic insecticides.
- The application of botanical pestcides will not cause harmful effects on plants.

The main advantage of botanicals relies in easy access of locally available plants for farmers and their less expensive nature. They possess efficacy against insects causing feeding deterrency, interruptions in larval development, moulting disruption, low fecundity and egg hatchability, interference with development and oviposition. Many plant derived substances have the capacity to influence the physiological and behavioural properties of insect pests and may contribute to the development of new natural pesticides. Alterations in nutritional parameters, high mortality especially in immature stages of insects are earlier reported for a wide group of phytophagous insects by Mitchell *et al.* (2004). These natural products are having varied range of biological activities including toxicity and growth regulation properties (Chiam *et al.*, 1999). Bowers (1992) also stated that eventhough these plant products are relatively weak in their action when compared to modern synthetics they are pest specific, readily biodegradable and non-toxic to higher groups of animals.

2.9. Secondary metabolites

The organic molecules synthesized by most plants without an obvious role in normal cell functioning are known as secondary metabolites. They have significant ecological functions in plants and protect them from being eaten by herbivores and against being infected by microbial pathogens. The pesticidal activity of plant materials and their extracts are associated with the presence of secondary compounds of plant origin which have no particular

functions in photosynthesis, growth or other aspects of plant physiology (Singh, 1993). Rattan (2010) reported that these secondary metabolites possess the capacity to kill, retard or accelerate development or interfere with the life cycle of pest and also function as defensive, which inhibits reproduction and other biological processes too.

Chowdhuri *et al.* (2007) stated that secondary metabolites involving alkaloids, flavonoids, terpenoids, phenolics, chromenes, essential oils and other minor chemicals are associated with a wide range of biological activities such as serving as attractants, deterrents, phagostimulants, antifeedants or modify oviposition, disruption of major metabolic pathways and rapid death.

Scientific literature shows that phytochemicals of plant origin contain a wide range of chemically active ingredients, which can interrupt most of the biological processes of insect pests and thus interfere its life cycle, spreading and damage they cause and thereby improve agricultural crop production. The increased production of secondary metabolites from different parts of a plant is mainly due to the high risk of pest inundation, saying that plants release these compounds as chemical weapons (Miresmailli and Isman, 2014). Sometimes these compounds can reduce the desirability of the plant and make it unfit for feeding (Hirayama *et al.*, 2007) or otherwise lure the pests towards poison and kill them (Tangtrakulwanich and Reddy, 2014).

The bioactive compounds of plant origin include alkaloids, terpenoids, phenols, flavonoids, steroids etc. which are generally thought to be involved in plant-insect interactions (Kubo, 2006) and are also mediating interactions between plants and their biotic environment and do not have apparent function in physiological or biochemical processes (Berenbaum, 2002). These plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics and nitrogen-containing compounds.

The terpenes or terpenoids are secondary metabolites of the largest, diverse class of water insoluble compounds. Certain terpenes show significant functions in plant growth, for example, gibberellins, the important plant hormones are diterpenes. However most of the terpenes are secondary metabolites considered to be involved in plant defenses acting as toxins and feeding deterrents to many herbivorous insects and mammals. Pyrethroids with striking insecticidal properties are monoterpene esters occurring in the leaves and flowers of *Chrysanthemum* sp.

Many plants exhibit the presence of mixtures of volatile monoterpenes and sesquiterpenes, commonly called as essential oils, gives a characteristic odour to their foliage. Prates and Santos (2002) revealed that essential oils are those plant derived compounds which are lipophilic in nature causing toxic interference in basic biochemical processes with physiological and behavioral consequences for the insects. Essential oils are considered as ecofriendly pesticides with diverse toxicity levels as ovicidal, larvicidal and repellent properties against various insect species (Cetin *et al*., 2004). Many higher plants serve as a reservoir of essential oils which in turn constitutes compounds with insecticidal activities, principally monoterpenes and their analogues in abundance. It has been earlier reported that these essential oils and their constituents were found to be potent source of botanical pesticides (Krishnappa *et al*., 2011). Essential oils found in glandular hairs projecting from the epidermis are having strong insect repellent properties.

Terpenoids have got much ecological interest since they have been causing larval mortality, significant growth inhibition, repellent and fumigant activity. These plant terpenoids along with phenols, flavonoids, steroids play a significant role in plant defenses against phytophagous animals (Kubo, 2006). Most of these compounds are having varying mode of actions, but many affect nervous system; for instance the plant origin compound,

monoterpenoids, may affect the nervous system on contact (Isman, 2000), nicotine affects acetylcholine receptors in the nervous system and also veratrine acts on membrane sodium channels of nerves (Rattan, 2010).

Phenols with their reduced carbon skeleton display antioxidant properties and also show significant role in photoprotection (Close and McArthur, 2002) and several other important properties for plant defense mechanisms. They are water soluble also, stored in vacuoles and are ubiquitous in the plant kingdom (Balasundram *et al.*, 2006). Plant phenolics are heterogeneous group of chemicals, consists of nearly 10,000 individual compounds. Phenolic compounds vary in their solubility in different solvents, for example some are soluble only in organic solvents, some are water-soluble carboxylic acids and glycosides and others are large, insoluble polymers. Schoonhoven *et al.* (2005) studied and recorded the importance of plant phenolic compounds on plant defense against herbivory.

War *et al.* (2012) reported that the high molecular weight phenolic compounds, tannins act as strong chelators in order to reduce the availability of metal ions to herbivores and also as astringents, which tends binding to dietary proteins and thus results in reduced nutrient absorption efficiency. Duffey and Stout (1996) earlier described that the chelation property of tannins lead to enzyme deactivation. Moreover, tannins also act as feeding repellents to a great variety of animals. Tannin containing plants will not be preferred by mammals such as cattle, deer, apes etc. High tannin levels in unripe fruits deter feeding on the fruits until it become mature for the dispersal of seeds.

Most of the plant secondary metabolites that possess nitrogen in their structure are synthezised from common amino acids, which include alkaloids and cyanogenic glycosides. Alkaloids possess striking pharmacological effects on vertebrate animals.

Alkaloids are regarded as the most important group of natural substances with very wide distribution in the plant kingdom (Bruneton, 1999), especially seen in families, Solanaceae, Papaveraceae, Apocynaceae and Ranunculaceae with more than 12000 identified compounds (Mithöfer and Boland, 2012). Akhtar *et al.* (2008) revealed the mode of action of alkaloids as neuro-muscular toxin and act both as contact and stomach poison. The activity of alkaloids as insectcides is not restricted to a single mode of action, for example nicotine targets the nicotinic acetylcholine receptors; colchicine shows inhibition of microtubule polymerization, preventing mitosis; caffeine causes insect paralysis through inhibition of phosphodiesterase activity (Mithöfer and Boland, 2012).

Other groups of nitrogenous protective compounds are cyanogenic glycosides and glucosinolates. They are not directly toxic but are readily broken down to release poisons, in which some compounds volatile in nature are released only by crushing. The poisonous gas coming out of cyanogenic glycosides is hydrogen cyanide (HCN) and so its presence deter insect feeding and herbivory. Glucosinolates are secondary metabolites characterictics of the order Brassicales. Schulze *et al.* (2002) noticed that for humans, glucosinolates have a pungent taste and are toxic to insects.

Saponins are plant secondary metabolites with amphiphilic nature possessing hydrophilic sugar moieties attached to a lipophilic group. They are triterpene steroid glycosides with soap like properties. They are water soluble and produce foam when shaken. Vincken *et al.* (2007) noticed that they are scarce, but widely distributed in 30 orders of plants. Chaieb (2010) investigated the mechanism of action through which saponins exhibit toxicity over insects by interaction with cholesterol, which is a precursor of ecdysteroids, a class of insect growth regulators and thus alter insect development through lengthening of larval stages or ecdysial failure.

Burt (2004) described that the induction of biological activity is not entrusted with a single mechanism of action but by the action of vast majority of chemical groups aiming at multiple targets in the cell or which may provoke toxic effects as larvicidal, pupicidal, adult emergence inhibition and several other bioactivities against insect pests.

Scientific literature shows that a lot of studies have used plant products for pest management and proved the entomotoxic properties of crude extracts from different plant species (Ulrichs *et al*., 2008; Baskar and Ignacimuthu, 2012; Packiam *et al*., 2013).

2.10. Toxic effects of botanicals

Scietific literature incorporates numerous publications reflecting the detrimental effects of diverse plant extracts on insect pests. Akhtar *et al.* (2008) suggested that a wide range of botanical extracts tested were found to be strong growth inhibitors, acutely toxic and active feeding deterrents against lepidopteran pests.

Sundar *et al.* (2000) studied and confirmed the insecticidal activity of azadirachtin based formulations against *S. litura.* Dadang and Ohsawa (2001) reported a significant reduction in *P. xylostella* population with application of certain plant extracts including *Alpinia galangal*, *Amomum cardamom*, *Cyperus rotundus* and *Gomphrena globosa* on cultivated cabbage reducing their damage to a great extent. Likewise, Sahayaraj (2002) also reported toxic effects on *S. litura* with the aqueous extract of *Calotropis* sp. Raja *et al.* (2003) noticed that ethyl acetate extract of *Artemisia nilagirica* expressed larval mortality powers on *S. litura.* Sedak (2003) described that *Adhatoda vasica* extracts showed insecticidal activities against *Spodoptera littoralis*.

Subashini *et al.* (2004) investigated the larval mortality of *H. armigera* caused by the hexane extract of *Dodonaea angustifolia*. Elumalai *et al.*

(2004) who worked on ethyl acetate leaf extract of *Acorus calamus* proved that it exhibited maximum larvicidal activity towards *S. litura.* Neem and *L. camara* extracts were reported to be used for reducing the damage caused by *P. xylostella* on cabbage leaves (Dong *et al*., 2005).

Pavunraj *et al.* (2006) noticed the insecticidal properties of hexane, chloroform and ethyl acetate extracts of *Excoecaria agallocha* towards *S. litura.* Senthil-Nathan and Sehoon (2006) observed the pesticidal properties of *M. azedarach* extracts against *Hyblaea puera.*

Malarvannan *et al.* (2008) conducted experiments with *Argemone mexicana* extracts and proved its toxic properties against *S. litura*. Mazoir *et al.* (2008) observed significant toxic activity of terpenoid derivatives of the major components of *Euphorbia resinifera* and *Euphorbia officinarum* latex on *S. littoralis.*

Furthermore, different solvent (petroleum ether, hexane) extracts of *Jatropha curcas* exhibited insecticidal activity against larvae of *H. armigera* (Aravinda *et al*., 2009). Sharma and Gupta (2009) evaluated the efficacy of aqueous extract of *M. azedarach*, *Nerium indicum* and *A. indica* and confirmed the higher mortality of larvae of cabbage butterfly, *Pieris brassicae*.

Abbasipour *et al.* (2010) evaluated and confirmed 100% insecticidal activity of *Peganum harmala* seed extract against diamondback moth, *P. xylostella.* Similarly, Haouas *et al.* (2010) conducted mortality studies with the methanolic extract of *Chrysanthemum macrotum* and proved its efficacy against *S. littoralis*.

Jeyasankar *et al.* (2011) isolated insecticidally active compound present in the leaves of *Syzygium lineare* and was assessed against fourth instar larvae of *S.litura* showing maximum activity more than that of the

positive control azadirachtin. Trindade *et al.* (2011) demonstrated the larval mortality of *P. xylostella* larvae caused by ethanolic extract of *Annona muricata* leaves.

Bhagat and Kulkarni (2012) reported the antifeedant and larvicidal activities of rhein isolated from *C. fistula* flower against lepidopteran pests *S. litura* and *H. armigera*. The larvicidal efficacy of leaf powder of *Calotropis procera* and *A. mexicana* against 4th instar of *H. armigera* was manifested by Deepali *et al.* (2013). The strong insecticidal activity of the ethanolic leaf extract of *A. muricata* proved to be effective against *P. xylostella* larva by Vanichpakorn *et al.* (2014).

Thushimenan *et al.* (2016) explored the potential of *Terminalia arjuna* and *Trachyspermum roxburghianum* against *H. armigera* as larvicidal agents. Ethyl acetate extract of *Duranta erecta* was analysed for its effectiveness and revealed its significant larvicidal activity against *H. armigera* and *S. litura* (Chennaiyan *et al.*, 2016).

For a variety of reasons botanical pesticides were considered as safe alternatives to synthetic insecticides. Besides causing mortality effects, these botanical insecticides, might have also caused repellency, oviposition deterrency, feeding deterrency and change in hormonal systems of several insect pests (Isman, 2006). All these features intensified the use of botanical pesticides for pest management (Copping and menn, 2000; Duke *et al.*, 2010).

2.11. Effect of botanicals on histopathology

For the proper understanding of metabolic changes brought about in an insect body histopathological studies are inevitable and are considered as the ultimate reason for changed physiological and biochemical functions initiated with the changes in the cellular levels of various tissues of insects coming under different orders. Scientific literature shows lot of works on the

histomorphology and ultrastucture of midgut of lepidopteran insects and these studies illustrate that the distribution and morphology of the epithelial cells may vary along different regions (Levy *et al*., 2004; Pinheiro *et al*., 2008).

Nasiruddin and Mordue (1994) observed histopathological changes like necrosis of midgut cells, rounding up of the cells and swelling of organelles; the connective tissue beneath the epidermal layer became enlarged with many invading cells or nuclei and the circular/longitudinal muscles became round and swollen in the midgut of *Schistocerca gregaria* and *Locusta migratoria* after treatment with azadirachtin.

Gerard (2002) described changes in the midgut epithelium of *Hofmannophila pseudospretella* associated with the introduction of keratin into the diet and observed differences in the size and activity of columnar cells in the middle part of midgut and goblet cells depending on food quality.

Literature studies shows that ingestion of toxic ingredients present in botanical pesticides can influence histomorphology of the midgut tissue of insects leading to disorders in the feeding, digestion and food absorption behaviours. Koul *et al.* (2004) noticed the involvement of rocaglamide isolated from *Aglaia elaeagnoidea* in the induction of cytotoxicity at non-specific cellular levels on *H. armigera.* The same mode of action was noticed in the case of Aglaroxin B and C isolated from the same plant on *H. armigera* and *S. litura* (Koul *et al*., 2005). Jing *et al.* (2005) conducted ultrastructural studies with diet incorporation of Jatropherol-I compound isolated from *J. curcas* seeds on silkworm larvae and showed that Jatropherol-I caused pathological changes in the midgut cells such as dilation in endoplasmic reticulum, withered microvilli and also the disappearance of secretory granules leading to alteration in metabolism of the cells.

Histological studies of the midgut tissue in the gypsy moth, *Lymantria dispar* larvae fed with leaves of *Robinia pseudoacacia* showed vacuolization of the cytoplasm, elongation of columnar cells and partial loss of microvilli (Perić-Mataruga *et al*., 2006).

Barbeta *et al*. (2008) studied the effect of plant cyclotides on the larval midgut of *H. armigera* and observed the disruption of plasma membrane in the epithelial cells with the formation of pores leading to cell swelling and lysis and suggested that ingestion of cyclotides results in marked changes in the midgut of lepidopteran pests. Electron microscopic studies were carried out on harmaline treated *Plodia interpunctella* midgut cells by Rharrabe *et al*. (2007) which showed cytoplasmic vacuolization, numerous autophagic vesicles and lysosomic structures, fragmentation of rough endoplasmic reticulum and microvilli, damage of plasma membrane, all these resulted in shedding of cytoplasmic contents into midgut lumen.

Jbilou and Sayah (2008) noticed severe histopathological changes on midgut epithelial cells such as vacuolization of the cytoplasm and occurrence of large intercellular space due to the effects of methanolic extract of *P. harmala* seeds on *T. castaneum* midgut cells through diet incorporation.

Adel *et al*. (2010) conducted studies to evaluate the histopathological effect of *Artemisia monosperma* on *S. littoralis* and observed cytotoxic changes like completely destroyed epithelial membrane; much elongated and extremely vacuolated epithelial cells with their cell boundary almost disappeared.

Rawi *et al*. (2011) demonstrated the histopathological effects of *A. indica* and *Citrullus colocynthis* extracts in the larval midgut of *S. littoralis* separately and noticed that extract of *A. indica* induced histological damage in the larval midgut, showing vacuolated epithelial cells and destruction of

nuclear content whereas extract of *C. colocynthis* caused degeneration of columnar epithelial cells and vacuolation.

Packiam *et al.* (2013) observed histopathological changes such as cellular shrinkage, necrosis, disorganisation of peritrophic membrane and epithelium, irregular nuclear arrangement etc. in the midgut of *H. armigera* when treated with Ponneem, a neem and pongam formulation.

Ranjini and Nambiar (2015) noticed histopathological changes such as vacuolization, morphometric changes in the columnar cells and destruction of microvilli with the treatment of leaf extracts of *C. infortunatum* and *Eupatorium odoratum* on the midgut tissue of sixth instar larvae of *O. exvinacea.*

2.12. Effect of botanicals on biochemical parameters

In addition to the mortality effects caused with the treatment of botanicals many authors have studied the influence of chemicals present in the botanicals on various nutritional indices of the insect pests, which gives an indirect evidence for identification of mode of action of plant derived compounds. Toxicity effects of botanicals can be directly assessed with the proper understanding of the various proportions of biochemical parameters (digestive and detoxification enzymes and energy reserves) in the body of insects.

Earlier many researchers have reported biochemical alterations of various components like carbohydrates, lipids, proteins etc as a result of pesticide treatment, which in turn disturb the internal metabolism of the insect, leading to their reduced activity or mortality. Proteins are the most important biochemical components or complex macromolecules of living cells, which are very essential for the proper development and growth of the insect including many vital biological functions like cell division, metabolism

and transportation of ions across membranes, cuticle melanization and sclerotization. Proteins are composed of amino acids bound together with peptide bonds. So any change in these components directly affects the development of an organism. Paramount insecticidal properties of many botanicals on the biochemical changes and functions have led to more proposed caustic works using botanicals for the control of insect pests.

Etebari *et al.* (2006) noticed that in physiological studies, determination of total protein is important because many of the insecticides lead to toxic effects and also reduce feeding efficiency which in turn minimises important vital components like proteins in the body. Finally, the weak energy metabolism leads to the death of the insects.

Many researchers noticed biochemical changes in lepidopteran pests with the treatment of botanicals. Li *et al.* (1995) reported the influence of azadirachtin in the haemolymph protein concentration of *S. litura*. There are reports on depletion of energy reserves after ingestion of neem oil in *Choristoneura rosaceana* (Smirle *et al*., 1996) and Jatropherol-I on *Bombyx mori* (Jing *et al*., 2005). Schmidt *et al.* (1998) investigated that treatment of azadirachtin on *S. littoralis* and *A. ipsilon* led to decrease in haemolymph protein composition. Moreover, *S. littoralis* larvae when treated with *Eucalyptus* oil and its combination with gamma radiation, exhibited reduction in the total protein content of midgut tissue (El-Naggar and Abdel-Fattah, 1999).

Rajkumar and Subrahmanyam (2000) experimented with azadirachtin for topical treatment of *H. armigera* and this effected in the reduction of cuticular protein content. This study also revealed considerable changes in the electrophoretic banding patterns of proteins indicating defective cross linking of chitin- proteins. Treatment of *Annona* seed extract in *S. litura* larva exhibited significant reduction of protein content during second and fourth

instars and also conspicuous variations in electrophoretic banding pattern of fourth instar larva (Boreddy *et al.*, 2000). Chitra and Reddy (2000) reported significant effects of *A. squamosa* seed extract on protein metabolism of *S. litura.*

It was described that the treatment of acetone extract of *M. azedarach* on sixth instar larvae of *A. ipsilon* caused reductions in total proteins, lipids, and carbohydrates of haemolymph (El-Shiekh, 2002; Abd-El-Wahab, 2002). Vijayaraghavan and Chitra (2002) analysed the activity of neem and *Annona* seed extracts in the reduction of protein content in *S. litura* larvae.

Huang *et al.* (2004) reported altered pupal protein levels of *S. litura* when fed with azadirachtin contaminated diet from fourth instar onwards. Moreover, when the proteins were electrophoretically separated, it was observed that 10 protein bands significantly affected indicating the effect of azadirachtin on protein expression. It was earlier reported the influence of azadirachtin on head protein of *H. armigera* by Neoliya *et al.* (2005, 2007).

Huang *et al.* (2007) conducted experiments with azadirachtin on *Ostrinia furnacalis* to assess its effect on protein profile and concluded that it significantly affected six proteins. Ulrichs *et al.* (2008) studied the efficacy of *Porterasia coarctata* leaf extracts in reducing the total proteins in the midgut tissues of *S. litura.*

Ethanolic extracts of *Ageratum conyzoides and Ageratum vulgaris* influenced the total head protein profile of *S.litura* (Renuga and Sahayaraj, 2009). Similary, a significant reduction of energy reserves, chiefly protein and glycogen contents was noticed in *P. interpunctella* followed with the ingestion of harmine, harmaline, 20-hydroxyecdysone or azadirachtin (Rharrabe *et al.*, 2007; Bouayad *et al.*, 2012).

Similar findings on reduced total proteins and carbohydrates in *S. litura* larva due to the effect of methanolic extracts of *Lantana wightiana* have been recorded (Rathi and Gopalakrishnan, 2010). Khosravi *et al.* (2010) studied the impact of methanolic extract of *A. annua* and reported reduction in the total protein, carbohydrate and lipid contents in the fifth instar larva of mulberry pyralid, *Glyphodes pyloalis*. Studies were conducted on the leaf webber larva, *C. binotalis* to prove the influence of the extracts of *Strychnos nuxvomica, V. negundo* and *Lippia nodiflora* on nutritional parameters (Vijayaraghavan *et al.*, 2010). Khatter and Abuldahb (2010) evaluated the potential of extracts from *R. communis* leading to a significant increase of protein content in the haemolymph of *S. littoralis* larva.

Khosravi *et al.* (2011) studied and reported a considerable decrease in the amount of total protein in the larvae of *G. pyloalis* treated with *A. annua* methanolic extract. Rawi *et al.* (2011) tested the potential of *A. indica* and *C. colocynthis* extracts on *S. littoralis* larvae which showed marked decrease of the total protein, glucose and lipid contents.

Medhini *et al.* (2012) studied the effect of *Calendula officinalis* on the nutritional physiological parameters of *S. litura* larvae by treating with leaf and flower extracts in various solvents and observed significant decrease of total protein content in haemolymph and midgut of larvae.

Bouayad *et al.* (2013) conducted experiments with Morrocan plants against *P. interpunctella* and found a significant reduction of reserve substances, especially the protein and carbohydrate contents after plant extract ingestion. The essential oil isolated from *Lavandula angustifolia* treated on the pyralid, *G. pyloalis*, showed reduced protein and carbohydrate content (Yazdani *et al.*, 2013).

2.13. Effect of botanicals on enzyme activity

The metabolic changes, whether induced by endogenous or exogenous factors often results in altered enzymatic activities and may cause variations in the physiological state of the organism.

There are many reports on altered enzyme activities in Lepidopteran pests associated with the treatment of botanicals. Ayyangar and Rao (1990) noticed disturbed ACP activity on *S. littoralis* when treated with azadirachtin. Rosenthal and Dahlman (1990) conducted studies to evaluate the efficacy of L- canaline, isolated from higher plants and it was found inhibiting aminotransferase in *Manduca sexta* larva. Lee (1991) investigated an inhibitory Glutathione S transferase (GST) activity in *Trichoplusia ni* and *Papilio polyxenes* due to the effect of plant phenols.

Feng *et al.* (1995) studied the esterase inhibitory activity of bark extracts of *Melia toosendan* in *S. litura* and *Melanoplus sanguinipes*. Koul *et al.* (1996) also revealed similar results in *S. litura* larva treated with azadirachtin. Extracts from *Ammi majus*, *Apium graveolens*, *M. azedarach* and *Vinca rosea* were found to inhibit ACP activity in *A. ipsilon* (Abo-El-Ghar *et al.*, 1996).

Likewise, Smirle *et al.* (1996) also reported inhibition in mid gut esterase activity in *Choristoneura rosaceana* treated with various doses of neem oil. Babu *et al.* (1996) also reported enhanced gut ACP activity and decreased ATPase activity in *H. armigera* upon treatment with azadirachtin. Ortego *et al.* (1999) conducted studies to evaluate the effect of terpenoids on digestive proteases and detoxification of enzyme activities of Colarado potato beetle, *Leptinotarsa decemlineata* larvae, when fed with treated potato leaves which showed reduced esterase activity. Furthermore, Smirle *et al.*, 1996) reported that neem possess the capacity to affect enzyme synthesis in insects.

Yerasi and Chitra (2000) studied and evaluated the effect of annonain on aspartate and alanine aminotransferase activities of *S. litura*. Hemming and Lindroth (2000) had reported an induction in esterases activity in the gypsy moth, *L. dispar* fed with a diet of aspen leaves supplemented with phenolic glycosides. Disturbed ACP activity was again reported in *S. littoralis* when treated with certain botanical extracts (Hassan, 2002).

Breuer *et al.* (2003) carried out studies in *Spodoptera frugiperda* which showed reduction in cholinesterase and NADPH cytochrome reductase activity after treatment with *M. azaderach e*xtract. Senthil-Nathan *et al.* (2004) studied the effect of neem extracts in *C. medinalis* and recorded marked changes in acid phosphatases (ACP), alkaline phosphatases (ALP) and adenosine triphosphatases (ATPase) activities.

Senthil-Nathan and Kalaivani (2005) reported decreased amount of ALP and LDH in the midgut of *S. litura* fed with azadirachtin treated *R. communis* leaves. Senthil-Nathan *et al.* (2005) demonstrated the efficacy of azadirachtin and nuclear polyhedrosis virus individually and in combination against *S. litura* in reducing activity of gut enzymes such as acid phosphatase, alkaline phosphatase, ATPase and lactate dehydrogenase.

Similarly, Senthil-Nathan *et al.* (2006 a) studied the impact of neem seed kernel and *V. negundo* extracts treated rice leaves on *Cnaphalocrocis medinalis* larva in combination with *Bacillus thuringiensis* and found that it reduced digestive enzyme activities (protease, amylase and lipase). Furthermore, lactate dehydrogenase activity was found to be inhibited in rice leaf folder by various neem limonoids and identified azadirachtin as a potent inhibitor (Senthil- Nathan *et al.*, 2006 b). Senthil-Nathan *et al.* (2006 c) also reported that digestive enzymes like protease and lipase enzymes of *C. medinalis* was found to be suppressed by extracts of *V. negundo* and *A. indica*. Likewise, Senthil-Nathan (2006) conducted studies to evaluate the

effect of *M. azaderach* seed extract treatment on rice leaves fed by *C. medinalis* resulted in reduction of acid phosphatase, alkaline phosphatase, ATP ase and lactate dehydrogenase activities.

Krishnan and Kodrik (2006) found considerably high superoxide dismutase (SOD) activity in midgut tissue of *S. littoralis* larvae after feeding with potato leaves containing high content of allelochemicals. It was proved that dysoxylum triterpenes possess the capacity to inhibit the activities of acid and alkaline phosphatases and ATPase in rice leaf folder (Senthil-Nathan *et al*., 2007). Inhibition of amylase activity was noticed in *P. interpunctella* when treated with harmaline (Rharrabe *et al*., 2007). Rharrabe *et al.* (2008) investigated the effects of neem derivative, azadirachtin on the fourth instar larvae of *P. interpunctella,* showing severe reduction in amylase activity.

Zibaee *et al*. (2011) recorded the capability of different pesticides in reducing the activity levels of α and β-glucosidases on treated *Hyphantria cunea* larvae. Adel *et al*. (2010) have been studied the biochemical effects of *A. monosperma* on the cotton leaf worm, *S. littoralis* and noticed the following observations such as significant inhibition in the activity of amylase and invertase enzymes; considerable reduction in the transaminase enzyme activity of both AST and ALT; potent inhibitory effect of phosphatase enzymes (ACP and ALP) activity.

Rawi *et al*. (2011) revealed biochemical effects of crude extracts in the level of enzyme activity, in which *A. indica and C. colocynthis* induced a marked decrease in ALT activity. Significant reduction of α-amylase activity in *Pieris rapae* treated with *A. annua* extract was reported by Hasheminia *et al.* (2011). Khosravi and Sendi (2013) reported that azadirachtin possess the capacity to reduce the protease activity as well as the level of α and β-glucosidases in *G. pyloalis* treated with neem pesticide (achook).

In addition to the effects on lepidopteran insects altered levels of various enzyme activities were reported in insects of other orders too by many researchers (Zibaee and Bandani, 2010; Younes *et al*., 2011; Sahayaraj and Sobha, 2012; Ghoneim *et al*., 2014). The altered biochemical changes, either enhancement or inhibition of the enzyme activity caused by any kind of pesticides usually depends not only on the insect species but also on its developmental stage, age, tissue, nature of the botanical, the solvent used for extraction and method of treatment (Hamadah, 2009).

CHAPTER III
MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. **Experimental organism: *Orthaga exvinacea* Hampson**

(Order: Lepidoptera; Family: Pyralidae)

The present investigation was carried out on sixth instar larva of the mango leaf webber, *Orthaga exvinacea.* The mango leaf webber larvae are gregarious in nature. Initially they feed on the mango leaves by scraping the leaf surface and later on make webbings between leaves at the ends of branches. A webbed cluster of leaves harbour several larvae (Cherian and Ananthanarayanan, 1943). Srivastava *et al.* (1982) reported that the caterpillars feed within the webbed cluster of leaves, leaving dry bits of leaves.

Life cycle of *Orthaga exvinacea*

The life cycle of *O. exvinacea* is found to vary in accordance with the seasons. The complete life cycle consists of egg, larval instars, prepupa, pupa and adult moth (plate III. 1). It may take about 45-52 days to complete the life cycle, with six or seven larval instars. The period of time for each of the developmental stages are varied according to changes in the availability of food, temperature and other climatic factors (Srivastava *et al.*, 1982; Rafeeq and Ranjini, 2011). During summer season the development was found to be faster with six larval instars and in other seasons the period is prolonged with seven instars. This confirms the findings of Nair (1986) that there are 5 to 7 moults in *O. exvinacea.*

The eggs laid by *O. exvinacea* vary greatly in their size and shape. The round or globular eggs are laid singly or in clusters and are firmly glued on either side of the midrib of leaves or even to the culturing vessels. Freshly laid eggs are cream coloured, subsequently turn to yellow and within 72 h it becomes dark brown in colour just before hatching. Immediately after hatching the larvae moves about actively. An incubation period of 2-4 days is generally required for the complete development of the egg, but this is slightly prolonged in other seasons. During summer season when the ambient temperature rises above 32°C, the eggs fail to develop.

Life cycle consists normally of six larval instars. The first instar larva measures an average length of 2.1 mm, width of 0.34 mm and it lasts for an average duration of 3 days. It stops feeding to moult into the second instar larva. The second instar larva differs from the first instar only in their larger size (2.65 mm) and the instar duration lasts for an average period of 3-4 days. The size of third instar larva almost doubles in its length, reaches to 4. 5 to 5 mm and becomes greenish yellow in colour. They remain gregarious and have an average instar duration of 3-4 days. Fourth instar resembles the third instar, but they are less gregarious and the instar period lasts for an average duration of 4 days. Fifth instar, which lasts for 3-4 days attains an average length of 2 cm and it marks the beginning of the voracious feeding stage, feeding on the whole leaf lamina instead of scraping the leaf surface. Sixth instar which lasts for 5-6 days reaches to a length of 2.2 cm and a width of 1.8 mm and are voracious feeders. The last is seventh instar, which is full-grown and measures 3cm in length and 3mm in width and has prominent webbing activity and lives for an average of 5.5 days.

The last instar stops feeding and enters into a prepupal stage, which is bulky, nonfeeding and sluggish in nature with pale brown colour. The prepupa constructs a cocoon out of their webbings and prefers to pupate

within its own silken webbing. The pupa is brown and differs in its size between male and female individuals, female being bigger than male. Generally the pupal period extends for 10-14 days and then adults emerge out. Pupal life also differs between male (10-13 days) and female (12-14 days).

Adult moths are medium sized with a wing span of 24-30 mm and their wings are greyish with dark and white patches on the forewings, kept folded one over the other and cover the dorsal part of the body. The female and male moths differ from each other with respect to their body size as well as in the shape of the abdomen. Males are smaller in size than females and their abdomen tapers posteriorly and ends in a tuft of long scales. The females are larger than males and their body is stouter with bulky abdomen, the tip of which is not covered with long scales. In nature the adult moths feed on nectar of flowers and are having a very short life span of 5-6 days during that time they mate and oviposit. In the laboratory, 50 % honey: water soaked in cotton wool was used for feeding. Adult moths have pre- oviposition period of 2- 3 days and they mate more than once, where single male can fertilize 5-6 females successfully. Mated females continue to lay eggs for 3-4 days.

Nature of damage

O. exvinacea is a major pest of mango trees causing heavy damage to them. The larva feed voraciously on the leaves of mango trees which they web together. These webbed cluster of leaves dry up soon and later drop down giving a burnt look to the trees. Huge trees with thick canopy harbour numerous larvae in webbed clusters of leaves. The pest attack is not easily noticeable during early larval stages. As the larvae grow and start feeding voraciously, more and more leaves are found to be webbed together and the infestation becomes prominent due to drying up of the leaves that are initially attacked. The larval attack spread very fast and they get distributed to adjacent twigs. The pest incidence is not uniform throughout the year and the

significant attack occurs during the favourable six months (January –March and October – December) (Singh and Verma, 2013).

3.1.2. Experimental plants:

The present study was carried out with two locally available plants, *Hyptis suaveolens* and *Vitex negundo* (Plate III.2)

3.1.2.1. *Hyptis suaveolens* Poit.

(Order: Lamiales; Family: Lamiaceae)

(Hindi: Wilayati tulsi; Malayalam: Bilati tulasi; Sanskrit: Bhustrena)

The fresh leaves of *Hyptis suaveolens* were collected from the nearby areas of Malabar Christian College, Calicut. The leaf extract was used for the experimental study. Identification of the plant was done by the Department of Botany, University of Calicut.

Hyptis suaveolens is a strongly aromatic herb, found in West Africa especially Northern Nigeria, Philippines and Tropical America. It is also distributed in Deccan Peninsula, North East India, Andaman and Nicobar Islands and South India.

It is a tall, branched herb, usually woody at the base reaching to a height of 0.4-2 m or occasionally upto 3 m with hairy stems and leaves. The tetragonal hispid stems are square in cross section. The hairy leaves are ovate, oppositely arranged, cordate and denticulate with small blue flowers. It possess companulate fruiting calyx, ribbed with five aristate teeth.

Hyptis suaveolens possess numerous phytochemical constituents which include alkaloids, terpenes, phenols, tannins and steroids. Earlier researchers reported the presence of ethereal oil, suaveolic acid, suaveolol, traces of hydrocyanic acid, sterol, campesterol, fucosterol, sesquiterpene alcohols and

fatty acids, monoterpenes, diterpenes and triterpenoids in this plant. Several studies regarding the phytochemical aspects of the plant revealed that it yields 0.06 % of greenish yellow ethereal oil and its further analysis indicated the presence of 1-sabinene, d-limonene, azulenic sesquiterpene and unidentified sesquiterpene and sesquiterpene alcohols. The essential oil isolated from *H. suaveolens* contains various components such as 1, 8-cineole (44.4 %), β-caryophyllene, β-pinene and camphene, sabinene (41.0 %), terpinen-4-ol (12.31 %) etc. Hydrocyanic acid is present in the root, stem and leaves of *H. suaveolens*. Seeds showed the presence of fatty oil (Chandran *et al*., 2016).

Many works in the past suggest that the plant has insecticidal properties. The plant was found to be effective as feeding deterrent and also possess repellent activity against insect pests of stored grains and mosquitoes. Earlier it was reported that various solvent extracts of *H. suaveolens* leaves showed insecticidal activity, repellent activity, larvicidal activity, ovicidal activity and also inhibits adult emergence. In East and West Africa, plants of the *Hyptis* genus are commonly used against mosquitoes.

3.1.2.2. *Vitex negundo* Linn.

(Order: Lamiales; Family: Verbenaceae)

(Hindi: Nirgundi; Malayalam: Karunacci; Sanskrit: Nirgundi)

Vitex negundo was collected from the Malabar Christian College Campus and nearby places of Kozhikode district, Kerala during the period from October to January in order to prepare leaf extracts for the present studies. Identification of the plant was done from the herbarium of Department of Botany, Malabar Christian College, Calicut.

It is a deciduous shrub naturally distributed in many parts of the world. The plant occurs throughout India, Afghanistan, Africa, Madagascar, China,

Philippines, Sri Lanka and also in Bengal, Southern India and Burma. It is widely planted as a hedge plant along the roads or between the fields and now it is very common in barren areas in villages, moist localities and in the deciduous forests, but it is usually not browsed by cattle.

V. negundo is a large aromatic much-branched shrub or a small slender tree with a height of 2-5 m, having thin and grey bark and its branchlets are quadrangular in nature. Leaves are palmately compound, 3-5 foliate; leaflets lanceolate, entire or rarely crenate margins, terminal leaflets 5-10 cm x 1.6-3.2 cm, lateral leaflets smaller with a very short petiole. Flowers bluish purple, small forming compound, pyramidal panicles. Drupes globose, succulent, black when ripe, 5-6 mm in diameter, invested at the base with enlarged calyx.

Earlier studies have shown that the major chemicals extracted from leaves are various alkaloids and glycosides (Ahuja *et al.*, 2015). Phytochemical studies of *V. negundo* have revealed the presence of several types of compounds, such as volatile oils, lignins, flavonoids, terpenes (triterpenes, diterpenes, sesquiterpenes) and steroids . The antioxidant potency of *V. negundo* was found to be very high because of the presence of poly phenolic compounds which have high antioxidant potential. The various solvent extractions of the plant contains many polyphenolic compounds, terpenoids, glycosidic iridoids negundoside, agnuside and vitegnoside, alkaloids nishindine ($C_{15}H_{21}ON$) and hydrocotylene ($C_{22}H_{33}O_8N$), isoorientin, flavonoids, vitexilactone, casticin, chrysoplenol D glucononitol, p-hydroxybenzoic acid, an amorphous glucoside ($C_{20}H_{26}O_{11}$), tannic acid, auacubin, 5-hydroxyl-3,6,7,3',4'- pentamethoxyflavone ($C_{20}H_{20}O_8$), vitamin C and carotene (all in leaves), lignin (seeds) and volatile oils (flowering twigs) etc. The freshly collected leaves of the plant yield a pale greenish yellow oil (0.04-0.07 %), which consists 22.5 % of aldehydes and ketones; 15 % of

phenolic derivatives and 10 % of cineol (Vishwanathan and Basavaraju, 2010).

In old days, the dry *V. negundo* leaves are traditionally introduced to the stored woolen garments as the leaves repel wool-destroying insects or worms especially during rainy season, the leaves of this plant are burnt to keep away mosquitoes as the smoke repels mosquitoes and other insects. *V. negundo* leaf and branch extracts have the potential to act as repellent, deterrent and also as antifeedant against field insects. Various solvent extracts of *V. negundo* possesses insecticidal, ovicidal, larvicidal activity, growth inhibition and morphogenetic effects against various life stages of noxious lepidopteran pests.

3.1.3. Chemicals and Equipments

3.1.3.1. Chemicals

Laboratory chemicals (analytical grade) and equipments (guaranteed grade) were purchased from local suppliers.

1. 2, 4- dinitrophenyl hydrazine (NICE)
2. Acetone (MERCK)
3. Acrylamide (MERCK)
4. Amino acid standard kit
5. Ammonium persulphate (MERCK)
6. Aniline (MERCK)
7. Anisaldehyde sulphuric acid
8. Araldite resin

9. Benzene (MERCK)

10. Bisacrylamide (MERCK)

11. Bouin’s fixative

12. Bovine serum albumin

13. Bromophenol Blue (MERCK)

14. Cadmium acetate (MERCK)

15. Calcium chloride (MERCK)

16. Chloroform (MERCK)

17. Citric acid (MERCK)

18. Coomassie brilliant blue (MERCK)

19. Copper sulphate (MERCK)

20. Dabur Honey

21. Delafield’s Haematoxylin

22. Diethyl ether (MERCK)

23. Disodium hydrogen phosphate (NICE)

24. Disodium phenyl phosphate (MERCK)

25. dL- alanine (MERCK)

26. dL-aspartic acid (MERCK)

27. DPX (MERCK)

28. Dragendorf’s reagent

29. Eosin stain
30. Ethanol
31. Ethyl acetate (MERCK)
32. Ferric chloride (NICE)
33. Folin-Ciocalteau reagent (MERCK)
34. Formic acid (NICE)
35. Glacial acetic acid (MERCK)
36. Glutaraldehyde
37. Glycerol
38. Glycine (NICE)
39. Hydrochloric acid (MERCK)
40. Hydrogen peroxide (MERCK)
41. Methanol (NICE)
42. Methyl benzoate (MERCK)
43. Monosodium hydrogen phosphate (NICE)
44. n- Hexane (MERCK)
45. Ninhydrin (MERCK)
46. Osmium tetroxide
47. Phenol (MERCK)
48. Potassium chloride (MERCK)

49. Potassium dichromate (MERCK)
50. Propylene oxide
51. Sodium bicarbonate (NICE)
52. Sodium carbonate (NICE)
53. Sodium chloride (MERCK)
54. Sodium dodecyl sulfate (MERCK)
55. Sodium hydroxide (NICE)
56. Sodium potassium tartarate (NICE)
57. Sodium pyruvate (MERCK)
58. Sodium tungstate (NICE)
59. Sulphuric acid (MERCK)
60. TEMED (MERCK)
61. Toluene (MERCK)
62. Toluidine blue
63. Tris-Base (NICE)
64. Tris-HCl (MERCK)
65. Uranyl- lead acetate stain
66. wax
67. Xylene (MERCK)
68. α- ketoglutaric acid (MERCK)

69. β -mercapto ethanol (MERCK)

3.1.3.2. Equipments

1. CAMAG HPTLC system
2. Capillary tube
3. Centrifuge (ROTEK)
4. Culture bottles
5. Digital camera
6. Drier
7. Electrophoresis Unit (GENEI)
8. Eppendorf tubes
9. GC-MS- Agilent 7890A Series
10. Glass slides and cover glass (BLUE STAR)
11. Glass trough (RIVOTEK)
12. Graduated Pipettes (REVIERA)
13. Hot plate (ROTEK)
14. Incubator (ROTEK)
15. Micropipettes (REVIERA)
16. Olympus light microscope
17. Oven (ROTEK)
18. Petri dish (MERCK)

19. Reagent bottles (RIVOTEK)

20. Rearing cage

21. Rotary Microtome

22. Separating funnel (REVIERA)

23. Slide warming table (ROTEK)

24. Soxhlet apparatus (RIVIERA)

25. Test tubes (REVIERA)

26. Tissue Homogenizer

27. TLC apparatus

28. Transmission electron microscope

29. Visible Spectrophotometer (SYSTRONICS)

30. Vortex mixture (ROTEK)

31. Watch glass

32. Water bath (ROTEK)

33. Weighing balance (ROTEK)

3.2. METHODS

3.2.1. Collection, Rearing and Standardization of culture of *Orthaga.*

The larvae and pupae of *O. exvinacea* along with its webbings were collected from infested mango trees in the field by hand picking method and reared in wooden rearing cages with wire gauze net on sides under laboratory conditions. *O. exvinacea* culture was maintained in the laboratory under

optimum conditions of temperature (28± 2° C) and relative humidity (70-80 %) by feeding with mango leaves. Initial stages of the larvae were kept in containers of 1500 ml capacity (18×12 cm) and later they were transferred to plastic troughs (40×13 cm) covered with muslin cloths. After pupation the pupa were collected and kept in rearing cages in a plastic tray. When adult male and female moths were emerged they were collected and kept in plastic containers in the ratio of 1: 1 and were fed with 50 % honey. Within 2-4 days creamy white eggs were laid on the sides as well as on the covering clothes of the containers. After 2 days the colour of the eggs changed from brown to black. Egg hatches to young larvae within 4 days. Tender mango leaves were provided for feeding the first instar larvae.

3.2.2. Preliminary phytochemical studies

Phytochemical studies involve solvent selection for phytochemical extraction based on two aspects, the solvent with maximum yield of extract and the most bioactive extract against *O. exvinacea* larva. To confirm the maximum yield of extract with a particular solvent, TLC studies were also performed.

3.2.2.1. Solvent selection- Yield of extract

Prior to massive soxhlet extraction of the plant materials with a suitable solvent, five solvents (Chloroform, ethyl acetate, methanol, ethanol and water) with differing polarity were taken into consideration for extraction using soxhlet apparatus in order to confirm a single solvent for complete extraction process based on the total yield of extract obtained in each solvent. The solvent extract with maximum yield was considered as the best solvent for extraction.

Total yield of extract was calculated using the following formula.

$$\text{Total extract yield, Y (\%)} = \frac{\text{Total mass of extract}}{\text{Total mass of sample}} \times 100$$

3.2.2.2. TLC

Thin layer chromatographic analysis of chloroform, ethyl acetate, methanol, ethanol and water extracts of both *H. suaveolens* and *V. negundo* were carried out to confirm the maximum yield of extract with these solvents.

Aluminium backed pre-coated Merck silica gel plates 60 F_{254} plate (10×10 cm) of uniform thickness of 0.2 mm were used for TLC studies. Applied 10 µl of test solutions on the TLC plate at the bottom region (1 cm above) in the form of bands using a microsyringe (100 µl). The solvent system used for separation of components in different solvent extracts was toluene: ethyl acetate in 8:2 ratio. Developed the plate in the solvent system in a twin trough chamber to a distance of 9 cm.

The visualization of the separated components was made possible with observation under UV light (254 nm and 366 nm). The chromatogram was derivatized with anisaldehyde sulphuric acid reagent (ANS) and dried at an oven temperature of 105˚C and was observed under 550 nm visible light. The number of compounds separated in each solvent extract was noticed and the R_f values were calculated using the following formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{distance travelled by solvent front of TLC}}$$

3.2.2.3. Screening for active solvent extract.

The different solvent extracts prepared were used for screening studies to find out the most active solvent extract against *O. exvinacea* larva and thereby fixing the most suitable solvent for further massive extraction process. In the screening studies, topical application method was used for application of 75 µl of each solvent extract on the larva. In this method,

specific small volumes of the test solutions were applied topically on individual insects dorsally and ventrally using a micropipette. After application of extract, the insects were supplied with fresh mango leaves for feeding. For both botanical treatments, the experiment with each solvent extract was repeated thrice with each set of treatment consisting of 8 larvae. The observations on mortality in each set were noticed after 6 h, 12 h and 24 h of exposure. The treatment with maximum mortality within 24 h of exposure was considered as the most active solvent extract against this organism. The solvent for taking this extract was fixed as the most suitable solvent for further extraction process. Both negative and positive control sets were maintained with each experiment set.

3.2.3. Phytochemical extraction, fractionation and toxicity evaluation

3.2.3.1. Preparation of leaf extracts and their fractions

The shade dried leaves of both plants were used for taking extractions. Methanolic extract of both plant leaves were prepared by soxhlet extraction method. 660 mg of both plant leaf powders was used for extraction separately. Using soxhlet apparatus 50 g of each leaf powder was extracted with 500 ml of 100 % methanol. After complete extraction, the extract was evaporated to 250 ml in an oven at a temperature of 45°C and was subjected for fractionation with different solvents using separating funnel. The solvents used were n- hexane, chloroform, ethyl acetate and 80% methanol. Different solvent fractions were collected in different containers and their volume ranged from 400- 500 ml. Each fraction was then concentrated to 25 ml.

3.2.3. 2. Toxicity evaluation

In order to find out the most toxic fraction of both botanicals toxicity evaluation was performed using different solvent fractions of methanolic extracts. For this, different solvent fractions were topically applied on the

larva (75 µl) and observations on mortality were noticed after 6 h, 12 h, and 24 h of treatment with each of the fractions. Along with each treatment set, a set of negative control larvae without any treatment and positive control larvae with solvent alone treatment were also maintained. Three sets of replicates were performed for each set of experiment, which consists of 8 larvae/set. The percentage of mortality was calculated using the following formula:

$$\text{Percentage of mortality} = \frac{\text{No. of dead insects}}{\text{Total no. of treated insects}} \times 100$$

3.2.3.3. Determination of LD_{50}

After the preliminary screening test with different fractions, the most toxic fraction of each botanical was selected for topical application on *O. exvinacea* larva. For this, the solvent present in the most toxic fraction of both botanicals was completely evaporated off and the final dry powder was dissolved in the least toxic solvent towards *O. exvinacea* larva so as to make 10 % stock solution for topical application. From this stock solution, 75 µl of different concentrations of the selected toxic fraction (20 µg, 25 µg, 30 µg, 35 µg and 40 µg of *V. negundo*; 60 µg, 65 µg, 70 µg, 75 µg and 80 µg of *H. suaveolens*) of leaf extracts mixed with 10 µl of 1 % soap solution as wetting agent were topically applied on experimental sets of sixth instar larvae using microsyringe. Five sets of experiments, each set consisting of 10 larvae were conducted along with negative control set (10 larvae each) with out any treatment and positive control set (10 larvae each), treated with 75 µl methanol mixed with 10 µl soap solution. Experimental and control sets of larvae were fed with fresh mango leaves. Mortality counts were made for every 12 h duration (viz., 12 h, 24 h, 36 h, 48 h and 60 h). The larvae that did not move while probing with a needle were considered dead. Percentage of mortality was calculated with the formula:

$$\text{Percentage of mortality} = \frac{\text{No. of dead insects}}{\text{Total no. of treated insects}} \times 100$$

The data on contact toxicity was analysed and the lethal dosage (LD_{50}) needed to produce 50 % mortality in the experimental sets of both *H. suaveolens* and *V. negundo* were determined by Probit Analysis method (Finney, 1971) and the significant differences between different groups were tested with Duncan's multiple range test (DMRT) (Duncan, 1955).

3.2.4. Phytochemical identification and classification of compounds

3.2.4.1. HPTLC analysis

To identify and characterize the nature of compounds present in the active fractions employed in toxicity studies, they were subjected for HPTLC analysis. Toxic fractions diluted in respective solvents were used as samples. Aluminium backed pre-coated Merck silica gel plate 60 F_{254} plate (10×10 cm) was used as the stationary phase and the solvent system used was Toluene: Ethyl acetate: Formic acid (7: 3: 0.3). Samples were applied on the plate using Camag automatic TLC sampler 4 attached to Camag HPTLC system. Applied 10 μl of test solutions on a precoated silica gel 60 F_{254} TLC plate (E. Merck) of uniform thickness of 0.2 mm in the form of bands with width 8 mm using Hamilton syringe (100 μl). Developed the plate in the solvent system in a twin trough chamber to a distance of 9 cm.

Visualization

Observed the plate under UV light at 254 nm, 366 nm and after derivatization at 550 nm (visible light).

- For Terpenoids: Derivatization of the chromatogram was done using Anisaldehyde sulphuric acid (ANS) as visualizing agent. The chromatogram was gently dipped in ANS solution and was kept in a

hot air oven (5 minutes) for complete evaporation of the solvent until the bands attained maximum colour intensity.

- For Phenolics: Chromatogram was derivatized with 10 % alcoholic ferric chloride solution as visualizing agent.
- For Alkaloids: Derivatization of the chromatogram was performed by spraying Dragendorff's reagent for visualization of bands.

Densitometric scanning of the chromatographic plates was done using Camag TLC scanner at 550 nm after derivatization.

3.2.4.2. GC-MS analysis

GC-MS analysis of the most toxic fraction of both plant extracts were carried out using an Agilent 7890A series GC system with 5975C- Mass Selective Detector (MSD) under the following conditions: helium as carrier gas, injection temperature: 250°C, ion-source-heating: 230°C, column used for analysis was 30 m × 0.25 mm bonded non-polar FSOT- RSL- 200 fused silica with a film thickness of 0.25 µm, samples were kept at 40°C for 5 min and heated at a rate of 5°C/ min to 280°C and the flow rate was 1.0 ml/min. Compounds were identified from GC-MS spectral data and confirmed this identification based on National Institute of Standards Technology (NIST) Library (Stein, 1990).

3.2.5. Preparation of leaf extracts for histological and biochemical studies

The collected plant leaves were washed thoroughly in tap water and shade dried at room temperature (28 ± 2°C) for about one month and powdered in a grinder. The leaf powders were then sealed in separate plastic covers and stored at about 4°C. Using soxhlet apparatus 50 g of leaf powder was extracted with 500 ml of methanol solvent. After complete extraction, the extract was evaporated in an oven at a temperature of 45°C. The weight of the

dried extract was taken and it was dissolved in appropriate volume of methanol to prepare 10 % stock solution and it was stored in air-tight glass containers in a refrigerator. From this stock, required concentrations (1 %, 2 %, 3 %, 4 % and 5 %) of the extracts were prepared by diluting with methanol.

3.2.5.1 Mode of application - Food treatment method

For the experimental study mango leaves treated with different concentrations of the extracts were provided to the sixth instar larvae for 48 h. For this the leaves were smeared with fixed volume (1.0 ml) of each concentration of extract and kept open at room temperature until the methanol had completely evaporated off. Control larvae were provided with mango leaves treated with same volume of methanol alone.

3.2.6. Histomorphological studies of midgut tissue

Prestarved experimental sixth instar larvae were fed with mango leaves treated with different concentrations of leaf extracts (1 %, 2 %, 3 %, 4 % and 5 %). Likewise, prestarved control larvae were fed with mango leaves, treated with methanol alone. After 48 h of feeding, the larvae were dissected out in insect ringer solution and the midgut tissue, cleared from adhering trachea was fixed in the alcoholic Bouin's fluid for 4 h. The tissue was then washed in 70 % alcohol and processed for making wax blocks. Serial sections of 5 micron thickness were cut using a microtome and slides were prepared. The sections were stained by routine histological staining techniques with Delafield's haematoxylin and eosin stains. After mounting in DPX, it was observed under the Olympus CX21i microscope. The effects caused by botanical pesticides were noted by changes in the normal histological structure and the images were photographed. Morphometric studies were carried out with these microphotographs by measuring the height and width of

columnar epithelial cells and their nuclei along with measurements of goblet cell and its nucleus using Olympus magnus pro software.

3.2.7. Ultrastructural studies of midgut tissue

Early stage sixth instar larva was used for collecting the midgut tissue for transmission electron microscopy. For ultrastructural studies, the most effective concentration (5%) of the leaf extract observed in the light microscopic studies was selected. The prestarved experimental larvae were fed with mango leaves smeared with 5 % concentration of botanicals. The prestarved control larvae were fed with methanol treated leaves. After feeding for 48 h, both sets of larvae were dissected out to collect midgut tissue and it was fixed using 3% glutaraldehyde in 0.1M phosphate buffer for 48 h. These fixed tissues were washed with phosphate buffer, incubated for 90 min with 1 % osmium tetroxide, after that dehydrated with different grades of alcohol (70 %, 80 %, 90 %, 95 % and 100 %) and two changes of propylene oxide, then the tissues were infiltrated over night in 1: 1 ratio of propylene oxide-resin mixture and embedded in araldite molds and kept at 60° for 48 h. The resin embedded samples were sectioned using an ultramicrotome (Leica EM U C6). Semithin sections of 5-10 nm thickness were cut, stained with 1% Toluidine blue and observed under Olympus CX21i microscope and then ultrathin sections were cut and stained with uranyl- lead acetate stain and observed under Transmission Electron Microscope (Hitachi H 500) and the images were taken.

3.2.8. BIOCHEMICAL ANALYSIS

3.2.8.1. Quantitative estimation of protein in different tissues

a. Midgut tissue

Sixth instar larvae were selected for collecting midgut tissue for quantitative analysis of total protein. The experimental larvae were fed with

mango leaves smeared with different concentrations (1 %, 2 %, 3 %, 4 % and 5 %) of the leaf extracts. The control larvae were fed with methanol smeared mango leaves. Before feeding, the larvae were prestarved for 3-4 h. Five sets of samples were taken for each concentration and each sample contain midgut tissues from 5 larvae. After feeding for 48 h, the experimental sets as well as the control set larvae were dissected out separately to collect midgut tissue samples. Water adhered to the tissue was blotted with filter paper and weighed. Then it was homogenized with 1 ml of ringer solution. From the sample, protein was precipitated with 80 % ethanol, centrifuged and the residue was dissolved in 1 ml of 0.1N sodium hydroxide by boiling. The protein in the sample was estimated by Lowry's method (1951) using visible spectrophotometer.

b. Fat body

For total protein estimation of fat body tissue the experimental sets comprising larvae fed with mango leaves treated with different concentrations of botanical extracts and a control set of larvae fed with methanol treated leaves were taken. Five sets of samples were taken for each concentration and each sample contained fat body tissues from 5 larvae. After 48 h of feeding the larvae were dissected out and fat body tissue was collected, cleared from trachea and water adhered was removed using filter paper. Each sample set was weighed and homogenized. Protein was precipitated from homogenized samples with 80 % ethanol, centrifuged and the residue was dissolved in 1 ml of 0.1N sodium hydroxide by boiling. The protein in the sample was estimated by Lowry's method (1951) using visible spectrophotometer.

Procedure

In the case of midgut tissue and fat body, 1 ml of protein sample was mixed with 5 ml of alkaline copper reagent and was allowed for incubation at

room temperature for 10-15 minutes. Then added 0.5 ml of diluted folin-ciocaltaeu reagent (1:1) and kept for another 30 min of incubation at room temperature itself. The blue colour developed was read in a visible spectrophotometer at 650 nm against a reagent blank. A set of BSA standards were also carried out in a similar manner.

c. Haemolymph

Experimental sets and control set of sixth instar larvae were prepared as in midgut and fat body estimation. 0.2 ml of haemolymph samples were collected by amputing thoracic legs using capillary tube into pre-chilled eppendorf tubes (kept in ice cubes) containing 0.5ml of 10 % sodium tungstate solution to prevent melanization. Five sets of samples were collected for each concentration and the total protein in the haemolymph samples were estimated using spectrophotometer by Lowry's method (1951).

Procedure

To the collected haemolymph, 0.5 ml of 0.66 N suphuric acid was added and centrifuged for 5 minutes. The residue was dissolved in 1 ml of 0.1N sodium hydroxide. This protein sample was used for estimating the total protein content as described in section 3.2.8.1.b.

The percentage of reduction in protein content in midgut tissue, fat body and haemolymph were calculated using the formula:

$$\text{Percentage reduction in protein content} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

3.2.8.2. Electrophoretic protein profiling of different tissues of larvae.

The electrophoretic protein profiling of different tissues- midgut tissue, fat body and haemolymph were performed by one-dimensional Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS - PAGE)

according to Laemmli (1970). Experimental and control set of larvae were prepared as in the case of protein estimation (section 3.2.8 a,b,c). Each set of experimental and control consisted of 10 larvae. After feeding for 48 h, haemolymph samples were collected from the test larvae in pre- chilled phosphate buffer containing tubes and were stored in frozen temperature until use. The larvae of each experimental set were dissected out in insect ringer separately, the midgut tissue and fat body were collected and weight of each sample was taken. 100 mg of tissue was homogenized in 1 ml of phosphate buffer (pH 7), centrifuged and supernatant was taken for analysis. For haemolymph sample, 100 µl was taken and 1 ml of phosphate buffer was added, centrifuged and supernatant was taken for analysis. The protein samples were mixed with equal volumes of sample buffer. Samples were heated in micro-centrifuge tubes for 3 min at 95°C in a water bath. The denatured sample can be stored at - 20°C for further use. SDS- PAGE analysis for diverse protein samples was carried out using 5 % of stacking gel and 12 % of resolving gel. The resolved proteins were visualized by Coomassie brilliant blue (R-250) staining as per standardized protocol for Laemmli's method. Substantial changes in the SDS-PAGE protein profile was observed in control and treated ones and was photographed. Molecular weight determination of protein was carried out and documented using gel documentation system.

3.2.8.3. Quantitative estimation of free amino acid concentration in different tissues

a. Midgut and fat body tissue

The supernatant collected after precipitating the protein from the homogenized midgut tissue and fat body during total protein estimation as described in section 3.2.8.a, b. was taken for the estimation of total free amino acids by Lee and Takahashi method (1966).

b. Haemolymph

Treatment of the experimental and control larvae was similar to the method described in 3.2.8. a. Haemolymph samples were collected in pre-chilled tubes and were kept in ice cubes to prevent melanization. Protein in the sample was precipitated using 80% ethanol, centrifuged and the supernatant was collected and total free amino acid concentration in the sample was detected using the method of Lee and Takahashi (1966).

Procedure

The supernatant left after protein precipitation and centrifugation was used for estimating the free amino acid concentration. To 0.5 ml of supernatant 0.5 ml of ninhydrin cadmium acetate reagent was added. To this 5 ml of distilled water was also added. Boiled the mixture in a water bath for 20 min. Then cooled and allowed to stand for 15 min and the colour developed was measured at 540 nm in a spectrophotometer. A set of glycine standards were also carried out in a similar manner.

The percentage of reduction in free amino acid content in midgut tissue, fat body and haemolymph were calculated using the formula:

$$\text{Percentage reduction of free amino acid content} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

3.2.8.4. Quantitative analysis of enzyme activity in different tissues.

Tissue preparation

The activity of enzymes- catalase (CAT), aspartate amino transaminase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP) and acid phosphatase (ACP) in midgut tissue, fat body and haemolymph were estimated for different time periods of 24 h, 48 h and 72 h. For this, the sixth instar larvae were fed with mango leaves, smeared with highest concentration,

5 % of botanicals. After feeding for 24 h, 48 h and 72 h, from each set of the larvae midgut tissue, fat body and haemolymph were collected as mentioned in section 3.2.8. a, b and c. A set of control larvae was maintained with each set of experimental larvae.

3.2.8.4.1. Catalase

The collected tissues were homogenized with 0.1M phosphate buffer solution, centrifuged and supernatant was used for catalase enzyme analysis by the method of Sinha (1972). The reaction mixture when heated in the presence of H_2O_2 results in the conversion of dichromate in the dichromate acetic acid reagent to perchromic acid and then to chromic acetate. The chromic acetate formed was measured at 620nm.

Reagents

1. Phosphate buffer - 0.01M, pH 7.0
2. H_2O_2 - 0.2 M
3. Potassium dichromate - 5%
4. Dichromate-acetic acid reagent - 1:3 ratio of potassium dichromate and glacial acetic acid was mixed. 1 ml of it was again diluted with 4 ml acetic acid
5. Standard H_2O_2 - 0.1 ml of 0.2 M H_2O_2 diluted with 100 ml of distilled water.

Procedure

0.1ml of enzyme sample was added to 0.9 ml of phosphate buffer and then 0.4 ml of H_2O_2 was added to this mixture. It was kept aside for 60 sec. To this 2 ml of dichromate acetic acid reagent was added and the tubes were kept in boiling water bath for 10 min. A set of H_2O_2 standards were also

carried out with blank containing reagent alone and the colour developed was read at 620 nm.

Catalase enzyme activities were expressed as IU/mg in the case of mid gut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.8.4.2. Aspartate aminotransferase (AST) and Alanine amino tranferase (ALT)

The different tissue samples collected were homogenized with phosphate buffer. Activities of AST and ALT were assayed by the method of Reitman and Frankel (1957). AST catalyses the transfer of an amino group from dL-aspartate (dL-ASP) to α-ketoglutarate (α-KG) to yield oxaloacetate and L-glutamate. Similarly, ALT catalyses the transfer of an amino group from dL-alanine (dL-Ala) to α-ketoglutarate to yield pyruvate and L-glutamate. The liberated oxaloacetate and pyruvate reacts with 2,4-dinitrophenyl hydrazine to form 2,4-dinitrophenyl hydrazone, which was read at 540 nm.

Reagents

1. Phosphate buffer – 0.1M, pH 7.5
2. Substrate- pH - 7.5

a) Aspartate transaminase: Dissolve 0.30 g of dL-aspartic acid and 50mg of α-keto glutaric acid in 100 ml of phosphate buffer.

b) Alanine transminase: To 20 ml of phosphate buffer 5.0 g of dL-alanine and 20 mg of α-ketoglutaric acid were added and dissolved in 100 ml of phosphate buffer.

3. DNPH: 200 mg of 2, 4-dinitrophenylhydrazine (DNPH) dissolved in 100 ml of hot 1N hydrochloric acid.

4. Aniline-citrate reagent: 50 g of citric acid in 50 ml of distilled water and to this added an equal volume of redistilled aniline.

5. Sodium hydroxide – 0.4 N

6. Standard pyruvate solution: 12.5 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.

Procedure

1 ml each of AST and ALT substrates were subjected for incubation for few minutes at 37°C. Then enzyme sample of 0.2 ml was added and again kept for incubation of 1 h for aspartate transaminase and 30 min. for alanine transaminase. In the control, sample was added only after incubation. After incubation added aniline-citrate reagent (2 drops) to both test and control experiments. The reaction was stopped by adding DNPH (2,4-dinitrophenylhydrazine) solution (1 ml) and the tubes were kept under room temperature for another 20 min. Then, 0.4 N sodium hydroxide (1 ml) was added to each tube. A set of pyruvate standards were also run in the same way and the colour developed was read at 540 nm.

ALT and AST enzyme activities were expressed in IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.8.4.3. Alkaline phosphatase (ALP)

The collected samples of different tissues were homogenized with bicarbonate buffer and the activity of ALP was analysed using the method of King and Armstrong (1934) in which disodium phenyl phosphate was used as the substrate. Disodium phenyl phosphate is hydrolysed by alkaline phosphatase liberating phenol, which reacts under alkaline condition with Folin-phenol reagent to form blue colour, which was estimated colorimetrically at 680 nm.

Reagents

1. Bicarbonate buffer–0.1M, (pH 10)
2. Substrate – 0.01M disodium phenyl phosphate salt solution
3. Folin-phenol reagent
4. Sodium carbonate – 10 %
5. Standard phenol solution – 5 μg/ml

Procedure

An incubation mixture was prepared with bicarbonate buffer (150 μmoles) and substrate-disodium phosphate (10 μmoles) in distilled water (2.9 ml) and was pre-incubated for 10 min at 37°C. Then enzyme sample (0.2 ml) was added to this and subjected for incubation at 37°C for 15 min. The reaction was stopped by the addition of 1.0 ml of Folin-phenol reagent. The whole suspension was centrifuged and the supernatant was taken. To this 10% sodium carbonate (2.0 ml) was added and the solution was incubated at 37°C for 10 minutes. A set of phenol standard solutions were also treated with Folin-phenol reagent and sodium carbonate. The blue colour developed was read at 680 nm.

ALP enzyme activities were expressed as IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.8.4.4. Acid phosphatase (ACP)

Tissue samples were prepared by homogenizing in citrate buffer and were used for Acid phosphatase estimation by Kind and King method (1954) in which 4-aminophenazone was used to determine the phenol liberated instead of the Folin-Ciocalteu reagent.

Reagents

1. Disodium phenyl phosphate – 218 mg/100 ml

2. Citric acid-sodium citrate buffer- pH- 4.9

3. Buffered substrate

4. Sodium hydroxide - 500 mmol/ l

5. Sodium bicarbonate – 500 mmol/ l

6. 4-Aminophenazone – 6 g/ l

7. Pottassium ferricyanide – 24 g/ l

8. Stock phenol standard – 1 g crystalline phenol/ l

9. Working standard -10 μ gram phenol/ ml

Procedure

A mixture of 1 ml of citric acid-sodium citrate buffer and 1 ml of disodium phenyl phosphate solution was used as buffered substrate and was incubated for few minutes in a water bath at 37° C. For experiment samples added 0.1 ml of enzyme sample and allowed the tubes for incubation for an hour. After incubation completed, 0.8 ml of sodium hydroxide and 1.2 ml sodium bicarbonate were added to both experiment and control samples and then added 0.1 ml enzyme sample to the control. To both of these, 1 ml of 4 – amino phenazone and 1 ml of potassium ferricyanide were added, after thorough mixing, colour developed was read at 520 nm against a reagent blank. A set of phenol standards were also carried out in a similar manner.

ACP activities were expressed as IU/mg in the case of mid gut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.9. Statistical analysis

In the toxicity evaluation studies, mean percentage of mortality was calculated. Toxic effects of leaf extracts were calculated by Probit Analysis (Finney, 1971) using the software SPSS. Significant differences between treatments were calculated by Duncan's multiple range test (Duncan, 1955). Morphometric studies were statistically analysed with ANOVA. Results of quantitative estimation of protein, amino acids and enzyme activity were subjected to statistical analysis using ANOVA and post hoc test (Scheffé test) (Scheffé, 1959) with SPSS 16 package. Results with $P<0.05$ were considered to be statistically significant.

CHAPTER IV

PHYTOCHEMICAL EXTRACTION, SEPARATION AND TOXICITY EVALUATION OF *H. SUAVEOLENS* AND *V. NEGUNDO*

4.1. Introduction

Phytochemistry is a swiftly expanding area of research with new approaches or techniques for the analysis of plant organic compounds (Harborne, 1989). In order to discover new bioactive compounds, plant extracts are simultaneously evaluated by chemical screening (Wink, 1999). Modern methods of extractions are based on chemical polarity and solubility. Thus, organic extraction always followed with a further analysis, required for purification and separation of compounds within the fractions by suitable methods. The extraction of an active biochemical compound from plants depends upon the polarity of the solvents used, i.e., polar solvents will extract polar molecules and non-polar solvents extract non-polar molecules (Ghosh *et al.* 2012). This can be achieved by using many solvent systems ranging from the most non polar (hexane/ petroleum ether, polarity index of 0.1) to that of most polar (water with polarity index of 10.2). Non polar solvents extract essential oils while polar solvents extract high molecular weight compounds such as proteins, glycans, etc. Whereas moderately polar (polarity index of 4.1) solvents such as chloroform and acetone mainly extracts steroids, alkaloids, etc. Furthermore, Ghosh *et al.* (2012) noticed that a range of maximum polarity to minimum polarity solvents were found to be used in many studies and at the same time moderately polar solvents also showed good results as reported by a few bioassays. All these suggestions are pointing the fact that the potency of extracted plant compounds depends on the type of

solvent used for extraction, indicating the difference in the chemo-profile of the plant species.

Plant products play a major role in evolving as an ecologically sound and environmentally acceptable insect pest management system. Natural products from plants are obtained either as pure compounds or as standardized extracts, for which, the source material like dried, powdered plant will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent like methanol or chloroform may be used as the initial extractant.

Biological screening of plant extracts is carried out to evaluate their potential for various activities. This requires development of simple, rapid and inexpensive biological assays. Further the potent plant extracts can be fractionated for isolation and identification of biologically active constituents. Some of the commonly used biological assays include antimicrobial assays, toxicity assays, insecticidal assays, antioxidant assays and antidiabetic assays. The insecticidal activity of plant extracts can be determined by direct contact application of the test agent on the test organism and calculating the number of survivals and percentage mortality.

Plants produce a very wide array of chemicals, some of which are known as secondary metabolites because they do not contribute to plant biochemistry or physiology. Many of these are released as volatile compounds in response to damage caused by insects and some of these volatiles directly kill or retard insects by surface contact and others indirectly harm insects by attracting natural enemies of the insects. Still other plant chemicals are ingested with plant material, such as leaves or roots. To gain more insight into the toxicity aspect of *H. suaveolens* and *V. negundo* on *O. exvinacea larva*, topical application method was selected.

Generally in toxicity studies, botanical crude extracts were found to be the most effective toxicity bioassay because of the synergistic activity of various chemical components present in the crude extract. However, for a proper understanding of the nature and class of components contained in the botanical extracts suitable separating methods were employed for fractionation of the methanolic extracts of both botanicals.

The present investigation was designed to find out the most suitable solvent for extraction and its thin layer chromatography, fractionation of the extracts, screening of the fractions to find out most toxic fraction and the determination of 50 % mortality (LD_{50}) of the effective fractions.

4.2. Materials and methods

The methods used for the phytochemical studies of *V. negundo* and *H. suaveolens* were described in sections 3.2.2.1., 3.2.2.2. and 3.2.2.3. In addition, botanical fractionation, evaluation of the toxicity of these fractions against *O. exvinacea* and determination of LD_{50} of the most toxic fractions of both botanicals were carried out by the methods given in sections 3.2.3.1., 3.2.3.2. and 3.2.3.3.

4.3. Results

4.3.1. Solvent selection

The most suitable solvent for performing soxhlet extraction was confirmed based on two aspects- the solvent with maximum yield of extract and also the most active solvent extract in causing mortality of *O. exvinacea* larva.

4.3.1.1. Yield of extracts from different solvents

The total yield extracted for both botanicals using the organic solvents along with water are listed in Table IV. 1.

Table IV. 1. Effect of solvents on yield of extraction of both *H. suaveolens* and *V. negundo*.

Solvent extracts	*H. suaveolens*		*V. negundo*	
	Yield of extract (g)	Percentage yield (%)	Yield of extract (g)	Percentage yield (%)
Chloroform	0.151	1.51	0.539	5.39
Ethyl acetate	0.600	6	0.199	1.99
Methanol	0.845	8.45	0.565	5.65
Ethanol	0.804	8.04	0.425	4.25
Water	0.187	1.87	0.536	5.36

The results pointed out that high extract yield for *H. suaveolens* (8.45 %) was obtained from extraction with methanol followed by ethanol (8.04 %) and ethyl acetate (6 %). Low extract yield was observed when the extraction was done using chloroform (1.51 %). For *V. negundo* also, the high extract yield (5.65 %) was obtained with methanol as the extracting solvent which was followed with chloroform (5.39 %) and water extracts (5.36 %). In this case the minimum extract yield was obtained with ethyl acetate (1.99 %). Among solvents selected for extraction, both plants exhibited high solubility with methanol and thus more yield of extract. Hence methanol was found to be the suitable solvent for taking soxhlet extraction of both plant materials.

4.3.1.2. TLC analysis of the different solvent extracts of *H. suaveolens* and *V. negundo*

Thin layer chromatographic analysis of chloroform, ethyl acetate, methanol, ethanol and water extracts of both *H. suaveolens* and *V. negundo*

were carried out to confirm the maximum yield of extract with these solvents and the corresponding TLC profile images are given in plate IV. 1 and IV. 2.

The movement of the different compounds (spots appeared on the TLC plate) present in the plant extracts were expressed by its retention factor (R_f) and those values were calculated for different compounds and are given in (Table IV. 2 and IV. 3).

Table IV. 2. R_f values calculated from TLC profile for the leaf extracts of *H. suaveolens* using the solvent system (toluene: ethyl acetate: formic acid) after derivatization (550 nm).

Chloroform		Ethyl acetate		Methanol		Ethanol		Water	
No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values
9	0.03 0.06 0.31 0.36 0.51 0.60 0.68 0.76 0.89	8	0.06 0.22 0.28 0.38 0.51 0.61 0.68 0.79	15	0.06 0.12 0.23 0.29 0.40 0.48 0.52 0.54 0.60 0.70 0.75 0.79 0.85 0.89 0.94	12	0.04 0.06 0.11 0.22 0.29 0.39 0.52 0.60 0.70 0.78 0.85 0.94	2	0.01 0.02

The chromatogram revealed varying number of bands for the solvent extracts. In the case of *H. suaveolens*; the chloroform extract showed 9 bands under visible light (550nm) corresponding to the presence of various compounds present in the leaf extract. Similarly, 8 bands for ethyl acetate extract, 15 bands for methanolic extract, 12 bands for ethanolic extract and 2

bands for aqueous extract were seen under visible light- 550 nm (Table IV. 2). Maximum separation of compounds was with methanolic extract followed by ethanol, chloroform and ethyl acetate extracts.

Table IV. 3. R_f values calculated from TLC profile for the leaf extracts of *V. negundo* using the solvent system (toluene: ethyl acetate: formic acid) after derivatization (550 nm).

Chloroform		Ethyl acetate		Methanol		Ethanol		Water	
No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values	No. of spots	R_f values
11	0.13	6	0.01	13	0.01	5	0.01	9	0.01
	0.31		0.39		0.15		0.47		0.04
	0.35		0.46		0.22		0.55		0.15
	0.43		0.54		0.31		0.72		0.26
	0.51		0.58		0.42		0.79		0.31
	0.61		0.73		0.50				0.34
	0.63				0.58				0.42
	0.65				0.63				0.58
	0.77				0.72				0.68
	0.85				0.78				
	0.92				0.83				
					0.89				
					0.93				

The chromatogram for *V. negundo* exhibited varying number of bands for the solvent extracts. In this case, the chloroform extract possessed 11 bands under visible light indicating the presence of various compounds present in the leaf extract. Likewise, 6 bands for ethyl acetate extract, 13

bands for methanolic extract, 5 bands for ethanolic extract and 9 bands for aqueous extract were observed under visible light- 550 nm (Table IV.3). Maximum number of components were obtained with methanolic extract followed by chloroform and water extracts.

4.3.1.3. Testing the toxicity of different solvent extracts

In order to find out the most suitable solvent for soxhlet extraction, the different solvent extracts of both *H. suaveolens* and *V. negundo* were tested on *O. exvinacea* larva for their efficacy in causing mortality which was determined for 6 h, 12 h and 24 h of exposure. For each time duration the mean percentage mortality was calculated for both treatments and control sets of larvae (both negative and positive) and are expressed in table IV. 4.

Table IV. 4. Showing the mortality of *O. exvinacea* larva exposed to different solvent extracts

Solvent Extracts		**Mean percentage mortality (%) during different periods**		
		6 h	**12 h**	**24 h**
Chloroform	*H. suaveolens*	70.8	91.7	100
	V. negundo	75	95.83	100
	Positive Control	16.7	20.8	37.5
	Negative control	-	-	-
Ethyl acetate	*H. suaveolens*	33.3	41.7	54.17
	V. negundo	50	58.3	75
	Positive Control	12.5	16.7	25
	Negative control	-	-	-
Methanol	*H. suaveolens*	66.7	75	83.3
	V. negundo	70.8	79.2	87.5
	Positive Control	-	-	-
	Negative control	-	-	-
Ethanol	*H. suaveolens*	29.2	37.5	58.3
	V. negundo	41.7	54.2	66.7
	Positive Control	-	8.33	12.5
	Negative control	-	-	-
Water	*H. suaveolens*	12.5	25	33.3
	V. negundo	16.7	29.2	41.7
	Positive Control	-	-	-
	Negative control	-	-	-

Values are mean percentage mortalities of three replicates.

Table IV. 4. provides the mean percentage mortality of *O. exvinacea* larva exposed to different solvent extracts of both *H. suaveolens* and *V. negundo* for different time periods by topical application method. The percentage of mortality observed for *H. suaveolens* solvent extracts (Chloroform, Ethyl acetate, methanol, ethanol and water) after 24 h of exposure are 100 %, 54.17 %, 83.3 %, 58.3 % and 33.3 % respectively whereas that of *V. negundo* showed 100 %, 75 %, 87.5 %, 66.7 % and 41.7 % of mortality respectively. For both botanicals, maximum toxicity was caused by chloroform extract which was followed by methanolic extract. For the positive control, some mortality percentage was noticed for chloroform (37.5 %), ethyl acetate (25 %) and ethanol (12.5 %) treatments except methanol and water treatments. Hence, in the solvent extract treatments, chloroform, ethyl acetate and ethanol might be contributing towards the toxicity along with the active ingredients of the botanicals.

Whereas for the positive control with methanol and water alone treatment there was no mortality during the entire exposure time, indicating that these solvents were least toxic to the organism. Moreover methanolic extract was found to be causing more toxicity to the larva than chloroform, ethyl acetate, ethanol and water extracts, so methanol can be used as an effective solvent for extraction of both botanicals. On contrary to the positive control there was no mortality noticed in the negative control larval sets.

4.3.2. Toxicity evaluation of fractions of methanolic extract

A preliminary screening study was conducted with different solvent fractions (n- hexane, chloroform, ethyl acetate and methanol fractions) separated from methanolic extracts of both botanicals by topical application (75 µl of each fraction/ insect) and the percentage of mortality obtained in the screening studies were calculated for 6 h, 12 h and 24 h of treatment, displayed in table IV. 5. Simultaneous with each experimental set of

treatment, a negative control set without any treatment and a positive control set with solvent alone for treatment were also maintained. From this study, it has been revealed that chloroform fractions separated from the methanolic extracts of both plants were found to be very effective as toxicants towards the larval instar of *O. exvinacea* (Table IV. 5). For both botanicals, 100 % mortality was shown by the chloroform fractions separated from the methanolic extract. All the other three fractions were causing minimum toxicity towards the larvae. The results for each positive control set also indicated that methanol was found to be the least toxic solvent among the selected ones for the study. There was no mortality observed in the case of negative control sets.

Table IV. 5. Mean percentage mortality of *O. exvinacea* larva exposed to different solvent fractions of methanolic extract of both botanicals.

Extracts		Mean percentage mortality (%)		
		6 h	12 h	24 h
n- Hexane	*H. suaveolens*	12.5	12.5	25
	V. negundo	16.6	20.83	29.2
	Positive Control	4.17	4.17	12.5
	Negative control	-	-	-
Chloroform	*H. suaveolens*	62.5	87.5	100
	V. negundo	66.7	91.7	100
	Positive Control	12.5	16.7	25
	Negative control	-	-	-
Ethyl acetate	*H. suaveolens*	12.5	25	25
	V. negundo	20.83	25	33.3
	Positive Control	8.33	8.33	12.5
	Negative control	-	-	-
methanol	*H. suaveolens*	-	-	12.5
	V. negundo	-	4.17	8.33
	Positive Control	-	-	-
	Negative control	-	-	-

Values expressed are mean percentage mortalities of three replicates

4.3.2.1. Determination of LD_{50}

Mortality studies with chloroform fraction separated from the methanolic extracts of *H. suaveolens* and *V. negundo* were carried out as elucidated in chapter III. section 3.2.3.3. Toxic effects of various concentrations of both botanicals on *O. exvinacea* larva at 12 h intervals to cause 50 % mortality by topical application method were noticed. Table IV. 6 show the mean percentage mortality of *O. exvinacea* to different doses of *H. suaveolens* during different exposure periods.

Table IV. 6. Mean percentage mortality of chloroform fraction of *H. suaveolens* on *O. exvinacea* larva treated for different periods.

Concentration (µg)	Mean percentage mortality (%)				
	12 h	24 h	36 h	48 h	60 h
60	22	32	34	38	40
65	26	36	40	42	42
70	40	42	46	52	52
75	40	44	46	54	54
80	42	44	44	52	62
Positive Control	0	0	0	0	0
Negative control	0	0	0	0	0

Values expressed are mean percentage mortality values of 5 replicates

In the case of *H. suaveolens*, the application of 60 µg, 65 µg, 70 µg, 75 µg and 80 µg concentrations for 60 h duration caused mortality of 40 %, 42 %, 52 %, 54% and 62 % respectively. Mean percentage mortality calculated for *H. suaveolens* was inferred to exhibit a dose dependent pattern. No mortality was noticed for both negative and positive control sets.

In the case of *H. suaveolens*, the mean percentage mortality for each treatment was statistically analysed with Duncan's multiple range test to find out the differences between treatment percentages in causing mortality. It was

found that two adjacent concentrations applied were not showing significant difference in causing percentage mortality. But increasing concentrations were found to be effective and significantly different from lower concentrations at 1% level of DMRT (Table IV.7).

Table IV.7. Level of significance of mortality effects caused by *H. suaveolens* extract on *O. exvinacea* larva at different concentrations analysed with DMRT

	Extract concentration	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan	6.50	5	4.2000			
	7.00	5		5.2000		
	7.50	5		5.4000		
	8.00	5			6.2000	
	8.50	5				7.4000
	Significance		1.000	.526	1.000	1.000

The data obtained for treatment with *V. negundo* was analysed and the mean percentage moratlity was calculated for each 12 h duration of exposure and are given in table IV. 8.

Table IV.8. Mean percentage mortality of chloroform fraction of *V. negundo* on *O. exvinacea* larva treated for different periods.

Concentration (µg)	Mean percentage mortality (%)			
	12 h	**24 h**	**36 h**	**48 h**
20	-	12	12	14
25	14	28	30	30
30	40	42	42	46
35	34	44	46	54
40	52	58	60	62
Positive Control	0	0	0	0
Negative control	0	0	0	0

Values presented are mean values of 5 replicates.

In the case of *V. negundo*, the application of 20 µg, 25 µg, 30µg, 35 µg and 40 µg for 48 h duration caused percentage mortality of 14 %, 30 %, 46 %, 54 % and 62 % respectively. The mean percentage mortality for the same was calculated, which showed a dose dependent pattern of increase in mortality. But there were no mortalities in the negative and positive control sets. From the data it was evident that *V. negundo* was showing strong toxicity with low dosage whereas *H. suaveolens* required a more increased concentration to cause half death. In the case of *V. negundo*, the mean percentage mortality for each treatment was statistically analysed with Duncan's multiple range test to find out the differences between treatment percentages in causing mortality. It was found that two adjacent concentrations used for treatment were showing significant difference in causing percentage mortality. Even slight differences in the concentrations were found to be very effective in causing mortality and significantly different at 1% level of DMRT (Table IV.9).

Table IV.9. Level of significance of mortality effects caused by *V. negundo* extract on *O. exvinacea* larva at different concentrations analysed with DMRT

	Extract concentration	**N**	**Subset for alpha = 0.05**				
			1	**2**	**3**	**4**	**5**
Duncan	2.00	5	1.4000				
	2.50	5		3.0000			
	3.00	5			4.6000		
	3.50	5				5.4000	
	4.00	5					6.2000
	Significance		1.000	1.000	1.000	1.000	1.000

4.3.2.2. Statistical analysis of data

The data on contact toxicity was analysed and calculated the probit mortality, regression coefficient, LD_{30}, LD_{50} and LD_{90} values for both botanicals through probit analysis and their significance studies were tested with Duncan's multiple range test and are expressed in table IV. 10 and figures IV. 1 and IV. 2.

Figure IV. 1. Probit analysis graph for the determination of LD_{50} value for *Hyptis suaveolens*

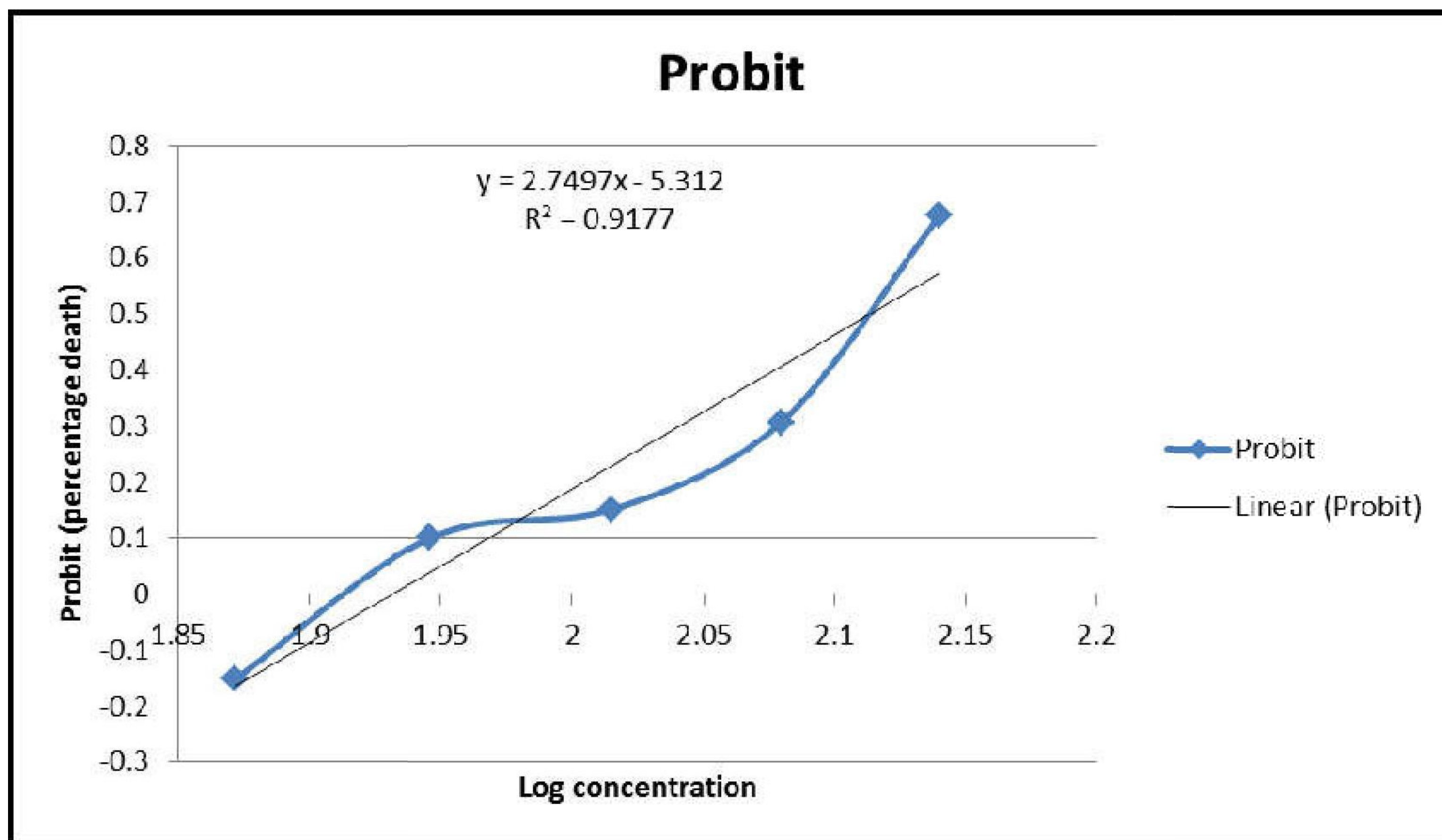


Figure IV. 2. Probit analysis graph for the determination of LD_{50} value for *Vitex negundo*

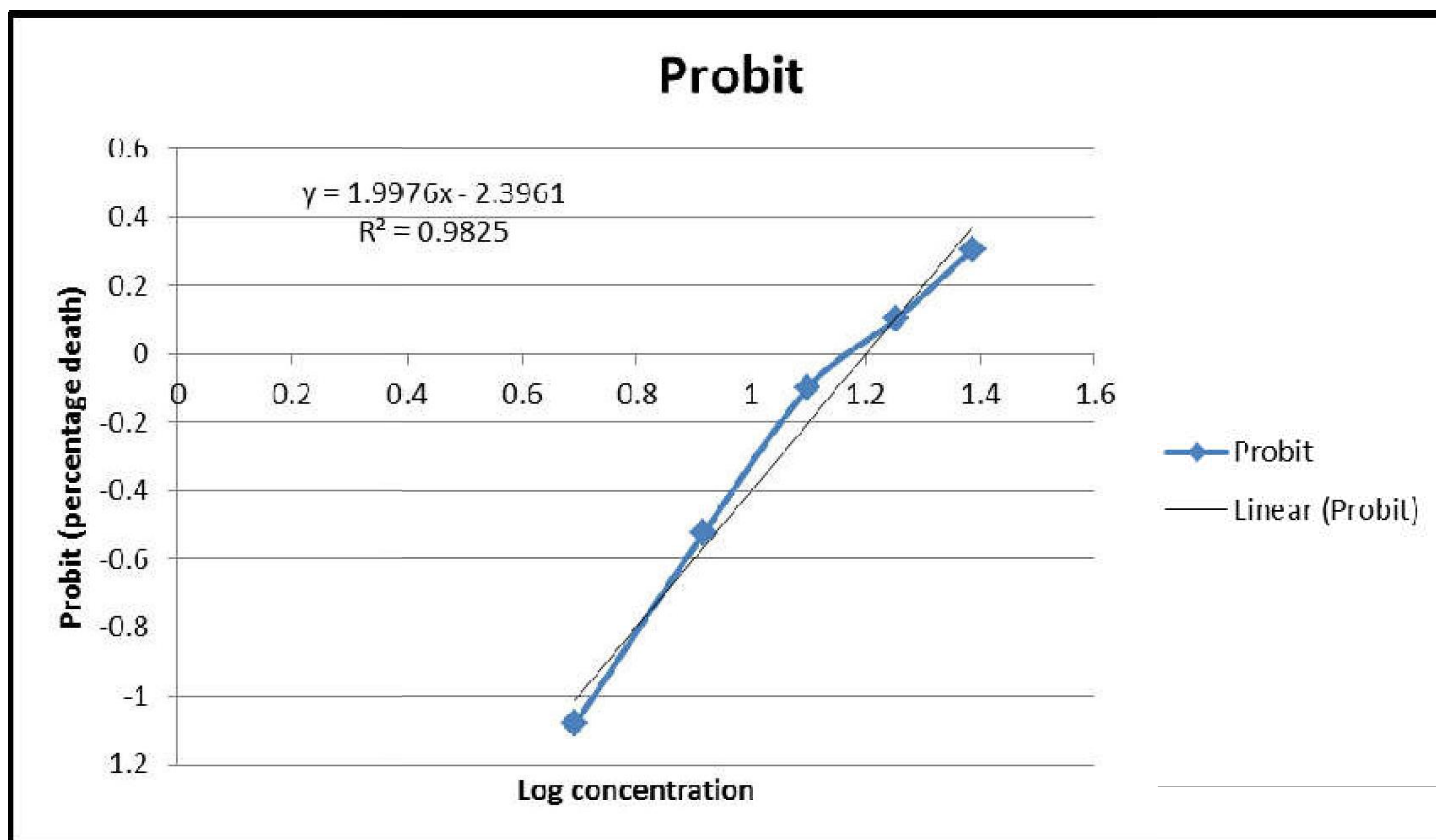


Table IV. 10. Probit analysis data on contact toxicity of chloroform fraction of *H. suaveolens* and *V. negundo* against *O. exvinacea*

Treatments	Regression equation	Regression coefficient		Slope	LD_{30}	LD_{50}	LD_{90}	P values
		R	R^2					
H. suaveolens	Y = 2.7497 x - 5.312	0.95	0.9177	2.7497	5.838	7.016	10.993	0.000
V. negundo	Y = 1.9976 x - 2.3961	0.99	0.9825	1.9976	2.539	3.316	6.367	0.000

The values expressed are probit analysed assessments of 5 dosages each having 5 replicates consisting of ten insects per replica. LD_{30}, LD_{50} and LD_{90} values were calculated to give 30, 50 and 90 percent mortality respectively. Y= Probit mortality; x= log concentration.

The data obtained from the toxicity studies on *O. exvinacea* larva by topical application of *H. suaveolens* and *V. negundo* were subjected to probit analysis for computing the regression equation to find out the LD_{50} values of the fractions. Larval toxicity resulted with the topical application of *H. suaveolens* exhibited a regression coefficient (R) of 0.95 and LD_{30}, LD_{50} and LD_{90} values of 5.838, 7.016 and 10.993 respectively. In this case, the toxic effect of *H. suaveolens* on the insect is highly significant ($P<0.01$). Correspondingly, the toxicity caused by topical application of *V. negundo* presented the value of regression coefficient (R) 0.99 and 2.539, 3.316 and 6.367 as LD_{30}, LD_{50} and LD_{90} values respectively. Here too, the toxic effect resulted with *V. negundo* was found to be highly significant ($P<0.01$) (Table IV.10).

Figure IV.1 indicates the toxicity of *H. suaveolens* against *O. exvinacea* larva upon topical application method. This graph displays the regression equation which correspond to the probit mortality (%) of Y= 2.7497 and log concentration of x = x - 5.312. The slope of the regression line 2.7497 designates that for each unit enhancement in the log concentration of *H. suaveolens* (dose) follows a corresponding increase in the mortality of larval insects.

Figure IV. 2. displays the toxicity of *V. negundo* on *O. exvinacea* larva by topical application method. This graph expresses the regression equation, symbolizes the probit mortality (%) of Y= 1.9976 and log concentration of x = x - 2.3961. The slope of the regression line 1.9976 implies that unit rise in the log concentration of *V. negundo* (dose) results in a consequent rise in the mortality of larval insects.

4.4. Discussion

In the present investigation, preliminary phytochemical studies concerned with the maximum yield of extract and the toxicity evaluation of different solvent extracts on sixth instar larva of *O. exvinacea* confirmed the efficacy of methanol as a proper solvent for both *H. suaveolens* and *V. negundo* for soxhlet extraction. Moreover, thin layer chromatographic studies of these solvent extracts also endorses this result in which the chromatogram revealed the presence of maximum number of components in the case of methanolic extract upon comparison with the other solvent extracts. Most of the phytochemical extractions were usually focused on studies with maximum yield of extract with different polar based solvents to fix a particular solvent for extraction process. For instance, selection of solvent system for executing plant material extraction has an influence on resultant efficacy upon toxicity evaluation (Yankanchi, 2009). Moreover, the selection of best solvent is much relied on the presence of type of components (whether polar or nonpolar) and their distribution in the herbal plant (Harborne, 1989). They reported much complementary results in that the extraction of herbal plants such as *Phyllanthus niruri*, *Orthosiphon stamineus* and *Labisia pumila* were made possible by considering the solvent with maximum yield of extract. Many researchers worked on phytochemical extraction with the same aspect so as to select solvent for extraction of various herbal plants (Markom *et al*., 2007; Razak *et al*., 2012; Azrie *et al*., 2014).

Moreover, the highest toxicity of chloroform extracts among the treatments may be due to the combined effect of active ingredients in the extract as well as the solvent, chloroform. Because in this treatment chloroform itself is showing some toxicity towards the larva. The methanolic extract (second highest mortality shown) was found to be exhibiting more

toxicity than chloroform extract, since the control set with methanol alone treatment revealed no mortality. It revealed that the toxicity might be due to the presence of active ingredients in the extract itself and not with the toxicity of methanol. It also confirmed that methanol was found to be the least toxic solvent for *O. exvinacea* larva.

In the present study, methanolic extraction was carried out for both *H. suaveolens* and *V. negundo* which were further fractionated with n-hexane, chloroform, ethyl acetate and methanol. These fractions were tested on *O. exvinacea* larva to findout the most toxic fraction along with negative and positive control groups. In this study, the most toxic fraction noticed to be the chloroform fraction of both *H. suaveolens* and *V. negundo*. The highest toxicity of chloroform fraction may be due to the combined effect of active ingredients as well as the solvent, chloroform. Although, the toxicity was resulted with the combined action, comparison between different fractions causing toxicity revealed that chloroform fraction was found to be the most toxic among them. Whereas, the positive control set with solvent alone treatment revealed that methanol was found to be the least toxic solvent for *O. exvinacea* larva, so methanol was chosen as a suitable solvent for preparing stock solution after complete evaporation of the chloroform in it.

The toxicity studies by topical application of *H. suaveolens* and *V. negundo* for different time intervals in order to calculate LD_{50} were showing strong contact toxicity against *O. exvinacea* larva. It was clearly seen that the extract of *H. suaveolens* at low concentration was found to be less toxic and so high concentration is required for causing mortality. However, in the case of *V. negundo* toxic effect was resulted with very low concentration itself. *H. suaveolens* on the other hand was active only at the highest concentration which however was not showing any instant action as in the case of *V. negundo*.

The data obtained from the toxicity studies indicate that toxic materials are present in both *H. suaveolens* and *V. negundo*. However, the chloroform fraction of *V. negundo* was found to be more effective than *H. suaveolens* in causing mortality at very low concentrations. Probit analysis and DMRT of mortality data revealed that the toxicity by topical application of *H. suaveolens* and *V. negundo* were highly significant ($P < 0.01$).

In the case of positive control larva, topical application with methanol alone caused no mortality for all durations indicating that the treatment of methanol on *O. exvinacea* was found to be non-toxic. Similarly, for negative control also no mortality was noticed in any of the larval sets indicating the healthy nature of larval forms.

The contact toxicity with *H. suaveolens* and *V. negundo* was noticed to be both concentration and time dependent. Mortality was triggered with the enhancement of both concentration of treatment and time of exposure of the larva. Among the treatments higher toxicity was exhibited by *V. negundo* than *H. suaveolens*. The slope of the regression line specifies the existence of a direct proportionality between unit increase in the concentration of the extracts and the resulting probit mortality of the insects. Dose dependent toxicity of plant extracts and oils etc. have been reported by many authors (Fitzpatrick and Dowell, 1981; Pathak and Tiwari, 2010).

Complementary to the present study, *V. negundo* extracts were found to be effective against a number of insects. *V. negundo* extracts and some indigenous plants possess larvicidal properties against *S. litura* (More *et al*., 1989) and *T. confusum* (Khanam *et al*., 1995). Similarly the extracts of *V. negundo* were effective against *E. vitella*, *Diaphania indica* and *Epilachna septima* (Kalavathi *et al*., 1991). Moreover, petroleum ether extracts of the leaves of *V. negundo* caused larvicidal activity against larval stages of *Cx. tritaeniorhynchus* (Karunamoorthi *et al*., 2008).

The present study revealed that *H. suaveolens* possesses larvicidal activity against *O. exvinacea* which is comparable with many results in which *H. suaveolens* possess larvicidal activity against larvae of *Ae. aegypti* (Amusan *et al*., 2005); *S. litura* (Raja *et al*., 2005); *S. zeamais* and *C. maculates* (Iloba and Ekrakene, 2006); cow pea borer, *M. testulalis* (Gbehounou, 2007); *H. armigera* and *S. litura* (Ivoke *et al*., 2009) and *T. granarium* (Musa *et al*., 2009). Complementary to the toxic effect noticed in the present study, *H. suaveolens* essential oil was reported to be toxic against *P. xylostella* larvae and *C. maculatus* adults (Kéïta *et al*., 2006; Tripathi and Upadhyay, 2009).

Moreover, much comparable results were reported by Pavunraj *et al.* (2014) in which the crude extracts of *H. suaveolens* leaves and their fractions were found to be exhibiting larvicidal activities against four lepidopteran pests namely *H. armigera, S. litura, E. vittella* and *L. orbonalis.*

CHAPTER V

PHYTOCHEMICAL PROFILING AND ANALYSIS OF TOXIC FRACTION OF BOTANICALS FOR IDENTIFICATION OF CHEMICAL CONSTITUENTS

5.1. Introduction

Plants, the chemical factories of nature, mysteriously treasured with valuable phytochemical constituents show promising biological activities towards agricultural pests. These biologically active ingredients, termed secondary metabolites are dispersed in various plant parts and are of three major groups: terpenes, phenols and alkaloids (Schoonhoven *et al*., 1998).

Terpenoids form the largest group of secondary metabolites, constitutes more than 40,000 distinct molecules and are ecologically important for their defense function in plants (Garcia and Carril, 2009). They are generally insoluble in water and their main function in plants is to protect or to attract beneficial organisms and defense against herbivory with their analogous structure to that of the molting hormone in insects which cause interference in the moulting process (Taiz and Zeiger, 2013).

Phenolic or polyphenolic compounds of plants are one of the most abundant groups of substances, with more than 8,000 phenolic structures recognized and broadly spread all over the plant kingdom (Harborne, 1989). This class of natural products is chemically a heterogenous group and possesses at least one aromatic ring with one free hydroxyl group or exists as its other derivatives such as esters or heterosides (Carvalho *et al*., 2007). Generally, the phenolic compounds are very unstable, easily oxidized and susceptible to degradation, with some soluble in water and others not. These compounds have many biological activities like attraction of pollinators and

seed dispersers and some others have antifeedant action on herbivores (Taiz and Zeiger, 2013).

Alkaloids are basic natural products seen in many plants which comprise the largest single class of secondary plant substances with more than 5,500 alkaloids identified (Okwu, 2005). They are cyclic organic compounds which contain one or more nitrogen atoms and are widely distributed in the plant kingdom (Gurib-Fakim, 2006). They are reported to be biologically very active and constitute towards plant defenses against phytophagous animals and are also insecticidal with varying mode of actions (Aniszewski, 2007).

In the present study, the presence of these classes of compounds in the toxic fraction of both botanicals were made possible through high performance thin layer chromatography (HPTLC). HPTLC profiling is an important qualitative analytical technique which has been employed frequently for ensuring the existence of certain chemical constituents. It is a very useful quality assessment tool for the analysis of a broad number of compounds present in the botanical materials efficiently. Usually the spots separated in HPTLC studies are well resolved and so it is more versatile than ordinary TLC methods. HPTLC fingerprinting is demonstrated to be a more accurate multipurpose method for plant identification, confirmation and characterization of the important medicinal plants (Kpoviessia *et al.*, 2008).

Gas Chromatographic- Mass Spectroscopic method is a most widely used technique in phytochemical analysis for the identification and quantification of volatile chemical constituents. It is a most compatible technique for recognizing unknown organic compounds in a complex mixture which can be determined by interpretation of mass spectrum. Usually the interpretation of the mass spectrum is made possible using the database of National Institute of Standards and Technology (NIST) library.

The present investigation deals with the development of HPTLC fingerprints of the most toxic fractions separated from methanolic extracts of both *H. suaveolens* and *V. negundo* which can be employed for identification, authentication and classification of chemical compounds. Since these fractions were showing larvicidal properties, identification of volatile chemical constituents by GC-MS analysis have also been carried out.

5.2. Materials and Methods

The materials and methods used for performing HPTLC profiling were described in the section 3.2.4.1. Whereas the requirements for GC-MS analysis were given in the section 3.2.4.2.

5.3. Results

5.3.1. HPTLC analysis

High performance thin layer chromatographic studies were conducted for toxic fraction of both *H. suaveolens* and *V. negundo* in order to identify the presence of toxic components. Qualitative analysis for the identification of the components of different bands was performed with spraying of specific reagents for visualization.

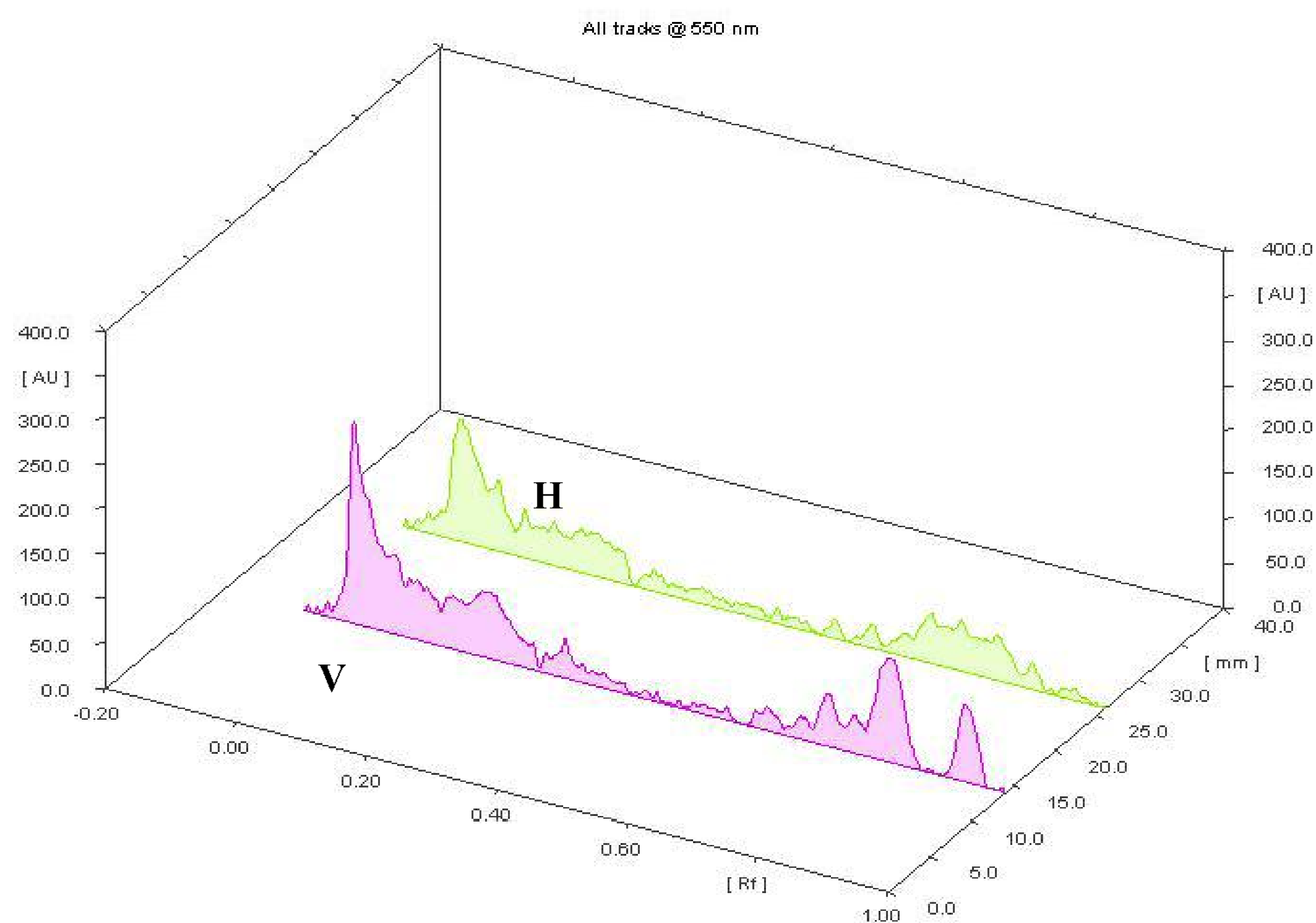
HPTLC profile of toxic fractions of both plants were studied for the presence of alkaloids using solvent system Chloroform: Ethyl acetate: Formic acid (5:5:1) which was detected under 254 nm, 366 nm UV light and 550 nm visible light (after derivatization with Dragendorff's reagent) and the respective chromatogram were shown in Plate V. 1. The HPTLC densitometric profiling of toxic fraction of both botanicals was illustrated in figure V.1. It is apparent from Figure V. 2, that 14 spots were got separated and visualized in the HPTLC fingerprint of *V. negundo* scanned at 550 nm visible light with varying R_f values ranging from 0.01 - 0.94 (Table V.1).

Whereas for *H. suaveolens* (Plate V.1) possessed 17 spots with R_f values ranging from 0.01- 0.95 (Figure V. 3 and Table V. 1). Alkaloids were detected in the regions of the chromatogram with orange- red coloured bands in the R_f range of 0.32, 0.57, 0.64, 0.69, 0.73, 0.82 and 0.94 for *V. negundo* (7 bands) and that for *H. suaveolens* showed 6 bands with R_f range of 0.30, 0.50, 0.58, 0.64, 0.69 and 0.73, indicating the presence of alkaloids in both plant toxic fraction.

Table V.1. Showing the number of spots with their R_f values (under 254 nm, 366 nm and 550 nm) and area in percentage (550 nm) for alkaloids present in the toxic fraction of *V. negundo* and *H. suaveolens*.

Alkaloids - R_f values							
V. negundo				***H. suaveolens***			
Under 254 nm	**Under 366 nm**	**Under 550 nm**	**Area in %**	**Under 254 nm**	**Under 366 nm**	**Under 550 nm**	**Area in %**
0.07	0.07	0.01	0.61	**0.13**	**0.19**	0.01	1.92
0.12	0.12	0.03	23.34	0.19	0.27	0.04	30.68
0.31	0.20	0.06	5.88	0.39	0.75	0.07	9.50
0.61	0.24	0.09	8.52	0.50	0.84	0.11	5.08
0.73	0.31	0.15	5.54	0.74	0.94	0.15	5.10
0.80	0.73	0.21	14.68	0.83		0.20	5.45
0.94	0.81	0.32	3.69			0.25	2.37
	0.94	0.57	0.65			0.30	3.50
		0.64	2.57			0.50	1.03
		0.69	1.84			0.58	1.96
		0.73	5.41			0.64	2.60
		0.77	3.08			0.69	3.56
		0.82	16.06			0.73	7.56
		0.94	8.14			0.78	6.86
						0.83	8.04
						0.89	2.82
						0.95	1.77

Figure V. 1. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for alkaloids scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 2. HPTLC fingerprint of toxic fraction of *V. negundo* for alkaloids after derivatization showing different peaks of phytoconstituents (550 nm).

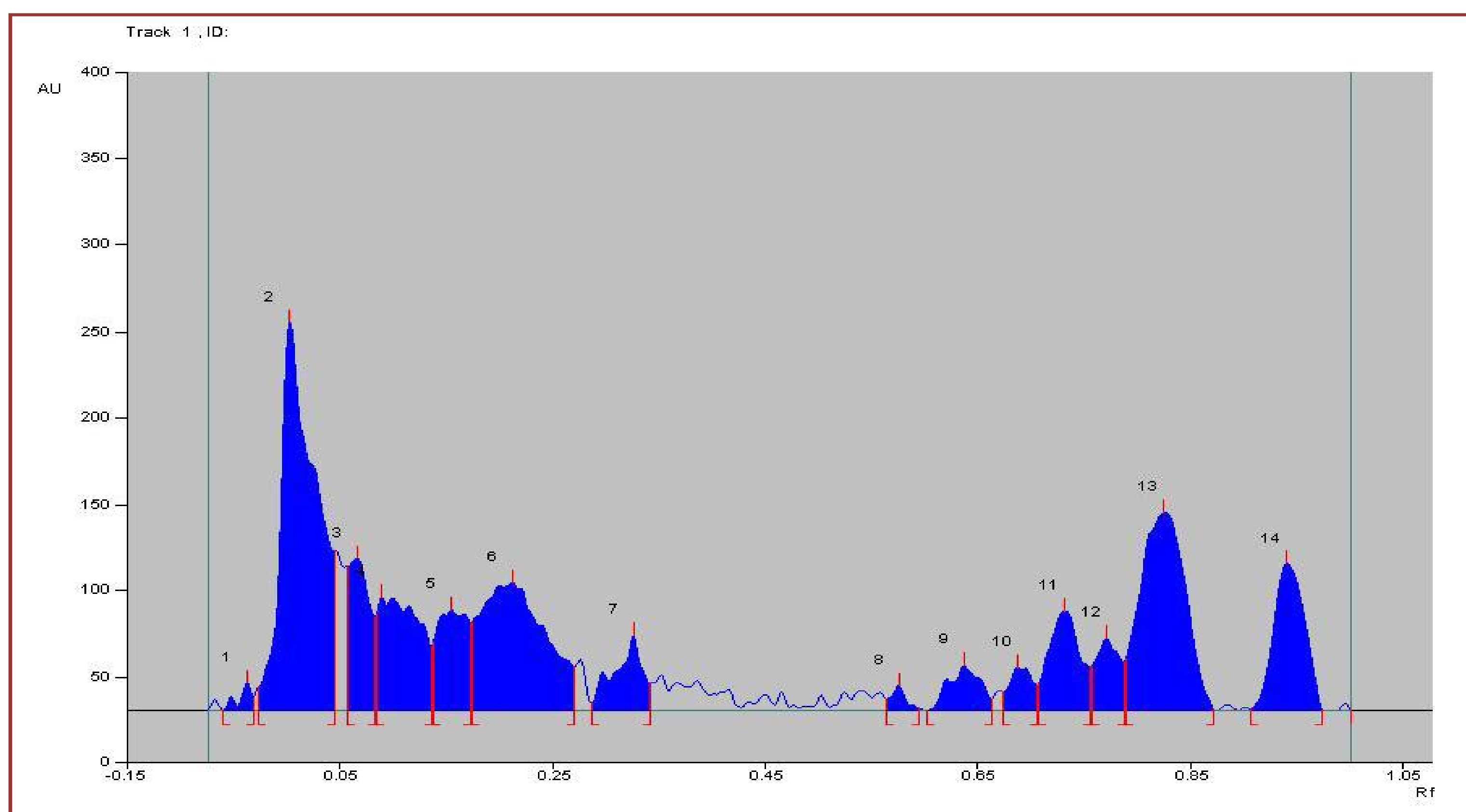
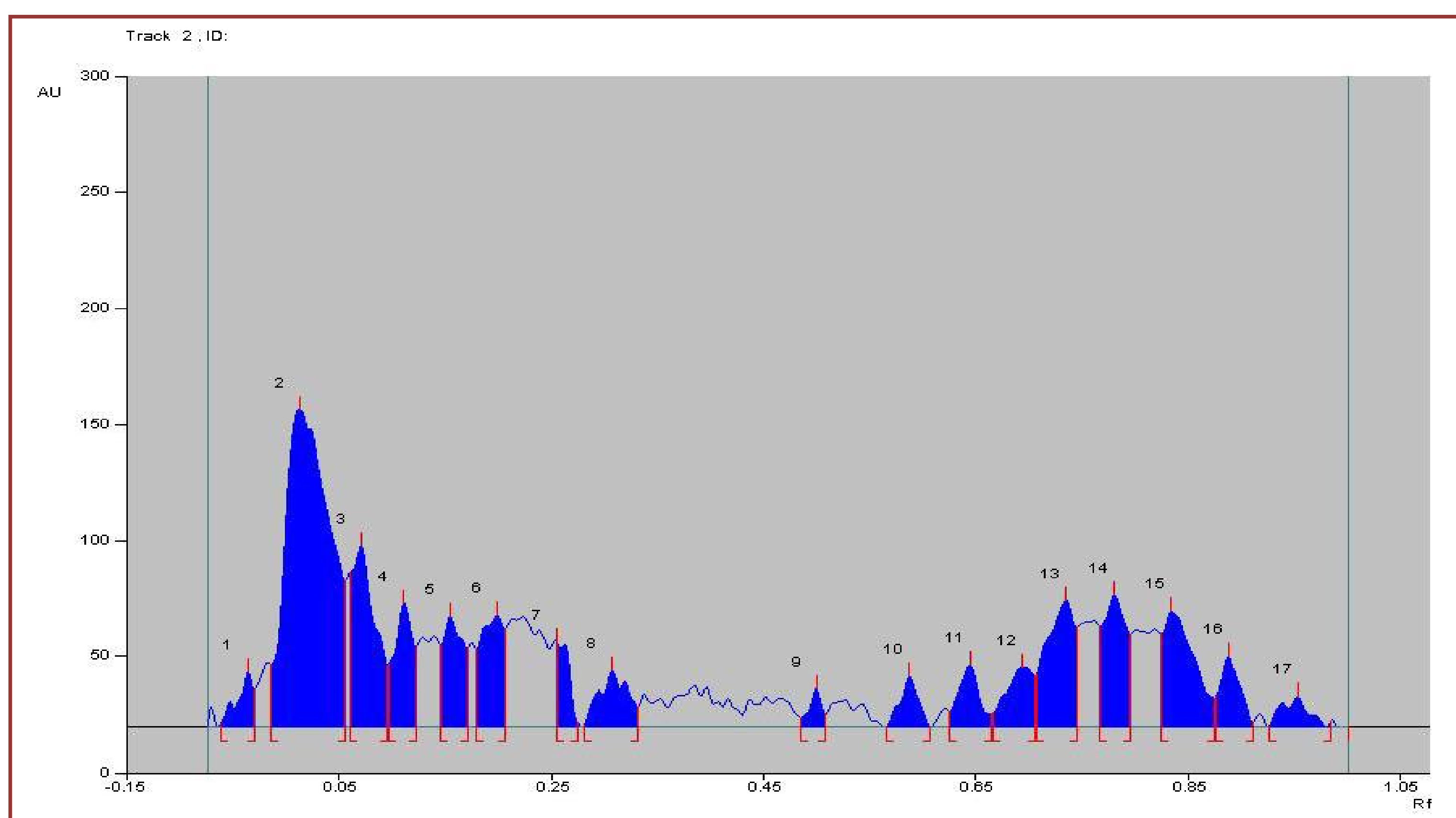


Figure V. 3. HPTLC fingerprint of toxic fraction of *H. suaveolens* for alkaloids showing different peaks of phytoconstituents after derivatization (550 nm).

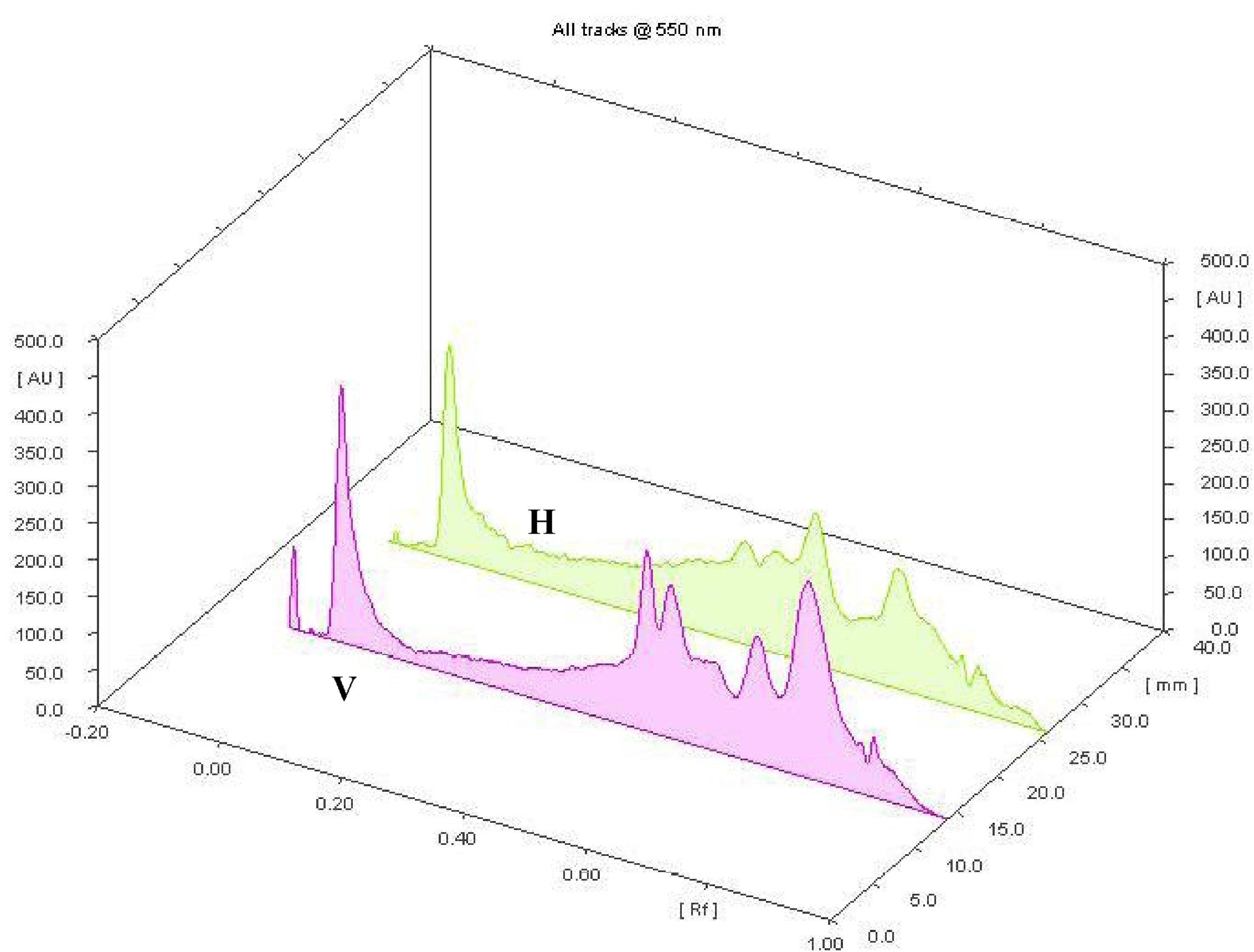


Similarly, HPTLC profiling of the same fractions were performed for the presence of phenolics using solvent system, Toluene: Ethyl acetate: Formic acid (5:5:1) and the developed chromatogram was detected under 254 nm, 366 nm UV light and 550 nm visible light (after derivatization with ferric chloride reagent) is illustrated in the Plate V. 2. The HPTLC densitometric profiling for the same was presented in Figure V. 4. The HPTLC fingerprint of *V. negundo* showed 6 peaks (Figure V. 5) with R_f values in the range of 0.13- 0.97 (Table V. 2). *H. suaveolens* possess 11 peaks (Figure V. 6) with R_f values ranging from 0.01- 0.97 (Table V. 2). The presence of phenolics were detected in the developed chromatogram with the presence of blue-green coloured bands in the R_f range of 0.63, 0.68, 0.90 and 0.93 for *V. negundo* (4 bands) and that for *H. suaveolens* showed 2 bands with R_f range of 0.65 and 0.77, indicating the presence of phenolics in toxic fraction of both plants.

Table V.2. Showing the number of spots with their R_f values (under 254 nm, 366 nm and 550 nm) and area in percentage (550 nm) for phenolics present in the toxic fraction of *V. negundo* and *H. suaveolens*

Phenolics – R_f values							
V. negundo				*H. suaveolens*			
Under 254 nm	**Under 366 nm**	**Under 550 nm**	**Area in %**	**Under 254 nm**	**Under 366 nm**	**Under 550 nm**	**Area in %**
0.35	0.37	0.13	95.05	0.44	0.37	0.01	12.76
0.42	0.40	0.63	0.83	0.52	0.46	0.12	61.53
0.45	0.45	0.68	0.65	0.62	0.48	0.33	8.94
0.50	0.48	0.90	1.85	0.71	0.55	0.48	2.61
0.58	0.52	0.93	1.00	0.89	0.59	0.60	1.44
0.68	0.60	0.97	0.62		0.64	0.65	1.08
0.87	0.68				0.68	0.68	2.18
	0.76				0.74	0.77	5.18
	0.87				0.88	0.90	0.83
						0.95	1.66
						0.97	1.60

Figure V. 4. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for phenolics scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 5. HPTLC fingerprint of toxic fraction *V. negundo* for phenolics showing different peaks of phytoconstituents after derivatization (550 nm).

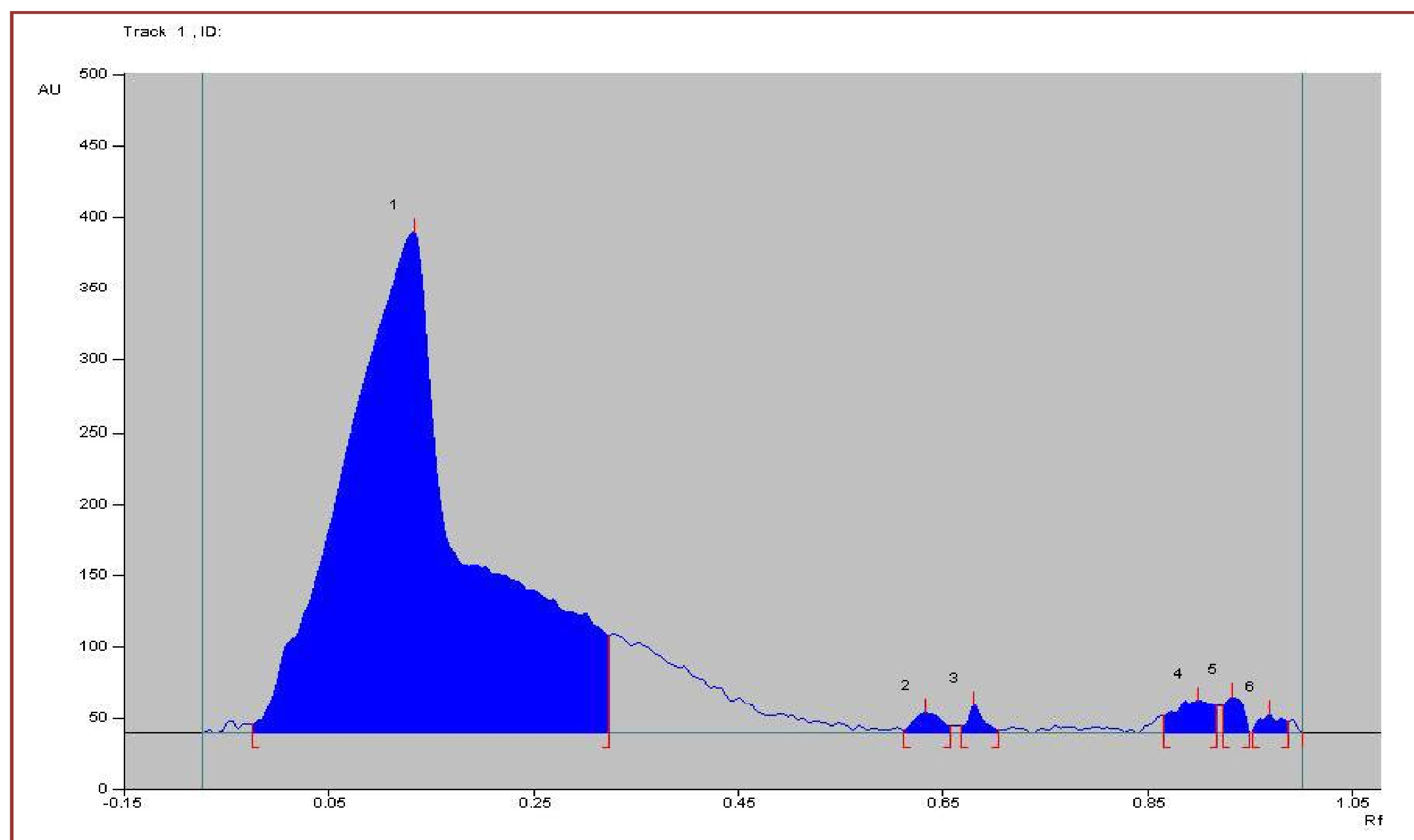
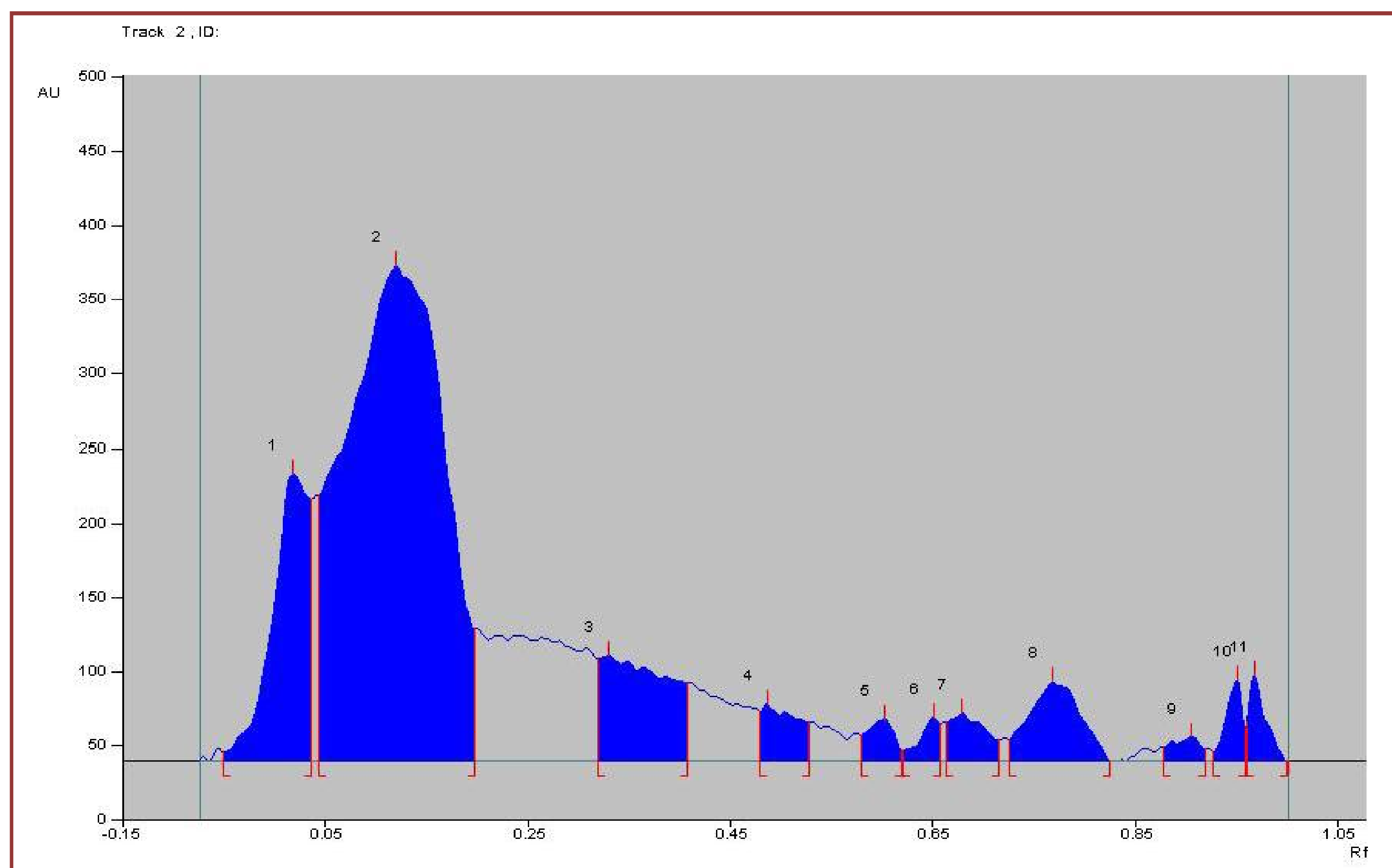


Figure V. 6. HPTLC fingerprint of *H. suaveolens* toxic fraction for phenolics showing different peaks of phytoconstituents after derivatization (550 nm).

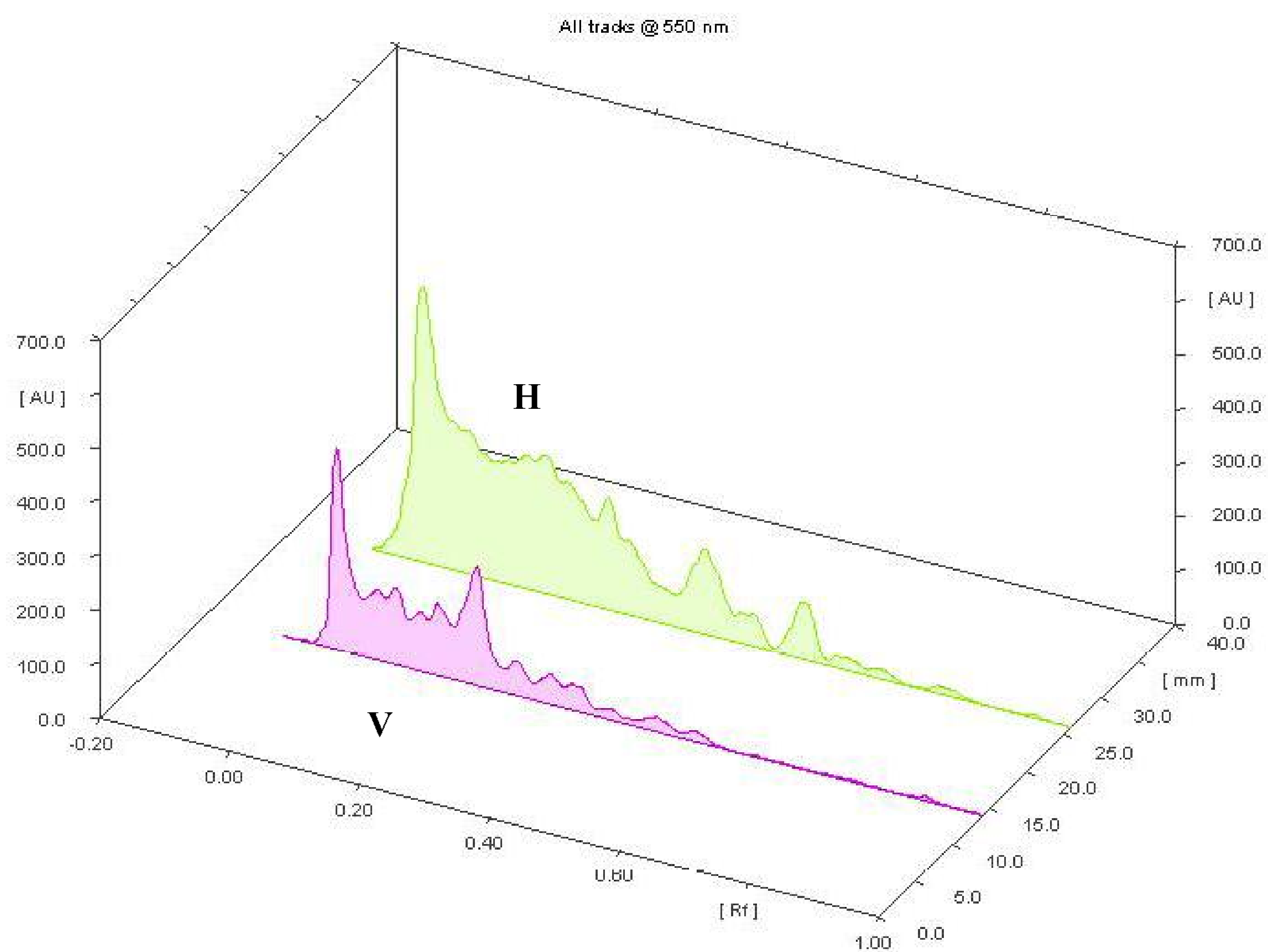


Likewise the presence of terpenoids were also confirmed with HPTLC profiling in the toxic fractions of both plants using solvent system, Chloroform: Ethyl acetate: Formic acid (5:5:0.5), detected under 254 nm, 366 nm UV light and 550 nm visible light (after derivatization with anisaldehyde suphuric acid reagent) is shown in the Plate V. 3. The HPTLC densitometric profiling of terpenoids of both botanicals was illustrated in Figure V. 7. The HPTLC fingerprint of *V. negundo* toxic fraction revealed 11 peaks (Figure V. 8) in the R_f range of 0.01- 0.56 (Table V. 3) and that of *H. suaveolens* possess 13 peaks (Figure V. 9) with R_f values ranging from 0.02-0.77 (Table V. 3). Terpenoids were detected with the presence of deep/light blue violet, blue, grey coloured bands in the chromatogram with R_f values of 0.16, 0.22, 0.28, 0.33, and 0.37 for *V. negundo* (5 bands) and that for *H. suaveolens* showed 6 bands with R_f range of 0.30, 0.34, 0.40, 0.51, 0.59 and 0.65, indicating the presence of terpenoids in both plant toxic fractions (Plate V.3).

Table V.3. Showing the number of spots with their R_f values (under 254 nm, 366 nm and 550 nm) and area in percentage (550 nm) for terpenoids present in the toxic fraction of *V. negundo* and *H. suaveolens*

Terpenoids - R_f values							
V. negundo				***H. suaveolens***			
Under 254 nm	**Under 366 nm**	**Under 550 nm**	**Area in %**	**Under 254 nm**	**Under 366 nm**	**Under 550 nm**	**Area in %**
0.09	0.13	0.01	26.19	0.07	0.14	0.02	27.50
0.13	0.15	0.07	9.29	0.43	0.16	0.09	12.03
0.18	0.18	0.10	9.48	0.48	0.20	0.14	10.42
0.25	0.23	0.13	6.50	0.55	0.26	0.18	11.1
0.31	0.36	0.16	10.05	0.65	0.37	0.23	9.02
0.37	0.42	0.22	20.78	0.70	0.46	0.30	8.13
0.42	0.54	0.28	4.81		0.54	0.34	6.1
0.54	0.65	0.33	4.50		0.60	0.40	6.19
0.59	0.68	0.37	3.81		0.64	0.51	2.57
0.91	0.91	0.50	2.99		0.73	0.59	3.69
		0.56	1.60		0.94	0.65	1.24
						0.70	1.08
						0.77	0.93

Figure V. 7. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for terpenoids scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 8. HPTLC fingerprint of toxic fraction of *V. negundo* for terpenoids showing different peaks of phytoconstituents after derivatization (550 nm)

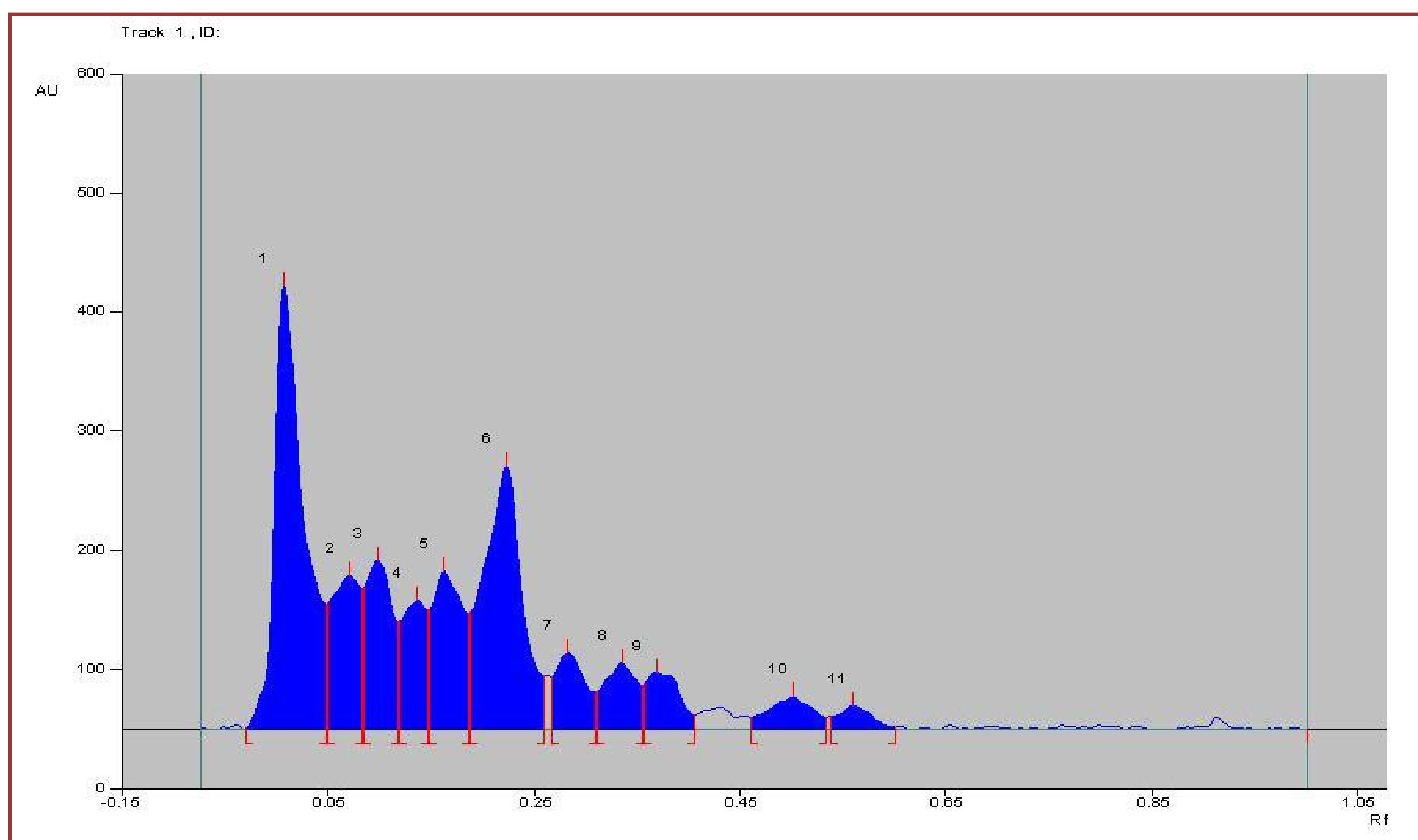
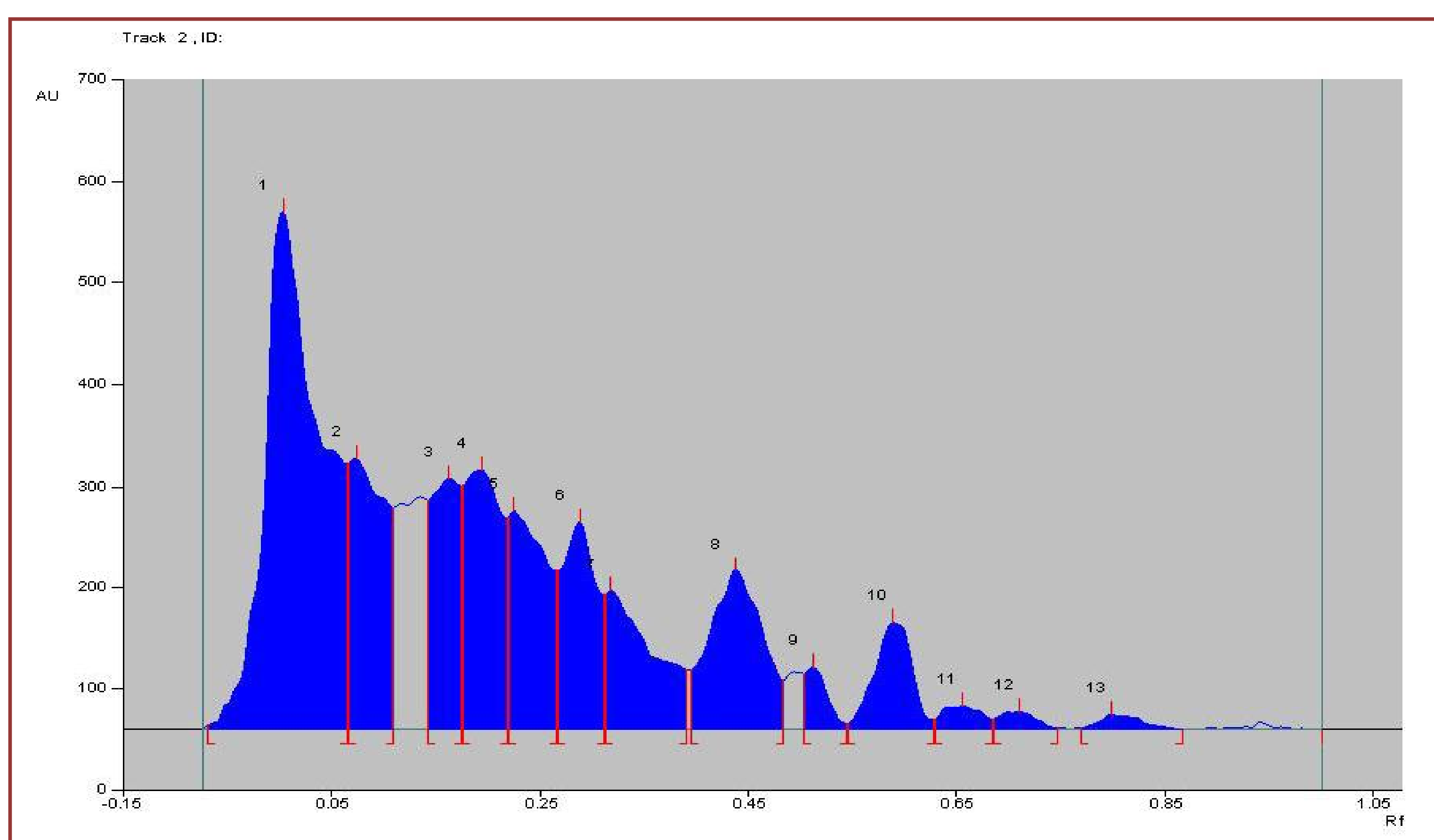


Figure V. 9. HPTLC fingerprint of toxic fraction of *H. suaveolens* for terpenoids showing different peaks of phytoconstituents after derivatization (550 nm)



5.3.2. GC-MS analysis

Toxic chloroform fraction of both *H. suaveolens* and *V. negundo* were further analysed by GC-MS for the identification of volatile chemical constituents and the spectral data of GC-MS analysis of chloroform fraction for *H. suaveolens* and *V. negundo* are shown in Plate V. 4 and Plate V. 5 respectively. From both toxic fractions, an array of chemical components were separated.

GC-MS analysis of *H. suaveolens* toxic fraction

In the toxic fraction of *H. suaveolens*, a total of 73 components were identified. The retention time, name, molecular formula, chemical class, molecular weight and area percentage of the components of this sample were determined and presented in Table V. 4. Among the constituents detected, the most pronounced constituent in *H. suaveolens* fraction is 12-O-Acetylingol 8-tiglate with an area of 8.420 % which is regarded as a diterpenoid compound. Second more prominent component was also a diterpenoid compound, 2-Cyclohexen-3, 6-diol-1-one, 2-[10-tetradecenoyl]- with an area of 7.939 % which was followed by compounds like diepoxy ester (5.864 %), diterpenoid (5.652 %) and phthalate diester (5.265 %) in the 3rd, 4th and 5th positions respectively. In addition, maximum number of peaks was noticed with phthalate diester (16 peaks) at varying retention time and area percentages. Moreover, GC-MS analysis of the toxic fraction of *H. suaveolens* revealed the presence of many other constituents like phenolics, fatty acid, steroid and other esters, ketones, sesquiterpenoids, heterocyclic compounds, flavones, alcoholic compounds, pyran and furan derivatives in varying quantities (Table V. 4).

Table V. 4. The phytochemical components identified in the toxic fraction of *Hyptis suaveolens*

Sl. No.	R.T Value	Name of the compound	Molecular formula	Chemical class	Molecular weight	Area %
1	24.07	Phenol, 2-methoxy-3-(2-propenyl)-	$C_{10}H_{12}O_2$	Phenol	164	0.476
2	25.29	Cyclohexanepropanoic acid, 3-hydroxy-, methyl ester	$C_{10}H_{18}O_3$	Hydroxy ester	186	0.472
3	26.22	2H-Pyran-2-one, 5,6-dihydro-4-(2-methyl-2-propen-3-yl)-	$C_9H_{12}O_2$	Pyranone derivative	152	1.015
4	26.39	Cyclopentaneacetic acid, 2-(hydroxymethyl)-3-methyl-, δ-lactone	$C_9H_{14}O_2$	d-lactone	154	0.096
5	26.76	3a,6-Methano-3aH-inden-4-ol, octahydro-, (3aα,4α,6α,7aβ)-	$C_{10}H_{16}O$	Terpenoid	152	0.290
6	26.98	Benzene, 1,1',1'',1'''-(1,6-hexanediylidene)tetrakis-	$C_8H_{12}O_2$	Aromatic hydrocarbon	140	0.193
7	28.13	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	Phenol	206	0.672
8	28.87	1-Isobutyl-7,7-dimethyl-octahydro-isobenzofuran-3a-ol	$C_{14}H_{26}O_2$	Isobenzofuran alcohol	226	0.433
9	29.92	Phthalic acid, ethyl pentadecyl ester	$C_{25}H_{40}O_4$	Phthalic ester	404	0.282
10	30.08	Cyclooctanol, acetate	$C_{10}H_{18}O_2$	Ester	170	1.121
11	30.29	2,2-Dimethyl-1-(3-oxo-but-1-enyl)-cyclopentanecarboxaldehyde	$C_{12}H_{18}O_2$	Ketonic aldehyde	194	0.334
12	30.67	Spiro[4.5]decan-7-one, 1,8-dimethyl-4-(1-methylethyl)-	$C_{15}H_{26}O$	Cyclic ketone	222	0.647
13	30.90	Octadecane, 1-isocyanato-	$C_{19}H_{37}NO$	Alkyl isocyanate	295	0.147
14	31.08	1-Piperidinecarboxaldehyde, 2-(1-formyl-2-pyrrolidinyl)-	$C_{13}H_{20}O_2$	Alkaloid	208	0.410
15	32.08	3-Methoxy-2,4,6-trimethyl-cyclohex-2-enone	$C_{10}H_{16}O_2$	Methoxy ketone	168	0.194

16	32.31	2-Cyclohexen-1-one, 2-hydroxy-3-methyl-6-(1-methylethyl)-	$C_{10}H_{16}O_2$	Ketone	168	0.482
17	32.59	Cyclohexanone, 3-(4-hydroxybutyl)-2-methyl-	$C_{11}H_{20}O_2$	Hydroxy ketone	184	1.185
18	33.22	12-Oxatricyclo[4.4.3.0(1,6)]tridecane-3,11-dione	$C_{12}H_{16}O_3$	Diketone	208	0.240
19	33.75	1,5,9,9-Tetramethyl-spiro[3.5]nonan-5-ol	$C_{13}H_{24}O$	Cyclic tertiary alcohol	196	0.592
20	33.80	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester	$C_{14}H_{22}O_3$	Epoxy ester	238	0.713
21	34.36	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	$C_{15}H_{28}O$	Bicyclic alcohol	224	0.629
22	34.49	Ethanone, 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-	$C_{13}H_{20}O_3$	Bicyclic hydroxy diketone	224	0.237
23	34.59	(1As-(1aα,4bβ,8as)-4a,8,8-trimethyloctahydrocyclopropa(d)naphthalen-2(3H)-one	$C_{14}H_{22}O$	Tri cyclic ketone	206	0.507
24	34.75	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-	$C_{13}H_{18}O_3$	Ketone	222	0.365
25	34.89	Phthalic acid, isobutyl propyl ester	$C_{19}H_{28}O_4$	Phthalate diester	320	0.376
26	35.18	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)]-	$C_{15}H_{26}O$	Bicyclic sesqui terpenoid	222	0.438
27	35.38	1,9-Dioxacyclohexadeca-4,13-diene-2-10-dione, 7,8,15,16-tetramethyl-	$C_{18}H_{28}O_4$	Cyclic diketone	308	0.218
28	35.90	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Phthalate diester	278	5.265
29	35.98	Acetic acid, 10,11-dihydroxy-3,7,11-trimethyl-dodeca-2,6-dienyl ester	$C_{17}H_{24}O_3$	Dihydroxy ester	276	0.073
30	36.09	Propionic acid, 3-(3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-4-yl)-	$C_{17}H_{24}O_3$	Alkaloid	276	0.662

31	36.74	1-Naphthalenone, 1,2,3,4,4a,7,8,8a-octahydro-2,4a,5,8a-tetramethyl	$C_{14}H_{22}O$	Cyclic ketone	206	1.031
32	36.83	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Phthalate ester	278	3.308
33	37.14	Phthalic acid, isobutyl 2-pentyl ester	$C_{17}H_{24}O_4$	Phthalic acid diester	292	1.954
34	37.36	3-Buten-2-one, 3-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl)-	$C_{14}H_{22}O_2$	Epoxy ketone	222	0.277
35	37.65	Widdrol hydroxyether	$C_{15}H_{26}O_2$	Bicyclic epoxy alcohol	238	0.188
36	37.78	Dibutyl phthalate	$C_{16}H_{22}O_4$	Phthalicacid diester	278	3.467
37	38.01	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{32}O_2$	Phthalate ester	256	0.484
38	38.11	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Phthalate ester	278	3.206
39	38.41	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalicacid diester	460	0.973
40	38.65	Phthalic acid, isobutyl 2-pentyl ester	$C_{17}H_{24}O_4$	Phthalicacid diester	292	0.755
41	38.79	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	$C_{22}H_{34}O_4$	Phthalate ester	362	1.482
42	39.06	Phthalic acid, di(2-methylbutyl) ester	$C_{18}H_{26}O_4$	Phthalicacid diester	306	0.306
43	39.45	4-Oxazolecarboxylic acid, 4,5-dihydro-2-phenyl-, 1-methylethyl ester	$C_{13}H_{15}NO_3$	Oxazole alkaloid ester	233	0.292
44	39.56	Dibutyl phthalate	$C_{16}H_{22}O_4$	Phthalicacid diester	278	1.105
45	39.75	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	Phthalate ester	334	0.697
46	40.40	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	Fatty acid	296	1.118
47	41.28	Phthalic acid, 2-cyclohexylethyl pentyl ester	$C_{21}H_{30}O_4$	Phthalicacid diester	346	0.588
48	41.95	1-Heptatriacotanol	$C_{37}H_{76}O$	Long chain alcohol	536	0.196

49	42.82	Cedran-diol, 8S,14-	$C_{15}H_{26}O_2$	Sesqui terpenoid	238	0.203
50	43.57	Propanoic acid, 2-(benzoylamino)-3-phenyl-, methyl ester	$C_{17}H_{17}NO_3$	Benzoyl amino methyl ester	283	0.697
51	44.91	Spiro[tetrahydrofuran-2,1'-decalin], 5-methyl-2'methylene-5',5',8a'-trimethyl-	$C_{18}H_{30}O$	Tri cyclic oxygen heterocyclic	262	1.817
52	45.65	Methanesulfonic acid, 2,4,7,14-tetramethyl-6-oxo-4-vinyl-tricyclo[5.4.3.0(1,8)]tetradec-3-yl ester	$C_{21}H_{34}O_4S$	Tricyclic sulphonic acid ester	382	0.476
53	46.75	1b,4a-Epoxy-2H-cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-	$C_{22}H_{32}O_8$	Tricyclic diepoxy ester	424	5.864
54	47.06	Spiro[tetrahydrofuran-2,1'-decalin], 5-methyl-2'methylene-5',5',8a'-trimethyl-	$C_{18}H_{30}O$	Tricyclic oxygen hetrocyclic compound	262	1.643
55	47.21	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	$C_{20}H_{34}O_2$	Macrocyclic dialcohol	306	1.305
56	47.48	1-Heptatriacotanol	$C_{37}H_{76}O$	Long chain alcohol	536	0.944
57	47.68	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	Phthalate ester	390	0.787
58	48.14	2-Cyclohexen-3,6-diol-1-one, 2-[10-tetradecenoyl]-	$C_{20}H_{32}O_4$	Diterpenoid	336	7.939
59	48.44	Ent-6beta,20-epoxy-6,15-dioxo-6,7-secokauran-7,1beta-olide	$C_{20}H_{26}O_5$	Diterpenoid	346	5.652
60	48.69	Acetic acid, 4,5-dihydroxy-10,13-dimethyl-3-oxohexadecahydrocyclopenta[a]phenanthren-17-yl ester	$C_{21}H_{32}O_5$	Steroid ester	364	2.270
61	48.94	4,8,13-Duvatriene-1,3-diol	$C_{20}H_{34}O_2$	Macrocyclic dialcohol	306	0.782
62	49.14	4,8,13-Duvatriene-1,3-diol	$C_{20}H_{34}O_2$	Macrocyclic dialcohol	306	1.061

63	49.77	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-, [1S-[1α(E),4aβ,8aα]]-	$C_{20}H_{32}O_2$	Bicyclic diterpenoid carboxylic acid	304	1.411
64	49.98	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-, [1S-[1α(E),4aβ,8aα]]-	$C_{20}H_{32}O_2$	Bicyclic diterpenoid carboxylic acid	304	2.891
65	50.27	Butyl 9,12,15-octadecatrienoate	$C_{22}H_{38}O_2$	Fatty acid ester	334	0.677
66	50.65	Phthalic acid, bis(7-methyloctyl) ester	$C_{26}H_{42}O_4$	Phthalate diester	418	0.905
67	51.90	1,2-Benzenedicarboxylic acid, diisodecyl ester	$C_{28}H_{46}O_4$	Phthalate ester	446	1.373
68	52.38	12-O-Acetylingol 8-tiglate	$C_{27}H_{38}O_8$	Tricyclic diterpenoid diester	490	8.420
69	52.87	3,9;7,8-Diepoxybicyclo[4.3.0]nonane-6β-ol-2-one, 9α-acetoxy-4-isopropenyl-5-methoxycarbonyl-1-methyl-	$C_{18}H_{22}O_8$	Bicyclic diester	366	3.089
70	53.89	1-Heptatriacotanol	$C_{37}H_{76}O$	Long chain alcohol	536	4.748
71	54.84	5,7-Dihydroxy-2-(3,4,5-trimethoxyphenyl)-4-chromanone	$C_{18}H_{18}O_7$	Flavone	346	0.914
72	56.18	5,7-Dihydroxy-2-(3,4,5-trimethoxyphenyl)-4-chromanone	$C_{18}H_{18}O_7$	Flavone	346	0.672
73	61.47	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,6,7-trimethoxy-	$C_{20}H_{20}O_8$	Flavon derivative	388	1.863

GC-MS analysis of *V. negundo* toxic fraction

In the toxic fraction of *V. negundo,* 49 constituents were identified and the retention time, name, molecular formula, chemical class, molecular weight and area percentage of those components were determined and presented in Table V. 5. In this case, the prominent component identified is 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester with an area of 8.525 % which is considered as a phthalate ester. The second identified constituent with highest peak was again the same compound phthalic acid, hexadecyl pentyl ester. Its area detected was 6.619 %. It was followed by compounds such as dibutyl phthalate (6.393 %), 1, 2-Benzenedicarboxylic acid, bis (2- methylpropyl) ester (5.522 %) and phthalate diester (4.918 %) in the 3rd, 4th and 5th positions respectively. These compounds include under the category of phthalate esters. Furthermore, maximum number of peaks was noticed for phthalate esters (22 peaks) with varying retention time and area percentages. Besides these compounds, GC-MS analysis of the toxic fraction of *V. negundo* revealed the presence of constituents like phenolics, fatty acid esters and some other esters, ketones, alkenes, sesquiterpenoids, nitrogen heterocyclic compounds, alcoholic compounds, azulene and morphinone derivatives in varying quantities (Table V. 5).

Table V. 5. The phytochemical components identified in the toxic fraction of *Vitex negundo*

Sl. No.	R.T Value	Name of the compound	Molecular formula	Chemical Class	Molecular weight	Area %
1	22.47	Cyclopentanecarboxylic acid, 4-tridecyl ester	$C_{19}H_{36}O_2$	Long chain alcohol ester	296	0.499
2	24.078	Eugenol	$C_{10}H_{12}O_2$	Phenol	164	0.847
3	28.12	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	2,4-ditertiary butyl phenol	206	0.967
4	29.92	Diethyl Phthalate	$C_{12}H_{14}O_4$	Phthalate diester	222	0.805
5	30.10	trans-5-Isopropyl-6,7-epoxy-8-hydroxy-8-methylnonan-2-one	$C_{13}H_{24}O_3$	Epoxy hydroxy ketone	228	0.512
6	31.01	6-(3-Hydroxy-but-1-enyl)-1,5,5-trimethyl-7-oxabicyclo[4.1.0]heptan-2-ol	$C_{13}H_{22}O_3$	Epoxy dialcohol	226	0.345
7	33.03	Azulene, 1,4-dimethyl-7-(1-methylethyl)-	$C_{15}H_{18}$	Azulene derivative	198	0.422
8	33.30	1-Cyclohexanone, 2-methyl-2-(3-methyl-2-oxobutyl)	$C_{12}H_{20}O_2$	Diketone	196	0.261
9	34.37	3-Octadecene, (E)-	$C_{18}H_{36}$	Long chain alkene	252	0.733
10	34.88	Phthalic acid, isobutyl propyl ester	$C_{15}H_{20}O_4$	Phthalate ester	264	0.752
11	35.08	Isoaromadendrene epoxide	$C_{15}H_{24}O$	Sesqui terpenoid	220	0.564
12	35.24	γ-Gurjunenepoxide-(1)	$C_{15}H_{24}O$	Sesqui terpenoid	220	0.931
13	35.45	{2-[1-(3,3-Dimethoxy-propyl)-vinyl]-5-methyl-cyclopentyl}-methanol	$C_{14}H_{26}O_3$	Diethoxy alcohol	242	0.398
14	35.90	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Phthalate ester	278	8.525
15	36.52	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	$C_{20}H_{30}O_4$	Phthalate ester	334	0.483
16	36.83	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)	$C_{16}H_{22}O_4$	Phthalate ester	278	5.522

		ester				
17	37.13	Phthalic acid, isobutyl 2-pentyl ester	$C_{17}H_{24}O_4$	Phthalate ester	292	3.574
18	37.78	Dibutyl phthalate	$C_{16}H_{22}O_4$	Phthalate ester	278	6.393
19	38.12	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Phthalate ester	278	6.619
20	38.42	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalate ester	460	1.860
21	38.65	Phthalic acid, 2-methylbutyl pentyl ester	$C_{18}H_{26}O_4$	Phthalate ester	306	1.157
22	38.77	Phthalic acid, butyl isohexyl ester	$C_{18}H_{26}O_4$	Phthalate ester	306	1.442
23	39.57	Dibutyl phthalate	$C_{16}H_{22}O_4$	Phthalate ester	278	2.447
24	39.76	Phthalic acid, 6-ethyl-3-octyl butyl ester	$C_{20}H_{30}O_4$	Phthalate ester	334	1.528
25	39.87	Phthalic acid, pentyl 2-pentyl ester	$C_{18}H_{26}O_4$	Phthalate ester	306	1.536
26	40.28	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	Fattyacid ester	294	0.774
27	40.42	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Fattyacid ester	296	1.620
28	40.53	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalate ester	460	0.951
29	40.69	Dibutyl phthalate	$C_{16}H_{22}O_4$	Phthalate ester	278	0.882
30	40.90	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	Fattyacid ester	298	0.397
31	41.28	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalate ester	460	1.316
32	42. 09	1-Eicosene	$C_{20}H_{40}$	Long chain alkene	280	0.785
33	43.56	Propanoic acid, 2-(benzoylamino)-3-phenyl-, methyl ester	$C_{17}H_{17}NO_3$	Aromatic benzyl amino ester	283	0.393
34	43.90	3-Pentyl-4,5,6,7-tetrahydro-1H-indazole	$C_{12}H_{20}N_2$	Bicyclic nitrogen heterocyclic compound	192	0.890
35	44.87	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	Long chain aliphatic alcohol	280	1.838
36	45.13	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-	$C_{20}H_{30}O$	Tricyclic alcohol	286	2.823

		octahydro-1,4a-dimethyl-7-(1-methylethyl)-				
37	45.48	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalate ester	460	1.062
38	45.67	Palustric acid	$C_{20}H_{30}O_2$	Carboxylic acid (Terpenoid)	302	3.206
39	45.86	Phthalic acid, decyl 2-pentyl ester	$C_{23}H_{36}O_4$	Phthalate ester	376	4.918
40	46.94	Phenol, 2,4-bis(1-phenylethyl)-	$C_{22}H_{22}O$	2,4 dibenzyl phenol	302	1.143
41	47.10	Phthalic acid, hexadecyl pentyl ester	$C_{29}H_{48}O_4$	Phthalate ester	460	3.909
42	48.35	6-Hydroxy-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-1-phenanthrenemethanol,	$C_{20}H_{30}O_2$	Tricyclic phenolic alcohol	302	3.265
43	49.33	Pregn-4-ene-3,20-dione, 16,17-epoxy-, (16α)-	$C_{21}H_{28}O_3$	Steroid	328	4.158
44	49.46	1-Heptatriacotanol	$C_{37}H_{76}O$	Long chain alcohol	536	1.352
45	49.55	6-Hydroxy-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-1-phenanthrenemethanol, (1α, 4aβ, 10a.alpha	$C_{20}H_{30}O_2$	Tricyclic phenolic alcohol	302	1.426
46	50.65	Phthalic acid, bis(7-methyloctyl) ester	$C_{26}H_{42}O_4$	Phthalate ester	418	2.385
47	51.87	1,2-Benzenedicarboxylic acid, diisodecyl ester	$C_{28}H_{46}O_4$	Phthalate ester	446	2.925
48	52.15	7,8-Dihydro-14-hydroxymorphinone	$C_{17}H_{19}NO_4$	Alkaloid	301	0.585
49	54.67	Cannabinol ester	$C_{23}H_{25}F_3O_3$	Phenolic ester	406	0.296

5.4. Discussion

HPTLC studies revealed the presence of various chemical classes of compounds, among them important constituent compounds identified were alkaloids, phenolics and terpenes. High performance thin layer chromatography of chloroform fractions exhibited large number of coloured bands along the entire track of running for alkaloids, phenolics and terpenoids of both plant extracts. For each type of compounds, spraying with various chemical reagents caused the development of characteristic colours for specific compounds and was used to identify the components. The discrepancy in the intensity of colours of the bands also designates significant variations in concentration of constituents present in the extract. HPTLC analysis of *H. suaveolens* and *V. negundo* toxic fractions revealed the presence of several toxic components in the two extracts (Plate V.1, V.2 and V.3). Chromatographic analysis for the identification of compounds in the fractions disclosed the chemical nature of active compounds such as alkaloids, phenolics and terpenoids present in both the plants and these compounds were supposed to be inducing toxicity in *O. exvinacea* larva. The presence of these secondary metabolites with insecticidal properties were isolated by many researchers from the various parts of these plants and were found to be corroborative with the present study results.

There are several evidences to demonstrate that the leaves of *H. suaveolens* possess many biologically active compounds such as essential oils, alkaloids, flavonoids, phenolics, saponins, terpenes and sterols (Prince *et al*., 2013). Moreover, they reported that the plant contains essential oils as chief constituents mainly in leaves, shoots and seeds. The presence of these vital compounds in the present study had been confirmed with the reports of many researchers (Mbatchou *et al*., 2010; Prasanna and Koppula, 2012). They revealed that different solvent extracts such as petroleum ether, chloroform,

methanol, ethanol, n-hexane and water extraction of various parts of *H. suaveolens* showed the presence of phytochemicals such as alkaloids, flavonoids, terpenoids and tannins. Furthermore, the presence of these active insecticidal compounds have been established by Prasanna and Koppula (2012) in which the leaves contained alkaloids (2.80±0.28 %), flavonoids (1.90±0.14 %), and tannins (5.50±0.074 %). Many researchers carried out phytochemical studies with *H. suaveolens* and they reported the presence of steroids, alkaloids, phenolics, tannins, terpenoids, saponins and cardiac glycosides (Pachkore and Dhale, 2011; Ayange-kaa *et al*., 2015).

There are many testimonies regarding the demonstration of phytochemical constituents present in *V. negundo* leaf extract to authenticate the present study results. In addition to leaves, the secondary metabolites are distributed throughout the plant which includes alkaloids, phenolics, flavonoids, glycosidic irridoids, tannins and terpenes (Rabeta and An-Nabil, 2013). It has been previously ascertained the presence of insecticidal compounds such as steroids, phenolic compounds, triterpenoids, saponins and tannins in the chloroform extract of *V. negundo* (Sahayaraj and Ravi, 2008). Moreover, the methanolic extract of *V. negundo* showed compounds like alkaloids, saponins and flavonoids through TLC analysis (Khan *et al*., 2012). Similarly, many reported comparable results showing the presence of similar active compounds present in both *H. suaveolens* and *V. negundo* (Meena *et al*., 2010; Vishwanathan and Basavaraju, 2010; Singh *et al*., 2011).

Generally, alkaloids, phenolic compounds and terpenoids are synthesized in plants or otherwise increase their synthesis in response to ecological and physiological stress conditions caused by herbivore attack. In the present study these vital compounds including alkaloids, phenolics and terpenoids were present in both *H. suaveolens* and *V. negundo* in varying concentrations. From HPTLC studies, it has been found that the toxic

chloroform fraction separated from methanolic extract of both *H. suaveolens* and *V. negundo* comprises a combination of compounds instead of a single compound and so, it is assumed that the insecticidal activity exhibited by these fractions are due to the cumulative effect of all compounds in composite. The larvicidal activity induced by both plant fractions might be due to the synergistic or independent participation of these compounds in causing toxicity against *O. exvinacea* larva. Since these plants are having important biologically active compounds like alkaloids, phenolics and terpenoids which have insecticidal activity they can also be included in the insect pest management programme.

In the present study, GC-MS analysis of the fraction of *H. suaveolens* showed that a total of 73 components are present in this sample. Whereas for *V. negundo*, 49 constituents were reported with varying amounts. Biological assay, using the chloroform fractions separated from methanolic extract of both *H. suaveolens* and *V. negundo* showed that both fractions are having the larvicidal property against *O. exvinacea* larva after topical application of these fractions. This may be due to the insecticidal property of the volatile components along with other insecticidal components present in these fractions.

Hyptis suaveolens possess many phytochemical constituents like alkaloids, terpenes, phenols, tannins and steroids with insecticidal properties and thus the plant has got increasing attention (Peerzada, 1997). Moreover, *V. negundo* also possess a number of phytochemicals like alkaloids, flavonoids, saponins, phenols, terpenoids and anthraquinones (Gangwar *et al.*, 2015; Kamalakannan *et al.*, 2015). Hence, in the present study toxicity of these plant extracts is due to the active phytochemical constituents present in the leaves of both *H. suaveolens* and *V. negundo*.

GC-MS analysis possibly will support to explore the biological activity of plant phytochemicals with the proper understanding of the chemical nature and structure of the constituent present in plant extracts. Toxic fractions of both botanical extracts constitute phenolic compounds, fatty acid, steroid and other esters, ketones, sesquiterpenoids, heterocyclic compounds, flavones, alcoholic compounds and aromatic compounds in general. Cumulative or synergistic activities of all these components of these plant fractions are assuring the toxic effects including larvicidal effects against *O. exvinacea*. Thus, it can be concluded that secondary metabolites present in the botanicals function cumulatively and will possibly bring about enhanced toxicity than the effects caused by the action of individual constituents. Anyhow, further studies are required for the proper understanding of the toxic action played by the phytochemical components present in these plant fractions. Moreover, for recommendation of these ecofriedly plant toxicants, in small or large scale field use for the control of this pest, practically needs more collaborative efforts.

CHAPTER VI

HISTOPATHOLOGICAL AND MORPHOMETRIC STUDIES IN THE MIDGUT TISSUE OF *ORTHAGA EXVINACEA* HAMPSON

6.1. Introduction

Generally, the digestive system of insects serves as the main physicochemical barriers towards toxic materials and pathogenic agents. If the toxic materials such as pesticides are taken by insects upon feeding they may cross these barriers and are supposed to damage the cellular architecture. Studies related with changes at the cellular and tissue level can be used as a direct measure of the physiological state of an organism in toxicity studies (Meyers and Hendricks, 1985). Histopathological changes is an indication of alterations in physiological or biochemical nature, which in turn bring about some changes in the metabolic functions and so histopathological studies considered to be reliable biomarkers of stress conditions. These histopathological changes occurred to the cells and tissues can be distinguished at two levels. The fine structure of the cells and tissues for normal and disrupted tissue structure have been made possible with light microscopic studies and electron microscopes were employed to study the ultrastructure of cell cytoplasm, organelles and membranes.

Histopathological studies possibly reveal the cellular disorganization and also associated morphometric changes of cell structure. Morphometric changes are significant in histopathological studies as it indicates the deformation of normal cell structure. Generally pesticide exposure brings about variations in the cellular morphometry of susceptible organisms. According to (Prasad and Roy, 2011), morphometric variations are phenotypic and there is no genetic relationship for such deformations. Hence,

morphometric studies offer an insight for the accurate understanding of the intensity of cytotoxicity.

Insect alimentary canal is differentiated into three main regions that include foregut (ectodermal), midgut (endodermal) and hindgut (ectodermal). In which, midgut is the major site for digestion and absorption of nutrients and is an important tissue where major structural changes occur when exposed to toxic substances due to physiological stress (Genta *et al*., 2006). Midgut is considered to be one of the most important areas in insect physiology for toxicity studies because alterations on it affect the growth and development of insects (Nisbet, 2000).

Generally, insect gut is made up of a single layer of epithelial cells, bounded by a basement membrane and striated muscle. The epithelial layer of the lepidopteran midgut is composed of four kinds of cells, i.e., columnar cells with a function of absorption and enzyme secretion, goblet cells for ionic homeostasis, endocrine cells with endocrine function and the regenerative cells for renewal of epithelium (Pinheiro *et al*., 2008). The midgut epithelial layer is separated from the gut lumen by a non-cellular structure called the peritrophic envelope or peritrophic membrane. The peritrophic membrane in the digestive tract has a fundamental role in the protection of the midgut from mechanical damage and it protect against toxic materials to the insect (Terra, 2001).

The present investigation was made to study the histopathological and morphometric changes occurred in the midgut tissue of *O. exvinacea* after the treatment of *H. suaveolens* and *V. negundo.* This study include,

a. Light microscopic studies

b. Morphometric studies

c. Ultrastructural studies using Transmission electron microscopy

6.2. Materials and methods

The materials and methods adopted for light microscopic and morphometric studies were described in section 3.2.6 and that for ultrastructural studies were given in section 3.2.7.

6.3. Results

6.3. a. Light microscopic studies

The histopathological changes noticed in the midgut tissue of *O. exvinacea* for the specified concentrations (1 %, 2 %, 3 %, 4 % and 5 %) of both *H. suaveolens* and *V. negundo* along with control midgut tissue were presented in plates VI.1 to VI.5.

➢ **Structure of Normal Midgut**

The transverse section of the midgut of *O. exvinacea* sixth instar larva showed normal cellular architecture of the insect midgut epithelium. Midgut epithelium consists of a single layer of epithelium placed on a basement membrane surrounded by an inner layer of circular muscles and an outer longitudinal muscle layer. The epithelium consists of four types of cells with distinct functions. They are columnar cells (absorption and secretion), regenerative cells (replacement and renewal of injured cells), secretory cells (endocrine) and goblet cells (transport of potassium and calcium ions) (Plate VI. 1. Fig. 1. b, c). 90% of the epithelium is made up of columnar cells, which are relatively long and form a regular and compact wall. An oval nucleus is located in the central part of each columnar cell with clear cytoplasm. The nucleus stains deeply and contains large number of small spherical chromatin granules (Plate VI. 1. Fig. 1.c, d). Regenerative cells are located basally in the form of group of cells without distinct cell boundaries and with small spherical nuclei containing sparse chromatin surrounded by a thin layer of

clear cytoplasm. In addition to the columnar and regenerative cells, pear-shaped calyciform or goblet cells are found interspersed among the columnar epithelial cells. The goblet cell has a round basal region which tapers into a narrow anterior region (Plate VI. 1. Fig. 1. c). The apical membrane of the cells is invaginated to form a large cavity with a basally located oval nucleus surrounded by granular cytoplasm along with numerous cytoplasmic extensions. The secretory cells are placed singly or in groups at the base of the columnar or goblet cells. The expanded apical surface of each columnar cell bordering the gut lumen is covered with microvilli which form the tight structure called brush border. Inner to the epithelium is a thin peritrophic membrane. It extends throughout the entire length of the midgut, as a transparent tube which encloses the food particles.

- **Observations for treatment with botanicals**

The histopathological observations for different concentrations of both *H. suaveolens* and *V. negundo* were given in Plates VI. 2 to VI. 5.

- **Observations of *Hyptis suaveolens* treated tissue**

1% *H. suaveolens* treatment: Transverse section of larval midgut treated with 1% concentration of *Hyptis* showed normal architecture of gut epithelium with highly active brush border, but the peritrophic membrane found to be totally disrupted (Plate VI. 2 .Fig. 2. a, b).

2% *H. suaveolens* treatment: The observed cytotoxic effects include tendency for vacuolization of columnar cell cytoplasm and also elongation of the nucleus of columnar cells with highly active brush border and it showed the presence of numerous secretory vesicles. Apical region of columnar cells seemed to be congested and there is initiation of sloughing off (Plate VI. 2. Fig. 2. c, d).

3% *H. suaveolens* treatment: Noticed histopathological changes were sloughed off brush border along with columnar cell and its nuclei, thus gut luminal area decrease occurred. Columnar cells and its nuclei found to be elongated, overlapped and congested at some regions along with numerous secretory vesicles (Plate VI. 2. Fig. 2. e, f).

4% *H. suaveolens* treatment: The cytotoxic effects noticed were prominent vacuolization in the epithelial layer along with apical movement of nucleus and also showed the presence of numerous secretory vesicles. Elongated upper part of the columnar cells found to be fused enclosing highly overlapped and congested nuclei with sloughing off of the brush border (Plate VI. 3. Fig. 3. a, b).

5% *H. suaveolens* treatment: The histopathological changes noticed include apical movement of nucleus of columnar cells and a tufty apical arrangement of the brush border with dense secretory activity. Apically, most of the columnar cell nuclei seemed to be fused (Plate VI. 3. Fig. 3. c, d).

- **Observations of *Vitex negundo* treated tissue**

1% *V. negundo* treatment: Transverse section of the larval midgut treated with 1% concentration showed normal architecture of the gut epithelium. The peritrophic membrane was found to be intact (Plate VI. 4. Fig. 4. a, b).

2% *V. negundo* treatment: The observed histopathological changes include elongation of epithelial cells, initiation of vacuolization, goblet cells were seemed to be large in size due to swelling, peritrophic membrane distorted and it pertains only in some areas (Plate VI. 4. Fig. 4. c, d).

3% *V. negundo* treatment: The cytotoxic effects noticed at this concentration were prominent elongation of epithelial cells and the apical movement of its nuclei, epithelial vacuolization in the cytoplasmic area and excessive swelling of goblet cells. Columnar cells were found to be highly congested and overlapped at the apical part towards the luminal area (Plate VI. 4. Fig. 4. e, f).

4% *V. negundo* treatment: Showed numerous histopathological effects in the epithelial layer such as appearance of alternative folds and infolds in the epithelial lining, overlapping of epithelial cells and detachment of epithelial layer from basement membrane. But, basement membrane was unaffected. Alternative folds and infolds in the epithelial layer overlap. In addition, columnar cells were noticed to be extremely vacuolated. Columnar cells with brush border were seemed to be sloughed off at some regions (Plate VI. 5. Fig. 5. a, b).

5% *V. negundo* treatment: Extreme cytotoxic effects noticed at this concentration include complete detachment of the epithelial layer from the basement membrane, a varied fashion of epithelial sloughing off with columnar cells forming large vacuoles with in the lumen such that luminal area found to be decreased. Excessively elongated columnar cells lost their identity forming large vacuoles in the lumen and the elongated nuclei moved towards the lumen (Plate VI. 5. Fig. 5. c, d).

6.3. b. Morphometric studies

The cytological parameters selected for the present study include height and width of columnar cell, columnar cell nucleus, goblet cells along with their nuclei in the epithelial layer. The morphological changes of these cell types observed from the light microscopic studies of both treatments were measured and presented in Table VI. 1 and Table VI. 2.

The morphometric calibrations for the treated tissues were compared with the control tissue measurements. The morphometric data of the histological sections showed significant differences among treatments and control. In the control midgut tissue, the columnar cells showed an average normal size of 759.6 µm × 75.24 µm and their nuclei size measured 97.07 µm × 54.78 µm (Table VI. 1). In the same tissue, goblet cells exhibited a size of 191.9 µm × 122.2 µm and their nuclei have 98 µm × 54.6 µm measurements.

Columnar cells and their nuclei possess varied increasing heights in the treatment of *H. suaveolens* whereas their width was found to be decreasing in all applied concentrations when compared to control measurements. At the same time, goblet cells with their nuclei were found to be enlarged in their size (both height and width) with respect to control cell size measurements. Unlike columnar cells, goblet cells were found to exhibit maximum height and width at high concentrations of the treatments applied (Table VI.1.). The number of goblet cells (Plate VI. 2. Fig. 2. b, d.) and their size along with their nuclei (Table VI. 1) were greater in the *H. suaveolens* treated larvae. In this botanical treatment, high percentage concentrations showed remarkable morphometric changes in which, besides causing elongation some degree of sloughing off also occurred at the apex of the epithelial cells and so there is no gradual increase or decrease in the height and width of the cells with their nuclei. After 3 % treatment, it was observed that there occurred gradual decrease in the height of columnar cells due to sloughing off of the epithelial layer. However, the morphometric changes noticed were found to be highly significant at 1 % level of significance in accordance with control cell sizes.

Table VI. 1. Showing the morphometric changes of the midgut epithelial layer of *O. exvinacea* caused by the treatment of *H. suaveolens*.

Treatments	**Columnar cell (in µm)**		**Columnar cell nucleus (in µm)**		**Goblet cell (in µm)**		**Goblet cell nucleus (in µm)**	
	Height	**Width**	**height**	**width**	**height**	**width**	**height**	**width**
control	759.6±8.64	75.24±1.15	97.07±0.33	54.78±0.65	191.9±19.82	122.2±6.9	98±2.94	54.6±0.91
1 %	2280.6±19.23	67.06±2.05	284±2.12	47.8±0.85	792.96±21.1	421.4±22.1	209.2±2.65	165.7±6.22
2 %	2490.5±21.23	58.08±1.47	297.8±0.7	46.58±1.34	1131±28.1	387.4±21.9	191.4±8.84	115.1±11.4
3 %	2092.8±26	64.45±2.07	319.4±1.75	43.66±1.29	903.3±17	423.4±16.3	239.1±9.59	133.3±9.76
4 %	1541.7±39.3	64.46±0.63	168.4±10.14	43.99±1.5	883.5±41.1	500.4±44.2	253.1±13.86	144.3±10.5
5 %	1533.4±12.96	61.55±2.21	223.4±5.58	42.79±2.75	1252±23.9	453.6±31.98	171.7±7.9	116.1±3.17
F- value	734.75	11.75	312.3	8.18	195.3	25.3	42.18	22.31
P- value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Measurements are expressed as mean ± S.E. Level of significance, $P < 0.01$.

Table VI. 2. Showing the morphometric changes of the midgut epithelial layer of *O. exvinacea* caused by the treatment of *V. negundo*.

Treatments	Columnar cell (in µm)		Columnar cell nucleus (in µm)		Goblet cell (in µm)		Goblet cell nucleus (in µm)	
	Height	**Width**	**height**	**width**	**height**	**width**	**height**	**width**
control	759.6±8.64	75.24±1.15	97.07±0.33	54.78±0.65	191.9±19.82	122.2±6.9	98±2.94	54.6±0.91
1 %	1570.8±5.09	80.02±0.99	271.2±10.2	49.08±0.97	329.3±13.8	161.6±3.6	164.2±6.64	81.48±5.11
2 %	1785.4±19.9	71.34±1.67	250.4±13.7	51.01±0.72	681.7±20.2	395.1±18.3	173.2±2.82	88.7±3.13
3 %	1028.7±10.4	50.35±1.6	284.4±3.28	35.07±0.87	315.4±16.5	133.6±3.96	126.8±4.36	83.67±3.4
4 %	2311.7±19.4	66.16±0.92	232.5±7.38	42.9±1.7	547.1±12.9	303.8±15.15	160.3±13.8	100±3.54
5 %			279.2±3.07	41.2±2.97	884.1±38.1	404.6±40.5	196.2±3.8	116.5±5.02
F- value	1931	76.44	81.52	21.88	141.6	44.97	25.88	29.75
P- value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Measurements are expressed as mean ± S.E. Level of significance, $P < 0.01$.

In the case of treatment with *V. negundo* elongation was maximum at higher applied concentration (4 %) (Table VI. 2). In addition, the 5 % treatment caused the complete detachment of epithelial cells from the basement membrane and so the measurement of epithelial cell size for 5 % treatment could not be carried out. In the 1 % treatment, it showed a slight increase followed with decrease in width of the cells in comparison to control. At 2 % treatment, eventhough there occurred slight increase in height of columnar cells, their width seem to be decreased. Columnar cell nucleus was found to have significant morphometric changes in which the height and width of the nucleus exhibited variation due to elongation with respect to control measurements. But goblet cells were showing more or less similar morphometric changes as noticed in the case of *H. suaveolens* treatment. i.e., the size of both goblet cell and nucleus in terms of height and width seemed to be increased with respect to control. Moreover, the maximum increase was noticed in the highest concentration (5 %) of the treatment percentages (Table VI. 2). The morphometric changes noticed were found to be highly significant at 1 %.

6.3.c. Ultrastructural studies

According to the intensity of histopathological changes of midgut tissue noticed in the light microscopy, the most effective concentration in bringing about cytotoxicity was found to be 5 % of both *H. suaveolens* and *V. negundo*. The ultrastructural changes of the midgut tissue of control and treated larvae were illustrated using transmission electron microscopy and were presented in Plates VI. 6 to VI. 8 and Plates VI. 9 to VI. 14 respectively.

- **Ultrastructural details of normal midgut tissue**

The electron microscopic studies on the midgut tissue of control sixth instar larvae showed normal histological structure of insect midgut consisting of an epithelial layer (comprised of columnar cells, regenerative cells, goblet cells and secretory cells which are not distinctly observed) which rests on a

basement membrane and outer to it a muscle layer composed of an inner circular and outer longitudinal muscle layer (Plate VI. 6. Fig. 1. a, b). General structure of outer muscle layers consists of circular muscle layer (Plate VI. 6. Fig. 1. c, d) and longitudinal muscle layers showing the presence of thick arrangements of actin and myosin filaments (Plate VI. 6. Fig. 1. e, f), which were found to be richly supplied with mitochondria. Epithelial layer was found to be consisting of numerous columnar cells (Plate VI. 7. Fig. 2. a.). The nuclei of columnar cells were enclosing condensed chromatin granules and are situated in the basal mid region of columnar epithelium (Plate VI. 7. Fig. 2. b.). Columnar epithelial cytoplasm also showed the presence of numerous mitochondria, rough endoplamic reticulum (RER) and secretory vesicles in the apical part (Plate VI. 7. Fig. 2. c.). Numerous smooth endoplasmic reticulum (SER) along with mitochondria and secretory vesicles were also present around the columnar cell nucleus (Plate VI. 7. Fig. 2. d.). Apical part of columnar cells exhibited various finger like projections, called microvilli which forms the brush border (Plate VI. 7. Fig. 2. e, f). Flask shaped goblet cells were present towards the basal region of the epithelium with a basally located oval nucleus (Plate VI. 8. Fig. 3. a). Inner cavity of goblet cells were found to be richly supplied with microvilli rich in mitochondria (Plate VI. 8. Fig. 3. b). Towards the basal part of columnar epithelial cells secretory cell with its secretions as secretory granules were also seen (Plate VI. 8. Fig. 3. c). Inner to the brush border, a protective layer of midgut is present termed as peritrophic membrane which separates the epithelial lining from direct contact with luminal food particles (Plate VI. 8. Fig. 3. d).

While feeding the treated food material, lot of degenerative changes has been occurred in the linings of the gut. The ultrastructural changes occurred for both plant treatments were given in Plate VI. 9 to VI. 14.

➢ Ultrastructural changes with *H. suaveolens* treatment

The ultrastructural changes noticed in the normal histomorphology of the midgut tissue of the sixth instar larvae upon treatment with 5% *H. suaveolens* were primarily located in basal and apical regions of columnar epithelium (Plate VI. 9. Fig. 4. a). Extensive vacuolization has been the prominent feature noticed in the basal region of the epithelial cells and thereby detachment of epithelial cell layer from the basement membrane were noticed (Plate VI. 9. Fig. 4. b). Large vacuoles were formed in between basement membrane and epithelial layer. Circular muscle layer also was found to be exhibiting very compactly packed myofibrils with very few mitochondria in the sarcoplasm (Plate VI. 9. Fig. 4. c, d). Longitudinal muscle layer had been detached from the circular muscle layer (Plate VI. 9. Fig. 4. b) and these longitudinal muscle fibres were exhibiting compact nature of myofibrils (Plate VI. 9. Fig. 4. e, f). Epithelial layer showed numerous goblet cells along with columnar cells with their nuclei intact indicating more secretory nature (Plate VI. 10. Fig. 5. a, b). Epithelial cells were found to be extensively elongated along with goblet cell (Plate VI. 10. Fig. 5. c). Goblet cells were found to be richly supplied with RER, mitochondria, secretory vesicles and abundant microvilli (Plate VI. 10. Fig. 5. d). Columnar epithelial cell cytoplasm also found to be richly supplied with cell organelles and abundant secretory vesicles (Plate VI. 10. Fig. 5. e). Apical part of columnar cell exhibited the presence of SER and mitochondria (Plate VI. 10. Fig. 5. f). Vacuolization was also noticed in the secretory cell cytoplasm along with columnar cell cytoplasm (Plate VI. 11. Fig. 6. a). Apical part of the epithelial cell showed blebbing of plasma membrane and numerous vacuoles (Plate VI. 11. Fig. 6. b). Brush border seemed to be highly secretory in nature (Plate VI. 11. Fig. 6. b). Columnar apical bleb was found to be richly supplied with SER and very few mitochondria (Plate VI. 11. Fig. 6. c, d). Peritrophic membrane was found to be totally disrupted.

- **Ultrastructural changes with *V. negundo* treatment**

The cytotoxic effects observed in the normal histological architecture of the midgut tissue of sixth instar larva with the treatment of 5% *V. negundo* were located throughout columnar epithelium and the muscle layers (Plate VI. 12. Fig. 7. a). Columnar epithelial cells along with their nuclei found to be displaced towards the luminal area due to apical migratory movement (Plate VI. 12. Fig. 7. a). Columnar cell layer was found to be completely detached from the basement membrane due to excessive vacuolization (Plate VI. 12. Fig. 7. b). Large vacuoles were formed in between basement membrane and epithelial layer. Circular muscle layer seemed to be reduced in its thickness and the sarcoplasm possess little or reduced cell organelles (Plate VI. 12. Fig. 7. c, d). Longitudinal muscle layer retains its structural integrity with the presence of actin and myosin filaments (Plate VI. 12. Fig. 7. e, f) but it has got completely detached from the circular muscle layer (Plate VI. 12. Fig. 7. a). Extensive vacuolization has been noticed in the epithelial layer, with large vacuoles inside the cytoplasmic area of columnar cells surrounding the nucleus (Plate VI. 13. Fig. 8. a, b). Columnar epithelial cells were found to be extensively elongated with their nuclei and also columnar cytoplasm possessed many vacuoles (Plate VI. 13. Fig. 8. c, d). Apical region and basal part of columnar cells were having very few cell organelles when compared to control. RER and secretory vesicles were very few in number in the columnar epithelium (Plate VI. 13. Fig. 8. e, f). Goblet cell was found to be completely degenerated with disrupted microvilli and shrunken mitochondria and RER totally absent (Plate VI. 14. Fig. 9. a, b). Apical part of columnar epithelium showed reduced cell organelles and degenerated microvilli (Plate VI. 14. Fig. 9. c, d). There are no remnants of peritrophic membrane.

6.4. Discussion

Feeding of the mango leaves treated with 5 % extract of both *H. suaveolens* and *V. negundo* caused much degenerative histopathological changes to the sixth instar larvae of *O. exvinacea.* While feeding treated leaves, the active principles present in the extracts affects the normal histological architecture of the gut. The general cytotoxic effects caused by the plant extracts include elongation and vacuolation of epithelial cells, detachment of the epithelial cells from basement membrane, epithelial sloughing off, thinning of the circular muscles and histolysation of epithelial cells which resulted in complete collapse of the gut epithelium. These pathological symptoms of treatment effects on the midgut may confirm the efficacy of botanicals in use as larvicide. These histopathological conditions effected with botanicals may be supported in the light of many acceptable views suggested by various researchers.

Histopathological studies on the gut resulted by the effect of various botanical pesticides have been undertaken by many scientists in insects of different orders including Lepidoptera (Woke, 1940) and revealed that administration of botanicals produced marked cytotoxic changes. The results of the present study correlated with the findings of Rawi *et al.* (2011) who noticed vacuolization and destruction of epithelial cells and their boundaries in the larval midgut of *S. littoralis* when treated with *A. indica* and *C. colocynthis* extracts, in which vacuolization resulted with *C. colocynthis* extracts also led to detachment of the columnar cell layer from the basement membrane. The occurrence of vacuoles was best explained by Salkeld (1951) stating that vacuoles may occur either as a result of cell elongation or due to excessive fat droplets which dissolve during fixation and dehydration process.

Similar cytotoxic effects were reported by several scientists. Packiam *et al.* (2013) noticed significant changes in the midgut of *H. armigera*

including disorganisation of peritrophic membrane and epithelium along with cytoplasmic vacuolization induced with the neem formulation, Ponneem. Similarly, Cottee (1984) reported histological changes like necrosis of cells, vacuolization of cytoplasm, reduction in the size of the nuclei and regeneration of cells in *S. gregaria* and *L. migratoria* when treated with botanical pesticides. Barbeta *et al.* (2008) and Correia *et al.* (2009) stated that the displacement of epithelial cells from the basal lamina and hypertrophy reduced the digestive capacity suggesting that these changes might be the result of cytoplasmic vacuolation, endoplasmic reticulum fragmentation and plasma membrane disruption, which was earlier reported in the midgut cells of *S. frugiperda* and some other lepidopterans.

In the present study, the botanical treatments affected the morphometry of the midgut epithelial layer significantly. Since the major affected part is the epithelial layer in the midgut tissue, the cytological parameters selected for morphometric studies were limited to the changes in columnar epithelial cells with nuclei and goblet cells with their nuclei. It is due to excessive elongation and related sloughing off, the epithelial cells were showing increased height and decreased width, similarly goblet cells are also enlarged in their size with increase in height and width of the same. Elongation of the cell also resulted in extended nuclear size of columnar cell types. But in the case of goblet cells, the nucleus was not very much elongated instead it had changed its size significantly when compared to control.

Many researchers reported morphometric changes associated with histopathological changes and presented various acceptable reasons for these cytotoxic changes. In the midgut tissue of *H. pseudospretella*, the differences shown in the size and activity of midgut columnar and goblet cells depend on the food quality (Gerard, 2002). The studies of Perić-Mataruga *et al.* (2006) recorded similar results to the present study in which significant morphometric changes in the size of columnar and goblet cells were noticed when fed with different food materials such as *Quercus cerris* leaves and

Robinia pseudoacacia leaves. It was also noticed that greater number and height of goblet cells and their nuclei were present in *Q. cerris* leaves fed larvae when compared with *R. pseudoacacia* leaves fed larvae. The feeding of unsuitable plant materials by insects led to midgut epithelial alterations in which the form and size of the nuclei change and cell height was also affected (Triseleva, 1995). Many scientists proved that differences in the feeding habits showed much correlation with the extent of morphological variations brought about in the digestive tract of insects (Lebrun, 1985; Caetano, 1988). These findings were complementary to the present study.

A more complementary result was reported by Ranjini and Nambiar (2015) in which significant morphometric changes in the height, width and diameter of the nucleus of columnar cells in the midgut tissue of *O. exvinacea* was noticed when treated with *C. infortunatum* and *E. odoratum*. It is assumed that the active principles present in both botanicals might have caused the histopathological and morphometric variations in epithelial layer which in turn might resulted in digestive and food absorptive disorders and also various other impaired physiological processes in the midgut tissue of *O. exvinacea*.

The ultrastructural changes in the midgut of *B. mori* such as disruption of the epithelial cells, extreme dialations in endoplasmic reticulum associated with vesiculations and shedding of ribosomes on treatment with Jatropherol-I compound isolated from *Jatropha curcas* seeds reflected turbulence in insect metabolism mainly the protein metabolism along with fluctuations in enzyme activities (Jing *et al*., 2005).

The histolysation in the gut lumen indicated the complete collapse of the gut. Based on light microscopic studies, the histopathological changes in insect midgut were found to be dose dependent. Therefore, the pathological symptoms of botanical treatment in the midgut tissue might confirm the efficacy of these botanicals to be used as larvicide. *V. negundo* was found to

be more effective in having insecticidal property, bringing about complete collapse of the gut.

The present study results such as blebbing of epithelial cells in the *H. suaveolens* treated larvae and complete destruction of peritrophic membrane in both plant treated tissues were found to be concomitant with the findings of Barbeta *et al.* (2008) who reported blebbing, swelling and ultimate rupture of the midgut epithelial cells and damaged peritrophic membrane in *H. armigera* when treated with plant cyclotides. The detruction of peritrophic membrane could be attributed to the fact that the primary site of most of the insecticidal compounds relied on this target (Hopkins and Harper, 2001).

Furthermore, it was reported that the occurrence of numerous cellular components in the apical part of columnar cells were involved in the elaboration of digestive enzymes and those in the basal region were meant for secretion and absorption of fluids and electrolytes (Terra, *et al.* (1996). The present study with *Vitex* revealed reduced number of cell organelles indicating altered physiological structure of the midgut.

Similarly, some identical histopathological changes like vacuolization of cytoplasm, rupture of plasma membrane leading to shedding of cytoplasmic contents into midgut lumen and in addition appearance of numerous autophagic vesicles and lysosomic structures, fragmentation of rough endoplasmic reticulum and microvilli in the midgut cells of harmaline treated *P. interpunctella* were noted by Rharrabe *et al.* (2007). Moreover, similar observations were made on midgut cells of predatory larvae *Ceraeochrysa claveri* when treated with neem oil (Scudeler and dos Santosa, 2013). The present research with the selected botanicals on the cytotoxicity of sixth instar larvae of *O. exvinacea* revealed that the histopathological changes resulted may ensure the capability of these extracts in causing the death of the insect when enters in tissues in adequate amounts.

CHAPTER VII

INFLUENCE OF BOTANICALS ON THE BIOCHEMICAL CHANGES OF TOTAL PROTEIN AND FREE AMINO ACID CONTENT IN DIFFERENT TISSUES OF *ORTHAGA EXVINACEA*

7.1. Introduction

The proteins are major structural organic components of living organisms with many vital functions. In living organisms, proteins perform important functions such as catalyzing metabolic reactions, replicating DNA, responding to stimuli and transporting molecules from one location to another (Chapman, 2012). Moreover, proteins are integral structural elements; serve as nucleoproteins for cell division and as enzymes or hormones which are wandering for the control of many biochemical processes in the cell metabolism. Nevertheless a lot of factors are involved in the control of protein synthetic machinery (Carlisle *et al*., 1987). In insects, under varying physiological conditions and different developmental stages, the synthesis, storage and utilization of proteins may vary with the influence of various hormones. Generally, the utilization of stored proteins happens under stress conditions like starvation. Recovery from the stress conditions necessitates high energy levels and most probably this could be resulted from protein catabolism.

Studies concerned with quantitative changes visualize the responses of organisms towards toxic principles, at the same time qualitative data is also found to be inevitable as it reveals the pathological conditions caused by the toxicants. Generally, quantitative change in the protein content is an indication of the physiological state of an organism. Moreover these proteins are considered to be important key elements within a cell since they are

known to be carrying out the duties specified by the information coded in genes. Therefore a correct knowledge on the qualitative changes in the protein profile of various tissues under different circumstances is essential. Mohammed and Hafez (2000) stated that one of the best methods to assess the polymorphic variation of protein in insects can be made possible with electrophoretic studies which help to reveal the chemical nature and the physiological functions of individual proteins.

The nutritional value of protein relies in its digestibility and qualitative or quantitative amino acid composition. Since amino acids are the building blocks of protein, they are very essential for the timely synthesis of proteins for various physiological activities. Besides this, amino acids are also important in the involvement of morphogenesis, facilitating neural transmission, phospholipid synthesis and energy production (Chen, 1985). Different insects have different requirements of amino acids and most insects derive some key amino acids from dietary proteins which are called essential amino acids that cannot be synthesized by insects. Whereas, the amino acids that insects can synthesize are termed non-essential amino acids. Altogether there should be the presence of 10 essential amino acids in spite of the fact that some 20 amino acids are needed for protein synthesis. The lack of even a single essential amino acid is enough for impairing the proper insect growth. Proteins or amino acids are always essential in the diet.

Midgut is a long straight tube constituting the major part of alimentary canal and alimentary canal in turn is differentiated into three main regions which include foregut, midgut and hindgut. In insect alimentary canal, the digestion and absorption of nutrients and also the secretion of digestive enzymes takes place in the midgut region and so it is considered as an important tissue affected by any kind of toxicants (Chapman, 1998). Moreover, midgut is considered to be the most significant area in insect

physiology and subsequently it has been chosen by many researchers for most of the biochemical studies intending to develop potential methods of insect pest control measures (Levy *et al.*, 2004). Many authors frequently studied the insect midgut region remarkably because any physiological changes on midgut upon meal intake, absorption and transformation have an impact on the growth and development of insects (Mordue and Blackwell, 1993; Nisbet, 2000).

The insect fat body is a mesodermal tissue primarily considered as a major organ of multiple metabolic processes with a meshwork of loose lobes suspended in the haemocoel and bathed in the insect haemolymph. The tissue is predominantly composed of metabolic storage cells, called adipocytes or trophocytes which are vacuolated, rounded or polyhedral cells, harbouring stored inclusions of proteins, lipids and glycogen. Fat body is serving as the principal site of intermediary metabolism and detoxification processes along with storage and excretion of glycogen, lipids and proteins. It is structurally well organized and suited to bring forth maximal exposure to the haemolymph and for both absorbing and releasing metabolites. Generally, the protein synthetic activity of fat body is very high in early growing larva and significantly reduced with the advance of larval life. The normal level of haemolymph protein concentration is directly associated with the synthetic and storage capacity of the fat body, since fat body is the major site of haemolymph protein synthesis and sequestration, in order to use in adult development (Ranjini and Mohamed, 2004).

The insect haemolymph is a greenish yellow circulating fluid that fills body cavity or haemocoel which contains a large number of haemocytic cells along with various organic and inorganic constituents. Normally, major part of haemolymph is water about 90%, which determines its total volume. The insect haemolymph acts as a bathing medium for various tissues or organs and

thus forms the meeting place of both the raw materials required and the products of various physiological activities of the insect body. Haemolymph is completely lacking a direct connection with the external environment and so any change in it reflects the physiological state of the animal's internal environment. Hence, the analysis of haemolymph may provide one of the most reliable data which can be used as an index of the physiological activity (Wyatt, 1961). Haemolymph comprises structurally and functionally heterogenous group of protein molecules such as storage proteins, vitellogenins, lipophorins, immunoglobulins, clotting proteins, tanning proteins, lysozymes, enzymes etc. (Miller and Silhacek, 1982). In Lepidoptera, fat body and midgut tissue are found to be important sources of protein. The insect haemolymph is also marked for its high titre of free amino acids (Chen, 1966) that occur in proteins which are also found in free form in the blood, extracellular fluid and within cells. The concentration of amino acids may be decreased with the utilization of amino acids for the synthesis of proteins and for the production of other nitrogenous constituents of the body according to the physiological needs.

The biochemical studies described in this chapter include the quantitative and qualitative estimation of total protein and free amino acid content in the midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea* after fed with botanicals treated mango leaves.

7.2. Material and methods

The materials and methods required for carrying out the quantitative estimation of protein are described in the section 3.2.8.1 and that for the electrophoretic studies are described in section 3.2.8.2. The methods followed for the analysis of amino acids are discussed in the section 3.2.8.3.

7.3. Results

The results of the present study include quantitative estimation of total protein and free amino acid contents and also the qualitative changes of proteins in different tissues of *O. exvinacea* sixth instar larvae. The results obtained were dependant on the concentration of both botanicals used for treatment and it clearly revealed the significant effect of both *H. suaveolens* and *V. negundo* in bringing forth biochemical changes in the larva of *O. exvinacea*. The present study results for altered total protein and free amino acid content in the midgut tissue, fat body and haemolymph for both botanical treatments along with control were given in tables VII. 1. to VII. 6.

7.3.1. Effect of botanicals on protein concentration

The total protein content of the sixth intsar larvae of both treated and control in the midgut tissue, fat body and haemolymph were estimated and the variations were presented in Tables VII. 1 and VII. 2 and Figures VII. 1 to VII. 3 and also the percentage of reduction in protein concentration were shown in Table VII. 3. Comparison of the results obtained for biochemical estimation of protein concentration revealed that *V. negundo* extract was more effective in reducing the protein content in all tissues than *H. suaveolens*. Statistical analysis of the data showed that the differences in the mean concentrations of protein between control and experimental sets of both the treatments were found to be highly significant. The data presented in Tables VII. 1 and VII. 2 indicate the varied concentration of protein in the treated samples, in which 5 % concentration of extracts were causing maximum effect in reduction of protein. The gradual decreasing protein concentration with increasing botanical concentration for midgut tissue, fat body and haemolymph were shown in figures VII. 1, VII. 2 and VII. 3 respectively.

Table VII. 1. Effect of leaf extract of *H. suaveolens* on protein concentration of midgut tissue, fat body and haemolymph of sixth instar larva of *O. exvinacea.*

Protein concentration		control	Concentration of leaf extract of *H. suaveolens*						
			1 %	2 %	3 %	4 %	5 %	F value	P Value
Midgut	mg/ml	2.94±0.032	2.8±0.075	2.28±0.153	2.05±0.125	1.7±0.163	1.46±0.052	139.3	0.000
	mg/g	18.48±0.89	17.52±1.8	17.28±4.81	15.69±4.37	12.11±1.27	10.3±0.98	6.702	0.000
	mg/larva	0.59±0.01	0.56±0.015	0.456±0.03	0.41±0.025	0.34±0.03	0.29±0.01	139.3	0.000
Fatbody	mg/ml	2.95±0.024	2.89±0.004	2.6±0.07	2.32±0.035	2.15±0.055	1.67±0.04	605.6	0.000
	mg/g	22.73±3.95	15.75±1.9	15.72±2.53	16.07±2.91	13.65±2.84	13.1±3.5	6.546	0.001
	mg/larva	0.59±0.005	0.58±0.001	0.52±0.014	0.46±0.007	0.43±0.011	0.33±0.008	605.6	0.000
Haemo-lymph	mg/ml	24.9±0.21	24.52±0.38	22.81±0.58	21.56±0.31	17.88±0.97	15.37±0.24	269.3	0.000
	mg/larva	3.56±0.03	3.51±0.05	3.26±0.08	3.08±0.04	2.55±0.14	2.2±0.03	269	0.000

The values are expressed as means of five determinations for each concentration with standard deviation (mean±standard deviation). The values were found to be statistically significant, $P < 0.01$. Total protein content was expressed in the units of mg/ml, mg/g and mg/larva for both midgut tissue and fatbody and for haemolymph it is expressed as mg/ml and mg/larva.

Table VII. 2. Effect of leaf extract of *V. negundo* on protein concentration of midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea*.

Protein concentration		Control	Concentration of leaf extract of *V. negundo*						
			1 %	2 %	3 %	4 %	5 %	F value	P Value
Midgut	mg/ml	2.94±0.032	2.67±0.14	2.06±0.145	1.62±0.12	1.36±0.145	0.97±0.07	216.5	0.000
	mg/g	18.48±0.89	18.06±2.26	15.87±2.58	14.56±3.73	9.35±1.61	8.43±1.37	18.02	0.000
	mg/larva	0.59±0.01	0.53±0.03	0.41±0.03	0.32±0.024	0.27±0.03	0.19±0.013	216.5	0.000
Fat body	mg/ml	2.95±0.024	2.57±0.1	2.16±0.04	1.8±0.061	1.64±0.06	1.47±0.02	507.2	0.000
	mg/g	22.73±3.95	19.86±7.15	15.84±2.72	14.56±2.35	14.5±3.43	13.8±3.6	3.715	0.012
	mg/larva	0.59±0.005	0.51±0.02	0.43±0.007	0.36±0.012	0.33±0.012	0.29±0.004	507.2	0.000
Haemo-lymph	mg/ml	24.9±0.21	21.82±0.58	18.65±0.96	16.03±0.62	11.22±1.11	3.7±0.54	549.1	0.000
	mg/larva	3.56±0.03	3.12±0.08	2.66±0.14	2.3±0.09	1.61±0.16	0.53±0.07	525.5	0.000

The values are expressed as means of five replicates for each concentration with standard deviation (mean±standard deviation). All values were found to be statistically significant, $P < 0.01$. Total protein content was expressed in the units of mg/ml, mg/g and mg/larva for both midgut tissue and fatbody and for haemolymph it is expressed as mg/ml and mg/larva.

Figure VII. 1. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in the midgut tissue of sixth instar larvae of *O. exvinacea.*

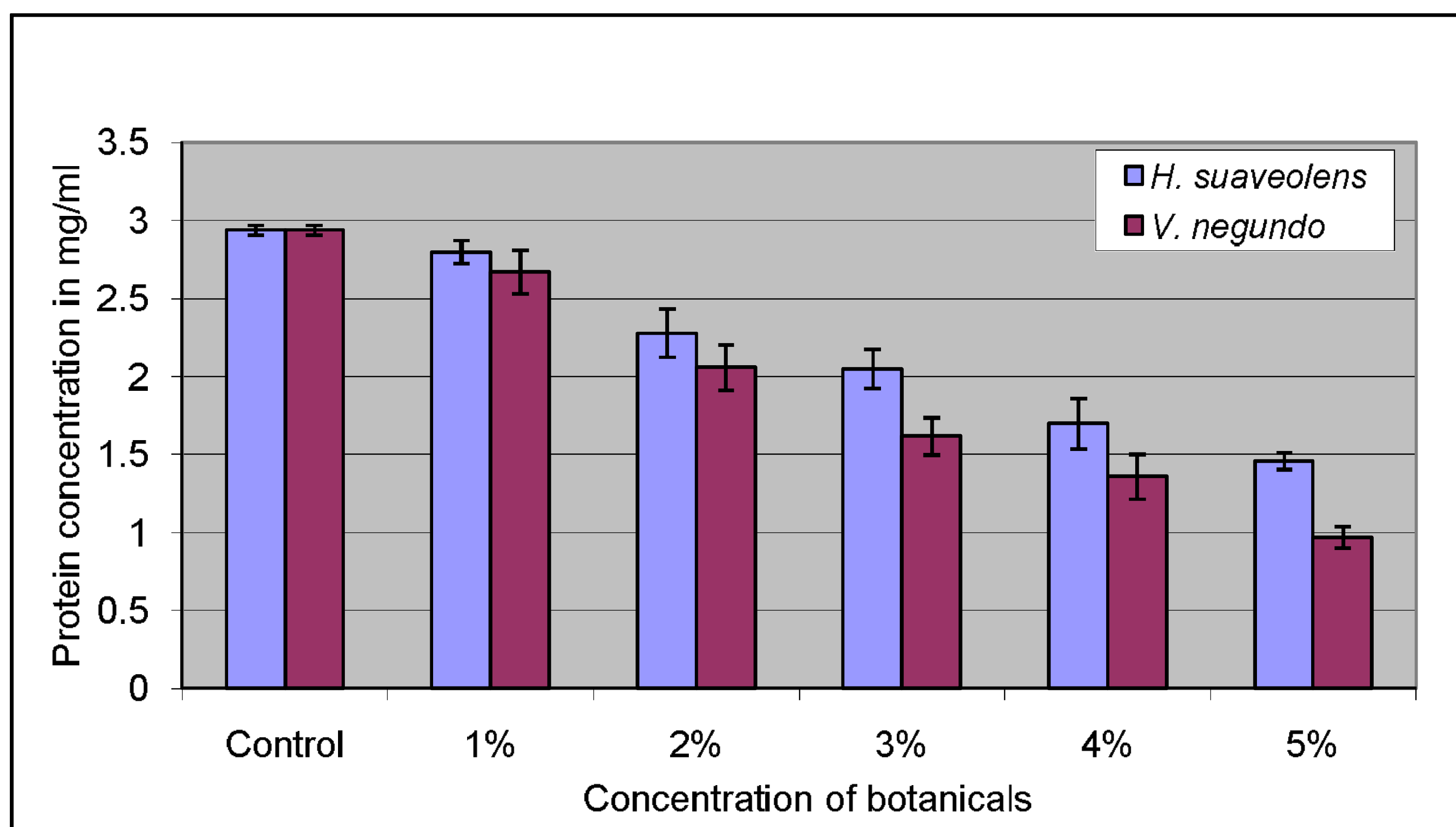


Figure VII. 2. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in fat body of sixth instar larvae of *O. exvinacea.*

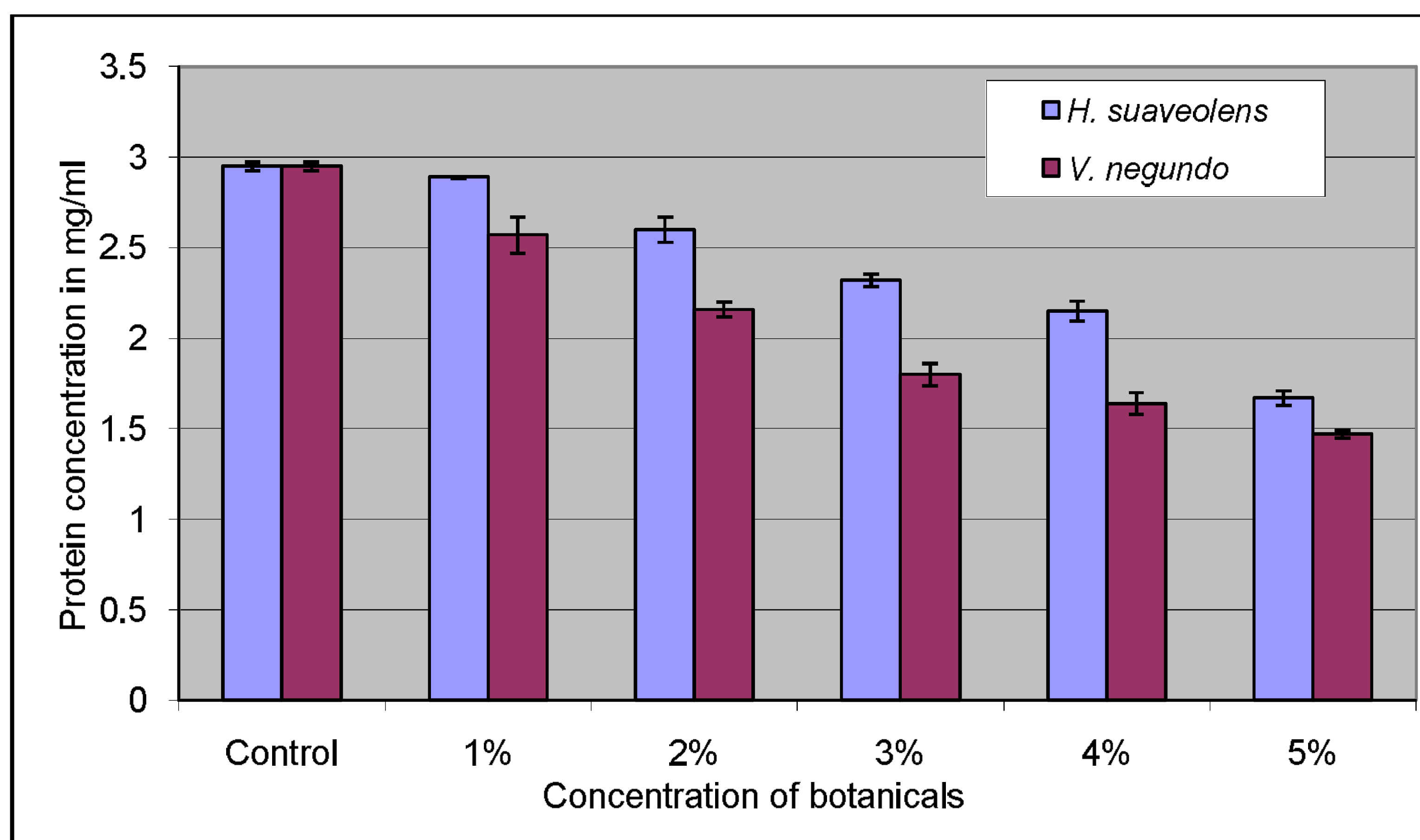


Figure VII. 3. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in haemolymph of sixth instar larvae of *O. exvinacea.*

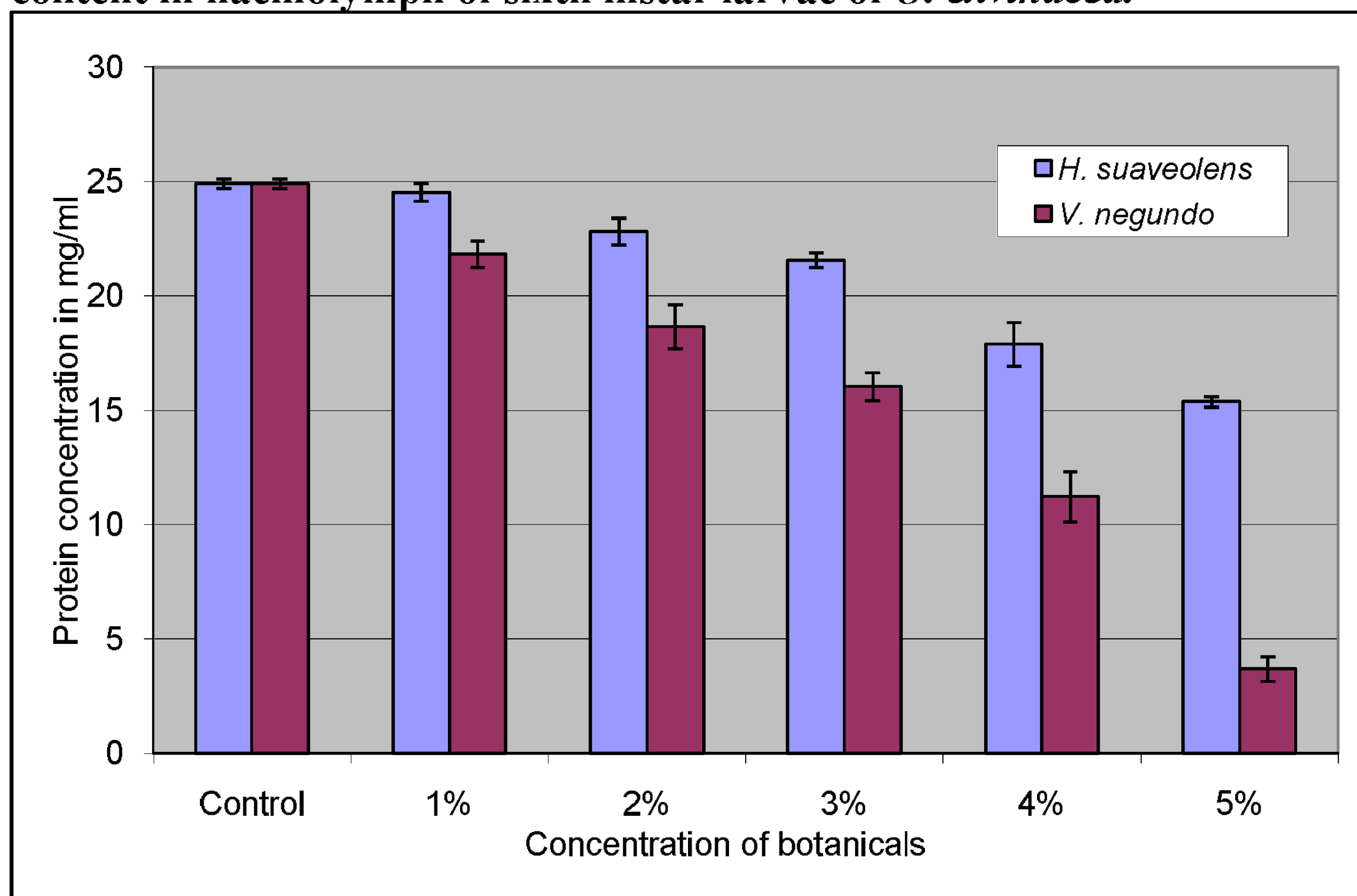


Table VII. 3. Consolidated table showing the percentage of reduction in total protein content over control with respect to mg/ ml mean values of protein concentration for both botanicals.

Conc. of botanicals	Percentage reduction of protein content (%)					
	Hyptis suaveolens treated			*Vitex negundo* treated		
	Midgut	Fat body	haemolymph	Midgut	Fat body	haemolymph
1 %	4.7	2.03	1.53	9.2	12.9	12.4
2 %	22.4	11.9	8.4	29.9	26.8	25.1
3 %	30.3	21.3	13.4	44.9	39	35.6
4 %	42.2	27.1	28.2	53.7	44.4	54.9
5 %	50.3	43.4	38.8	67	50.2	85.1

For 1 % treatment of *H. suaveolens*, the midgut tissue showed only 4.7 % reduction of protein concentration when compared to control midgut tissue while that of *V. negundo* exhibited 9.2 % of reduction to that of control larval tissue. In the case of samples treated with 5 % of *H. suaveolens*, the

percentage of reduction noticed in the midgut tissue was 50.3 %, whereas when it was treated with 5 % of *V. negundo* there was 67 % reduction to that of control tissue. The common feature noticed here was gradual increase in the percentage of reduction in protein concentration along with increasing concentration of botanicals (Table VII. 3).

Similarly the differences obtained for fat body include 2.03 % reduction of protein content at 1 % concentration of *H. suaveolens* treatment which was gradually changed in accordance with increasing concentration of botanicals and reached to 43.4 % reduction at 5 % of *H. suaveolens*. Whereas that of *V. negundo* caused reduction in protein content from 12.9 % to 50.2 % as the botanical concentration has been increased from 1 % to 5 % (Table VII. 3).

In the case of haemolymph too, reduction in protein concentration was noticed according to increasing concentration of botanicals. For *H. suaveolens* treatment, the haemolymph protein concentration at 1 % treatment showed only 1.53 % of reduction, which was gradually altered with increasing treatment concentration and reached to 38.8 % of reduction for its 5 % treatment. Whereas for *V. negundo*, the protein concentration was found to be 12.4 % reduced and for its 5 % treatment, the maximum reduction of protein concentration noticed was 85.1 % (Table VII. 3).

The results clearly depict that very low concentration (1 %) of both botanicals was not that much effective to cause a significant reduction of protein concentration. However, low concentrations also caused a minimum reduction in the protein concentration for both plants. Moreover, considerable difference was noticed between the two extracts and a dose dependant reduction in the mean protein concentration was also observed. Furthermore, the percentage of reduction in protein content among the botanicals was found to be more in the case of *V. negundo*.

7.3.2. Electrophoretic studies

Electrophoretic analysis of proteins in midgut tissue, fat body and haemolymph of both control and treated sixth instar larva of *O. exvinacea* with *H. suaveolens and V.negundo* are given in Plates VII. 1 to VII. 6.

- **Protein profiling of midgut tissue treated with *H. suaveolens*.**

The electrophoretic studies revealed that the intensity and distribution of protein bands were different for each concentration of the plant extract when compared to control. Control midgut tissue showed the presence of 6 protein bands of molecular weights in the range of 38 kDa to 119 kDa. Protein of molecular weight 90 kDa seen in control was found to be present in all the treatments of *H. suaveolens* with slightly altered molecular weights in the range of 80-95 kDa. The low molecular weight protein, 38 kDa present in control found to be of common occurrence in all the treatments of *H. suaveolens* with slightly altered molecular weights in the range of 37-45 kDa. Whereas, some of the high molecular weight proteins observed in control showed variation in distribution among treated percentages in which 77 kDa protein was absent in the treatments 2 % and 3 % and also 57kDa protein present in control showed its presence only in 4 % and 5 % treatments. Similarly, protein of molecular weight, 119 kDa was noticed in all treated percentages except 3 % and 68 kDa protein present in control was also present in 2 %, 4 % and 5 % except 1 % and 3 % treatments (Plate VII. 1).

- **Protein profiling of midgut tissue treated with *V. negundo*.**

In this case, control midgut tissue showed the presence of only 2 protein bands of molecular weights 17 kDa and 22 kDa. These two low molecular weight proteins present in control showed its occurrence in all applied treatments. The obtained low molecular weight proteins 22 kDa and 17 kDa were considered to be characteristic in both treated and control groups

with their presence. Excluding 1 %, all other higher treated concentrations (2 %, 3 %, 4 % and 5 %) showed the presence of additional protein bands with high molecular weights, in which protein in the range of 76-77 kDa was present in all these four treatments. Besides this, 3 %, 4 % and 5 % treatments exhibited protein in the range of 56-57 kDa. Moreover, a high molecular weight protein ranging from 163-164 kDa was found to be present in 2 %, 3 % and 5 % treatments which were not observed in the control tissue protein pattern (Plate VII. 2).

- **Protein profiling of fat body treated with *H. suaveolens***

The protein profile of control fat body tissue exhibited 7 bands of molecular weights ranging from 34 kDa to 101 kDa. The protein bands in the range of molecular weights 34 kDa to 75 kDa were present in all treatments with an exception in which the high molecular weight protein 101 kDa was found to be absent in 1 %, 2 %, 3 % and 4 % treatments. However, the appearance of new protein bands with molecular weights such as 95 kDa, 87 kDa, 86 kDa were present only for the treatments 1%, 2 % and 3 % respectively, which were found to be lacking in the control. Moreover, in 5 % treatment in addition to these protein bands an additional low molecular weight protein of 36 kDa was also present (Plate VII. 3).

- **Protein profiling of fat body treated with *V. negundo* .**

In this electrophoretic profile, control has 39 kDa to 128 kDa molecular weight proteins in 7 bands. All 5 treatments have similar bands with control in the range of 39 kDa to 128 kDa with slightly altered molecular weights except 95 kDa in 2 % and 47 kDa in 4 % and 5 % treatments. The protein of molecular weight, 39 kDa was of common occurrence in both the control and treated groups. Treatment with 1 % and 2 % showed the presence of a specific high molecular weight protein, 114 kDa and at the same time 4 % also indicated the presence of high molecular weight protein, 104 kDa. Moreover, all five concentrations of the treatments exhibited low molecular weight proteins in the range of 27 kDa to 35 kDa with varied distributions which were found to be lacking in the control tissue. Among these, 31 kDa protein was occurred generally in all treatments from 1- 5 % of *V. negundo* (Plate VII. 4).

- **Protein profiling of haemolymph treated with *H. suaveolens***

Protein profile of haemolymph in both control and treated groups were separated electrophoretically, in which control larvae showed protein bands of molecular weights in the range of 35 kDa to 105 kDa. The protein bands of molecular weights 63 kDa, 90 kDa and 105 kDa present in control were also occurred in all five sets of treatments with altered molecular weights. Moreover, bands in the range of 35-55 kDa were also showing their presence in all the five sets of treatments with varied distribution patterns. In addition, 85 kDa protein present in control was found to be absent in all treatments (Plate VII. 5).

- **Protein profiling of haemolymph treated with *V. negundo***

Haemolymph of control larvae showed the presence of 7 protein bands in the molecular weight ranging from 31 kDa- 90 kDa. In this treatment, appearance of high molecular weight proteins such as 145 kDa, 131 kDa, 154 kDa and 146 kDa proteins were present in 1 %, 2 %, 4 % and 5 % treatments respectively. These bands were found to be completely absent in control. 90 kDa protein of control was found to be present in all treatments with slightly modified molecular weights. In addition, three protein bands of control (68 kDa, 49 kDa and 33 kDa) with their range of molecular weights between 68-73 kDa, 49- 55 kDa and 33 kDa were found to be of common occurrence in all 5 sets of treatments with varying intensities. Moreover, 37 kDa protein of control was found to be present in all treatments except 5 % (Plate VII. 6).

7.3.3. Effect of botanicals on amino acid concentration

The effect of both botanicals on free amino acid content in all the three tissues observed were presented in Tables VII. 4 and VII. 5 and Figures VII. 4 to VII. 6. The percentage of reduction in the amino acid concentration was shown in Table VII. 6. The maximum reduction in the amino acid content was noticed in haemolymph samples treated with 5 % of both *V. negundo* (97.8 %) and *H. suaveolens* (96.1 %). From the tabulated data, it was evident that the percentage of reduction was found to be increased with increase in concentration of botanicals.

Statistical analysis of the data for free amino acid content indicated that the differences in the mean concentrations of free amino acid content between control and experimental sets of both the botanical treatments were found to be highly significant. The data displayed in Tables VII. 4 and VII. 5 exhibited significant changes in free amino acid content in the treated samples. Among the treatments, 5 % concentrations of both extracts were causing maximum effect in the reduction of free amino acid content. The gradual decreasing amino acid concentration with increasing botanical concentration for midgut tissue, fat body and haemolymph were shown in figures VII. 4, VII. 5 and VII. 6 respectively.

Table VII. 4. Effect of leaf extract of *H. suaveolens* on amino acid concentration of midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea*.

Amino acid concentration		Control	Concentration of leaf extract of *H. suaveolens*						
			1 %	2 %	3 %	4 %	5 %	F value	P Value
Midgut	mg/ml	24.25±0.45	19.4±0.3	16.42±0.27	14.85±0.17	13.42±0.2	6.3±0.51	1559	0.000
	mg/g	145.6±1.71	127.9±1.6	116.3±1.67	104.9±1.13	96.13±1.75	45.2±1.41	2451	0.000
	mg/larva	4.85±0.09	3.88±0.03	3.3±0.05	2.97±0.03	2.68±0.04	1.26±0.1	1612	0.000
Fat body	mg/ml	15.98±0.41	11.67±0.29	9.92±0.2	9.21±0.22	6.27±0.31	3.76±0.2	1133	0.000
	mg/g	74.6±1.03	54.24±0.41	48.33±0.85	46.19±0.18	35.4±0.76	22.02±0.26	3576	0.000
	mg/larva	3.2±0.09	2.33±0.06	2.00±0.014	1.84±0.04	1.25±0.06	0.75±0.04	959.5	0.000
Haemo-lymph	mg/ml	51.32±0.69	27.62±0.65	16.14±0.41	11.3±0.22	3.61±0.27	1.99±0.33	1209	0.000
	mg/larva	6.41±0.03	3.45±0.03	2.08±0.02	1.41±0.01	0.45±0.01	0.25±0.014	1220	0.000

The values are expressed as means of five replicates for each concentration with standard deviation (mean±standard deviation). All values were found to be statistically significant, $P < 0.01$. Free amino acid content was expressed in the units of mg/ml, mg/g and mg/larva for both midgut tissue and fat body and for haemolymph it is expressed as mg/ml and mg/larva.

Table VII. 5. Effect of leaf extract of *V. negundo* on amino acid concentration of midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea*.

Amino acid concentration		Control	Concentration of leaf extract of *V. negundo*						
			1 %	2 %	3 %	4 %	5 %	F value	P Value
Midgut	mg/ml	24.25±0.45	13.42±0.14	9.63±0.16	6.73±0.17	4.52±0.2	3.07±0.06	5659	0.000
	mg/g	145.6±1.71	53.68±0.44	50.41±0.56	41.87±0.31	36.66±1.11	31.62±0.39	11130	0.000
	mg/larva	4.85±0.09	2.68±0.03	1.93±0.03	1.35±0.04	0.91±0.04	0.61±0.01	5988	0.000
Fat body	mg/ml	15.98±0.41	8.08±0.09	7.59±0.03	7.02±0.08	6.37±0.04	6.04±0.08	2167	0.000
	mg/g	74.6±1.03	51.56±0.79	47.94±0.7	42.14±0.42	39.35±0.53	36.27±0.52	1992	0.000
	mg/larva	3.2±0.09	1.62±0.02	1.52±0.007	1.4±0.02	1.27±0.005	1.2±0.015	1986	0.000
Haemo -lymph	mg/ml	51.32±0.69	28.04±0.64	14.14±0.86	8.66±0.39	3.02±0.16	1.21±0.09	1587	0.000
	mg/larva	6.41±0.03	3.50±0.027	1.77±0.04	1.08±0.02	0.38±0.007	0. 15±0.005	1595	0.000

The values are expressed as means of five replicates for each concentration with standard deviation (mean±standard deviation). All values were found to be statistically significant, $P < 0.01$. Free amino acid content was expressed in the units of mg/ml, mg/g and mg/larva for both midgut tissue and fat body and for haemolymph it is expressed as mg/ml and mg/larva.

Figure VII. 4. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in the midgut tissue of sixth instar larvae of *O. exvinacea.*

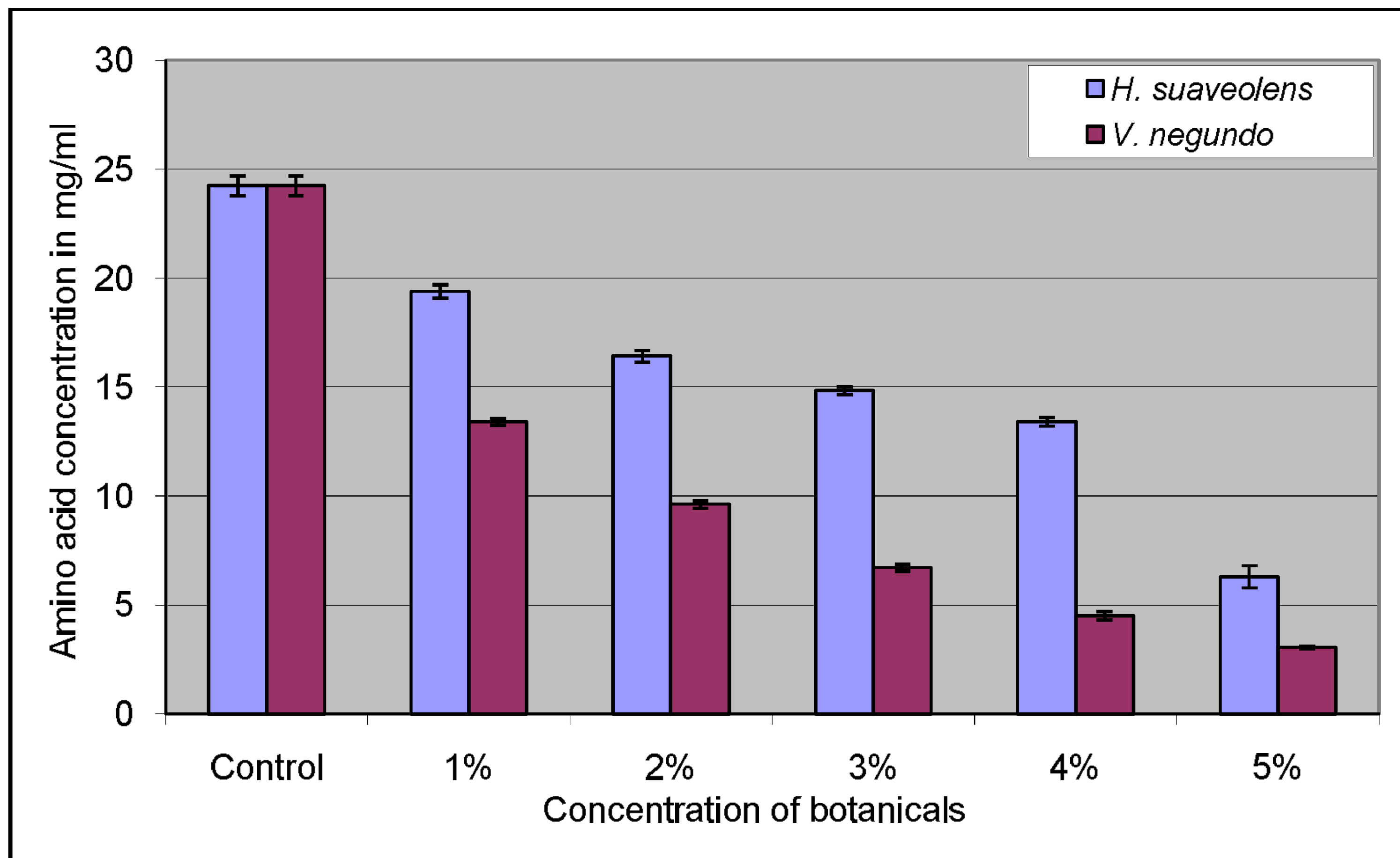


Figure VII. 5. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in fat body tissue of sixth instar larvae of *O. exvinacea.*

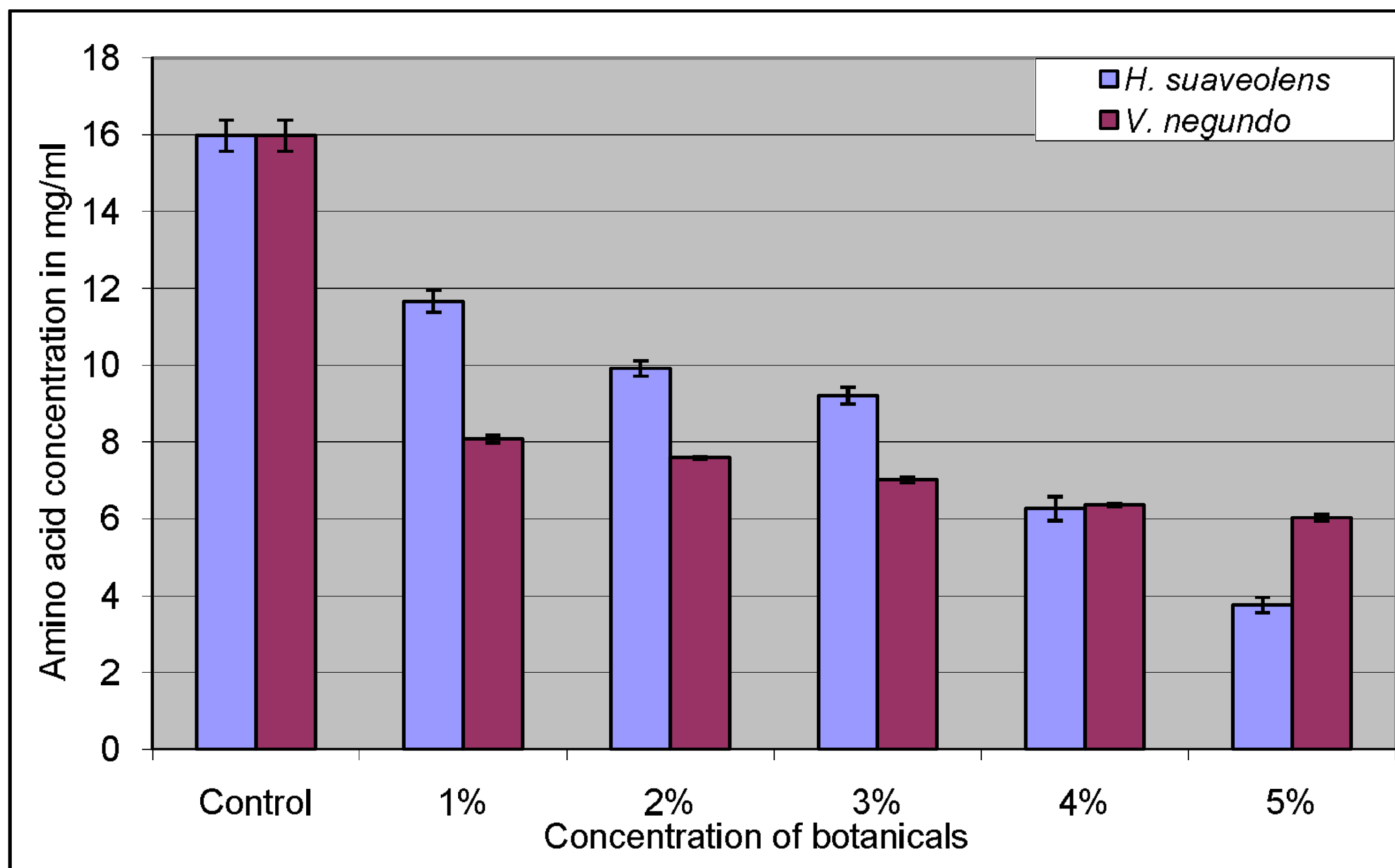


Figure VII. 6. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in haemolymph of sixth instar larvae of *O. exvinacea.*

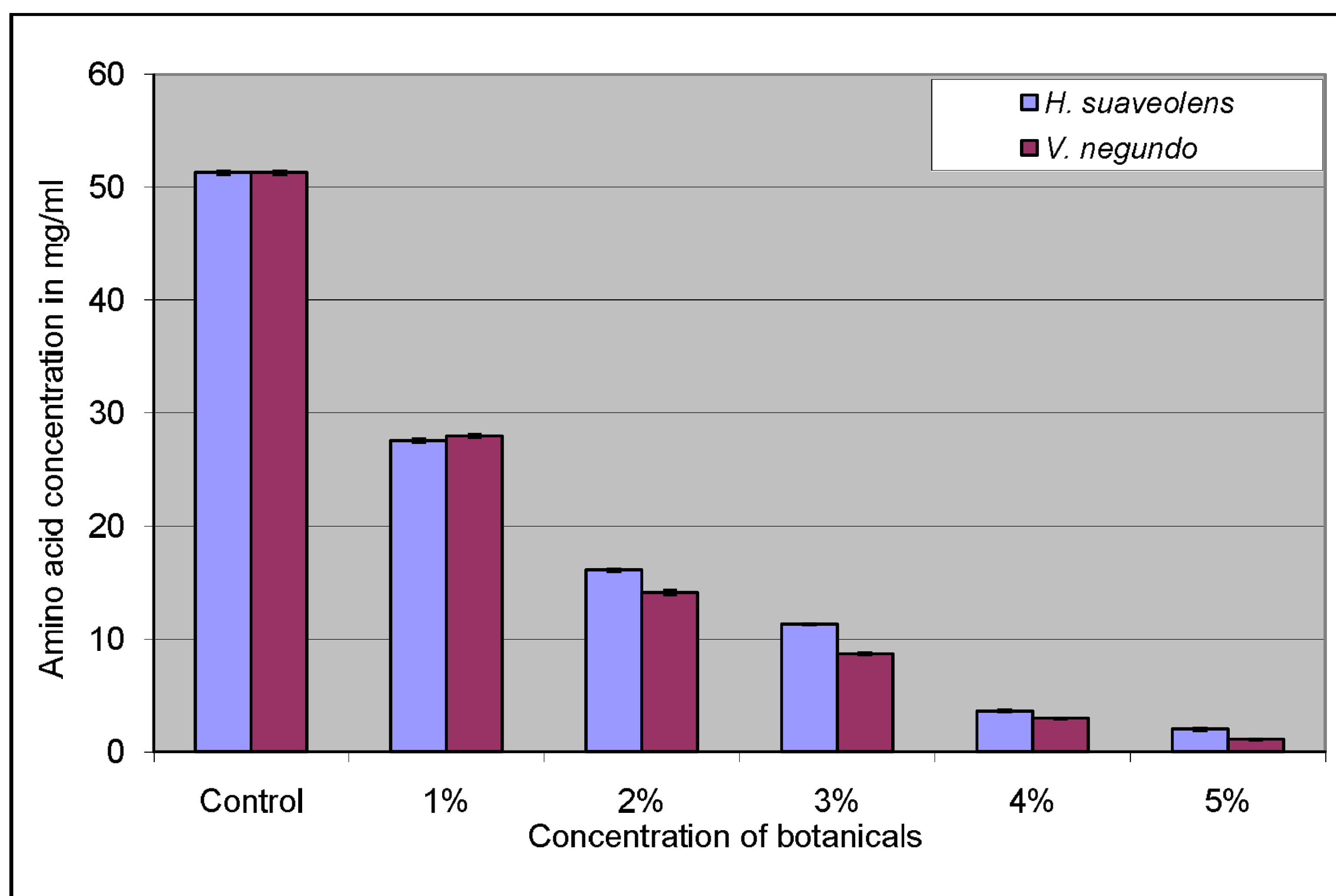


Table VII. 6. Consolidated table showing the percentage of reduction in total amino acid content over control with respect to mg/ ml mean values of amino acid concentration for both botanicals.

Conc. of botanicals	Percentage reduction of amino acid content (%)					
	Hyptis suaveolens treated			*Vitex negundo* treated		
	Midgut	Fat body	haemolymph	Midgut	Fat body	Haemolymph
1 %	20	27	46.2	44.6	49.4	45.4
2 %	32.3	37.9	68.6	60.3	52.5	72.5
3 %	38.8	42.4	78	72.2	56.1	83
4 %	44.6	60.8	93	81.4	60.1	94.1
5 %	74	76.5	96.1	87.3	62.2	97.8

In the midgut tissue, 1 % treatment of *H. suaveolens*, caused 20 % reduction of amino acid concentration to that of control midgut tissue and upon 5 % treatment resulted in 74 % reduction in amino acid concentration. Similarly, *V. negundo* exhibited 44.6 % of reduction to that of control larval tissue in the 1 % treatment itself and when it was treated with 5 %, it caused 87.3 % reduction with that of control tissue. There is a general tendency of gradual increase in the percentage of reduction in amino acid concentration along with increasing concentration of botanicals (Table VII. 6).

Likewise, the changes noticed for fat body include 27 % reduction of amino acid content at 1 % of *H. suaveolens* treatment which was gradually altered with increasing concentration of botanicals and caused 76.5 % reduction at 5 % of *H. suaveolens*. While that of *V. negundo* triggered percentage reduction in the amino acid content from 49.4 % to 62.2 % with increasing botanical concentration from 1 % to 5 % (Table VII. 6).

In the case of haemolymph, 46.2 % reduction of amino acid concentration was noticed for treatment with 1 % *H. suaveolens* which was gradually altered with increasing treatment concentration and resulted in 96.1 % of reduction with 5 % treatment. For *V. negundo,* the amino acid concentration was found to be reduced for 45.4 % and with its 5 % treatment caused 97.8 % reduction (Table VII. 6).

7.4. Discussion

The present study results clearly revealed a significant reduction potential in all the three kinds of tissues. The observed maximum decrease in protein and amino acid content occurred at the higher concentrations of both plant extracts. The results correlated with the findings of Rathi and Gopalakrishnan (2005) who reported reduction of the total protein content in the midgut tissue of *S. litura* after treatment with the leaf extracts of

Synedrella nodiflora. Quantitative changes in total proteins noticed in the larvae of *T. confusum* due to the impact of Vetiver oil and ethanol extracts of *Calotropis procera* have been reported by El-Bermawy and Abdel-Fattah (2000) was also found to be in agreement with the present study results.

Much comparable results concerned with depletion in protein concentration of midgut tissue, fat body and haemolymph in various insects reported were congruent with the results of the present study. The total protein concentration of haemolymph and midgut content in *C. binotalis* was significantly reduced with the treatment of *S. nuxvomica*, *V. negundo* and *Lippia nodiflora* (Vijayaraghavan *et al.*, 2010).

Similarly, decreased haemolymph protein concentration was noticed with the treatment of azadirachtin on *S. littoralis* and *A. ipsilon* (Schmidt *et al.*, 1998). Moreover, there were reports on reduced haemolymph protein content in *H. armigera* with the treatment of *A. annua* and *A. conyzoides* oil (Padmaja and Rao, 2000) and azadirachtin on *Corcyra cephalonica* (Jadhav and Ghule, 2003). In addition, significant decrease of total protein content in haemolymph and midgut of *S. litura* larvae with leaf and flower extracts of *Calendula officinalis* had been reported by Medhini *et al.* (2012).

Complementary results of decreasing protein content was reported by Vijayaraghavan and Chitra (2002) in *S. litura* larvae with the treatment of neem and *Annona* seed extracts and in *H. armigera* with the treatment of azadirachtin by Neoliya *et al.* (2005, 2007); in the fifth instar larvae of *G. pyloalis* with methanolic extract of *A. annua* (Khosravi *et al.*, 2010) and in *C. cephalonica* with powdered leaves of *L. camara* and *C. inerme* (Morya *et al.*, 2010).

It was observed that there occurred maximum reduction in the concentration of amino acid content in the haemolymph when treated with

both plant extracts, whereas in the case of protein concentration, maximum reduction was resulted with the treatment of *V. negundo*. Anyhow, *H. suaveolens* also caused depletion in the protein concentration to ascertain its insecticidal properties.

However, reduction of total protein and amino acid content in all these tissues by botanicals can be interpreted in the light of some acceptable suggestions. The reduced protein concentration might be due to the consumption of stored protein for repair of damaged cells and tissue organelles. So, proteins play a key role in compensatory mechanisms during insecticidal stress conditions and thus encounter the toxic impacts of pesticides (Kumar and Gopal, 2001).

Moreover, the decreased protein content associated with pesticide treatment can be attributed to increased neuromuscular activity of treated larvae which prompt higher energy requirements and depletion of protein level provoked diversification of energy, to meet the impending energy demands under conditions of toxic stress (Jagadeesan and Mathivanan, 1999).

Similarly, Etebari and Matindoost (2004) explained the cause of depletion in the total protein content might be due to breakdown of protein into free amino acid under the toxic effect of insecticides or due to the inhibition of protein synthesis by the lack of adequate energy for protein synthesis (Saxena, 1989). It was also noticed that azadirachtin induced disturbance in the hormone that regulate the protein synthesis in *S. gregaria* which caused lowering of protein content (Rao and Subrahmanyam, 1986). It was also reported that the reduction in protein content may be due to catabolism of protein into amino acids or breakdown of protein for liberation of energy or the direct effect of botanicals on the amino acid transport of the cell (Ahmed *et al*., 1993).

Polymorphic variations were noticed with regard to the number of protein bands in the midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea* with different treatments. The fluctuation in the normal banding pattern in various sets could be due to difference in the physiological activities of these experimental larval sets under treatment of differential concentration of the botanical extracts. In general, the results of protein profiling studies revealed that there occurred appearance and disappearance of proteins of varying molecular weights and also there was formation of new polypeptides in the high and low molecular weight ranges.

The changes of protein contents in the developmental stages of insects is an indication of their involvement in metabolism which reveals the balance between synthesis, storage, transport, deprivation of structural and functional proteins during autogeny as well as their response to certain ecological and physiological conditions (Ranjini and Mohamed, 2004).

Many researchers studied and reported the nature of haemolymph protein pattern during development in several insects (Rajagopal and Basker, 1993; Archana and Nath, 1995). These electrophoretic studies revealed that both the concentration of protein along with the number of amino acid sequences representing protein bands were found to be varied during development. Generally increased protein concentrations were noticed during the larval stages and was decreased in the pupal stages because of their morphogenetic functions in different organs (Archana and Nath, 1995).

Electrophoretic studies reported by many researchers were found to be in agreement with the results of the present study. Similar to the present work report on protein profile, Rajkumar and Subrahmanyam (2000) reported considerably modified electrophoretic banding patterns of cuticular proteins upon treatment with azadirachtin in *H. armigera.* There occurred significantly affected protein bands in *S. litura* when fed with azadirachtin indicating the

effect of azadirachtin on protein expression (Huang *et al*., 2004). Similarly, Huang *et al*. (2007) also noticed variations in the protein banding pattern of *Ostrinia furnacalis* with azadirachtin treatment.

It was noticed that the effect of some dried plants on the protein configuration of *Oryzaephilus surinamensis*, in which treatment with ginger extract divides body proteins to smaller subunits resulting in the appearance of more low molecular weight proteins, at the same time treatment with hail reduced the polypeptide fractions of moderate and low molecular weights leading to the appearance of new set of high molecular weight proteins and also the shammar treatment affected the low molecular weight proteins which caused the formation of new polypeptide bands (Al-Qahtani *et al*., 2012). An aqueous extract of *Koelreuteria paniculata* seeds affect electrophoretic separation of midgut proteins in the fourth instar larvae of *Anticarsia gemmatalis* with the presence or absence of protein bands (Martins *et al*., 2012).

Many researchers described the possible reasons for the alteration of electrophoretic banding pattern. According to Al-Qahtani *et al*. (2012) the occurrence of variations in protein bands might be due to inducing effect of pesticides on the enhancement of transcription of certain sequences involved in the development of resistance or detoxification mechanism for the intoxication of insecticide. In other words, this can be best explained as these bands are normally present in untreated individuals in an obscure form and are most likely triggered with the addition of insecticide (Mohammed and Hafez, 2000).

Generally, living organisms respond at cellular levels to unfavorable conditions such as heat or stress condition including exposure to insecticides by expressing specific set of stress proteins which are induced as a result of damage to the cells by the toxicant. In the present study the disappearance of

electrophoretic band might be due to the stress experienced by the organelles at higher concentration of botanicals and the related tissue damages. This clearly indicates that pesticide exposure may cause initial induction followed by the increase in the synthesis of tissue enzymes to neutralize the deleterious effect of pesticides as a part of the repair mechanism or may result in the synthesis of certain stress induced proteins involved in response to botanical intoxication.

Amino acids are essential ingredients or intermediates of protein synthesis and upon its degradation, byproducts appear in the form of different nitrogenous substances. Generally, the level of amino acids and some nitrogenous compounds possess important functions on osmotic stress and so any change in free amino acid indicate toxic impacts at the tissue level. Many supporting results documenting the altered amino acid composition were evidently reported for many botanicals.

The results of present study were in agreement with the findings of Reddy *et al.* (1993) who reported reduction in the total amino acid and protein content with the treatment of *Annona* extract on *Dysdercus koenigii* and also with Gnanamani and Dhanasekaran (2014) reported greater decline of essential amino acids in larvae fed with *Catharanthus roseus* and *Eucalyptus globulus* plant extracts. Complementary results with decreasing total protein and amino acid content in *An. stephensi* larvae treated with *A. squamosa extracts* were recorded by Senthilkumar *et al.* (2009). This decrease can be attributed to increased neuromuscular activity of treated larvae which resulted in higher demands for energy. Henceforh, more free amino acids entered into the Tricarboxylic Acid Cycle and get oxidized. This could be the reason for the drastic reduction in the free amino acid content also. Since amino acids have an accelerating role in moulting of insects (Pandey *et al*., 1986),

reduction of amino acids cause impacts in physiology and moulting process inducing morphological abnormalities in treated insects (Chen, 1966).

The reason for depleted amino acid and protein content may be attributed to the maintenance of balanced "amino acid pool" in insects by amino- transferases (Meister, 1957). In addition, it was suggested that the changed transaminase levels is an indication of anabolism or catabolism of protein (Mordue and Goldsworthy, 1973). It was clearly revealed fact that the transaminase (ALT and AST) enzymes were responsible for energy production using free amino acid content and served as a strategic link between the carbohydrates and protein metabolism and were known to be altered during various physiological and pathological conditions (Azmi *et al.*, 1998).

However, it may be concluded that the decrease in tissue proteins and amino acids under pesticidal stress could be due to many reasons such as their involvement in the formation of lipoproteins which were employed for repair of damaged cell and tissue organelles and to overcome high energy demands under stress (Ghosh and Chatterjee, 1989), which were noticed in different tissues of *O. exvinacea* during botanical treatment.

CHAPTER VIII

EFFECT OF BOTANICALS ON ENZYME ACTIVITIES IN THE MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF SIXTH INSTAR LARVA OF *ORTHAGA EXVINACEA* HAMPSON

8.1. Introduction

Enzymes are globular proteins with high specificity and selectivity for the type of reaction they catalyze. They facilitate the rate of biochemical reactions by many folds, faster than the relative noncatalyzed reactions. Generally enzymes are concealed within the plasma membrane or inside the cells of their origin and they do not leak out of the cell due to the plasma membrane integrity. Stress conditions cause inhibition of ATP production which will result in loss of integrity and the associated disruption of plasma membrane leading to their hyperactivity and an increase in cell damage. All these changes ultimately results in increased production of free radical and oxidative damage and the perforated membrane discharges most of its contents depending on the extent of damage. Extreme tissue damage may cause release of transaminases and also the activation of detoxifying enzymes, phosphatases etc. According to the tissue damage noticed in the histopathological studies of the present work, a biochemical study was planned to analyze the activities of aspartate amino transferases (AST), alanine amino transferases (ALT), acid phosphatases (ACP), alkaline phosphatases (ALP) and also the catalase activity to ascertain the stress condition. According to Wigglesworth (1972) these biochemical macromolecules have the capacity to control metabolic processes of the organisms, thus any change in the enzyme activities also would affect the physiological state of the organism. Henceforth, enzymes are considered as bioindicators of various aspects of metabolism including the physiological,

biochemical and metabolic defects in various tissues of animals. The quantitative assessment of enzyme activity in an organism is considered as an authentic record for easy understanding of the stress imposed on the organism through identification of disturbances in its metabolic pathways.

Generally tissue damage leads to the induction of oxidative stress resulting in the accumulation of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and superoxide radicals (•O_2-), all these bring about oxidation of lipids, proteins, carbohydrates and nucleic acids as a result of the obligatory dependence of oxidative metabolism on molecular oxygen (Mittler, 2002). Insects possess an antioxidant system (Felton and Summers, 1995) for protection from these ROS which consists of enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). The SOD possesses the capacity to convert superoxide radical into H_2O_2 and molecular oxygen whereas CAT and GPx prevent the accumulation of H_2O_2 in the cell, in which catalase is an enzyme converts the potentially harmful oxidizing agent, hydrogen peroxide into water and diatomic oxygen and thereby reducing stress.

Transaminases include aspartate aminotransferase (AST) also known as glutamate oxaloacetic transaminase (GOT) and alanine aminotransferase (ALT) or glutamate pyruvic transaminase (GPT). Amino transferases are catalyzing various biochemical reactions involved in the maintenance of balanced 'amino acid pool' in insects (Meister, 1957). According to Mordue and Goldsworthy (1973), transaminase enzymes are the key enzymes involved in the formation of non-essential amino acids, in the metabolism of nitrogen waste and also in gluconeogenesis, henceforth the change in transaminase levels have been correlated with anabolism or catabolism of protein. Moreover, transaminases act as a strategic link between carbohydrates and protein metabolism for the production of energy. They are

mainly related with the transamination of glutamate, aspartate and alanine and thus these enzymes were found to be altered under various physiological and pathological conditions like tissue damage which may provoke elevated transaminases level in the circulation (Etebari *et al.,* 2005).

Phosphatases are detoxifying hydrolytic enzymes active at specific pH and are involved in the production of ATP. Phosphatases include ACP and ALP mediating the hydrolysis of phosphate monoesters under acidic or alkaline conditions respectively and are usually termed as phosphomonoesterases (Bai *et al*., 1993). They transfer phosphate from the nucleotides which in turn used for the biosynthesis of chemical energy in the form of ATP to overcome the toxic stress (Naveed *et al*., 2004).

ACP is considered as the lysosomal marker enzyme (Ferreira and Terra, 1980) which is active in both gut and malpighian tubules and its abundant level is probably associated with the disintegration of tissues and organs leading to total cell damage (Sahota, 1975). It is believed that increase in acid phosphatase and alkaline phosphatase activities accentuate energy break down pathway from normal ATPase system which includes transphosphorylation reactions to enhance the phosphate pool for synthesizing higher energy compounds such as adenosine triphosphate (ATP), ATP ase and genetic materials (DNA or RNA) (Hollander, 1971).

ALP is termed as brush border membrane marker enzyme (Wolfersberger, 1984) occur primarily in tissues with active membrane transport especially intestinal epithelium, malpighian tubules and haemolymph (Caglayan, 1990). Its major function is to release phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes. Generally, cytolysis of tissues during the insect development is confined to the activity of ACP and ALP and they have many other roles also during insect development such as acting as hydrolases during the final stages

of digestion, gonad maturation and metamorphic moults (Rhadha and Priti, 1969). Their activities are very less at larval moulting stage and progressively increased after moulting (Miao, 2002).

The present study described in this chapter were biochemical analysis for the quantitative changes of different enzymes activities such as catalase, AST, ALT, ACP and ALP in the midgut tissue, fat body and haemolymph of sixth instar larvae of *Orthaga exvinacea* after fed with botanicals treated mango leaves at different time durations (24 h, 48 h and 72 h).

8.2. Materials and methods

The materials and methods adopted for the quantitative analysis of enzyme activities for catalase, ALP and ACP were discussed in sections 3.2.8.4.1, 3.2.8.4.3 and 3.2.8.4.4 respectively. The methods for the activities of AST and ALT were described in the section 3.2.8.4.2.

8.3. Results

Results obtained for the enzyme activities in midgut tissue, fat body and haemolymph of last instar larvae of *O. exvinacea* were displayed in Tables VIII. 1 to VIII. 3.

8.3.1. Enzyme activity of midgut tissue

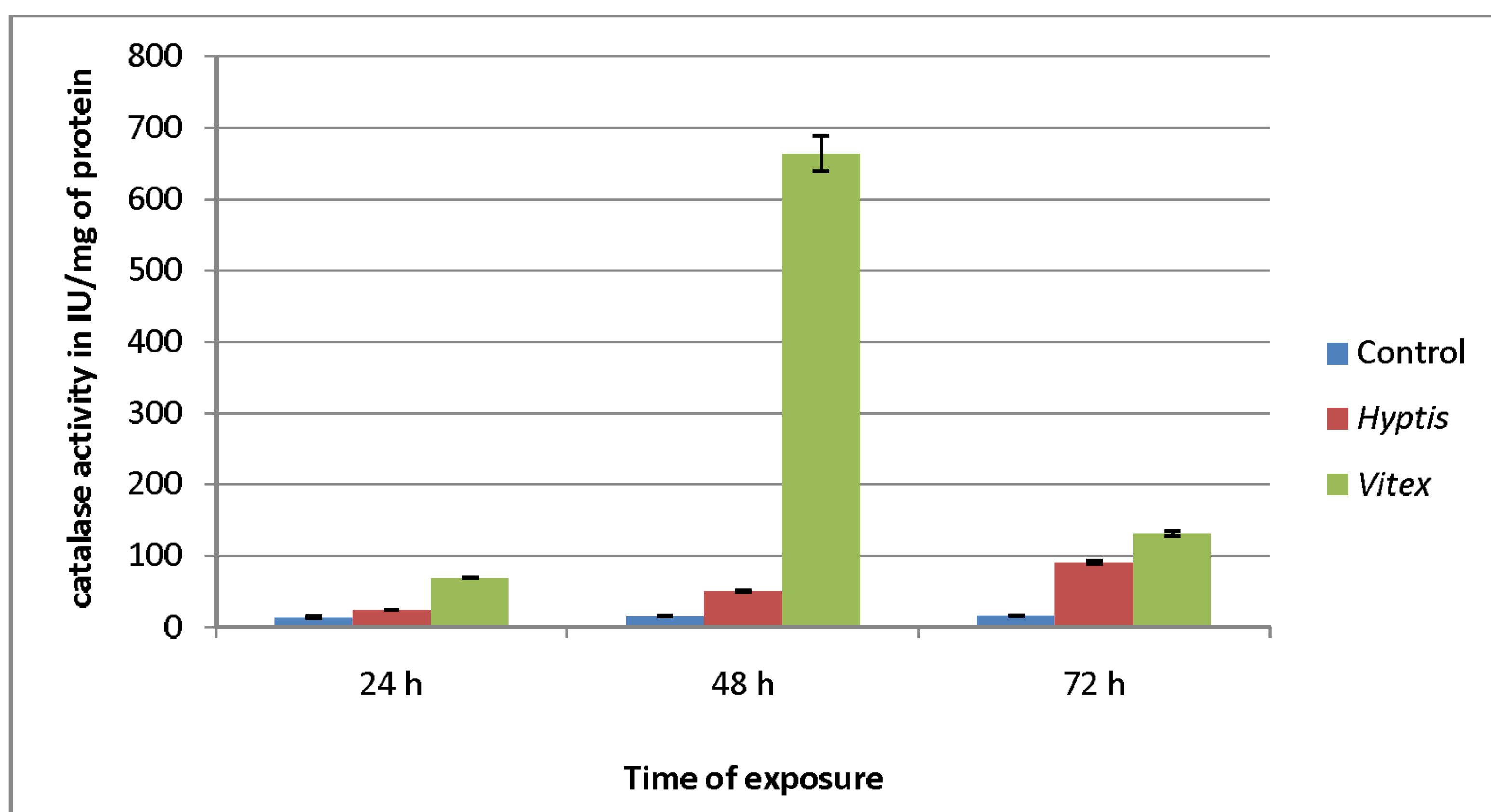
The results obtained for the effect of both botanicals on catalase, AST, ALT, ACP and ALP activities in the midgut tissue of *O. exvinacea* at different time intervals were presented in Table VIII. 1 and Figures VIII.1 to VIII. 5. The mean values for different enzyme activities were represented in the table values. The percentage of various enzyme activities in the midgut tissue were presented in Table VIII. 2 and Figure VIII. 6.

Catalase: Normally, the specific activity of catalase in the untreated tissue showed slight increase in its activity ranging from 13.72 IU/mg, 15.2 IU/mg and 16.32 IU/ mg in accordance with different time of exposure 24 h, 48 h and 72 h (Table VIII.1). Catalase activity in both botanical treatments were found to be altered with increasing time of exposure (Figure VIII. 1).

H. suaveolens treatment caused a significant increase in the specific activity of catalase enzyme on 24 h (23.87 IU/mg), 48 h (50.2 IU/mg) and 72 h (90.93 IU/mg) of exposures (Table VIII. 1). On comparison between treated and control, the percentage of enzyme activity for 24 h of exposure reached nearly 74 % increased activity to that of control and further more than two folds and four folds of increase with that of control was recognized when the larvae were subjected to 48 h and 72 h of exposure respectively (Table VIII. 2 and Figure VIII. 6).

For *V. negundo* treatment, the specific activity of catalase enzyme showed 69.12 IU/mg, 663.7 IU/mg and 131.2 IU/mg on 24 h, 48 h and 72 h of exposure respectively (Table VIII. 1), it exhibited general increasing trend when compared to the values of control. The specific activity was maximum at 48 h of exposure and thereafter at 72 h of exposure, a slight declining tendency was noticed in the specific activity of catalase (Figure VIII. 1). The percentage of enzyme activity revealed four times, 42 times and 7 times of increased activities to that of control on exposure for 24 h, 48 h and 72 h durations respectively (Table VIII. 2). Though there was increase in the activity of catalase on 72 h of exposure, the activity was noticed to be slightly decreased in comparison with the catalase activity for 48 h of post treatment (Figure VIII. 6).

Figure VIII. 1. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on catalase activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.



Transferases (AST/ALT): Significant increase in the activity of AST and ALT values with respect to time of exposure caused by both botanical treatments were observed.

AST: The AST activity for the control tissue with different time periods was not significantly altered, which remains in the range of 12.12 IU/mg. Alteration in the AST activity of both treated and control were expressed in Figure VIII. 2.

AST activity in *H. suaveolens* treatment caused a specific activity of 13.04 IU/mg, 24.23 IU/mg and 40.64 IU/mg after 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). Similar to specific activity of AST, the percentage of enzyme activity was found to be significantly increasing with time of exposure. On 24 h exposure, a very slight increase was noticed for aspartate aminotransferase (7.59 %). Later on, at 48 h and 72 h of exposure a drastic increase of 97.8 % and beyond 2 folds of increase to that of control respectively was noticed in the midgut tissue (Table VIII. 2 and Figure VIII.6).

For *V. negundo* treatment, the specific activity of AST enzyme was found to be 11. 62 IU/mg, 33.7 IU/ mg and 55.8 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 1). The specific activity was found to be showing an initial slight decrease followed with significantly increased activity with increasing time of exposure (Figure VIII.2). The percentage of enzyme activity was indicating a slightly decreased activity of -4.12 % to that of control on 24 h of exposure and later considerable increase in percentage of enzyme activity was noticed in accordance with treatment time. i. e., 175 % and more than 3 folds increase to that of control was noticed on 48 h and 72 h of exposures respectively (Table VIII. 2 and Figure VIII.6).

ALT: ALT activity showed a progressive elevation in its activity for both botanical treatments according to different time of exposures. The specific activity of ALT in the untreated tissue showed slight increase in its activity ranging from 17.1 IU/mg to 18.54 IU/mg when the exposure time was increased from 24 h to 72 h (Table VIII.1). Comparison of specific activities of both treatments and control were given in figure VIII. 3.

In the case of *H. suaveolens* treatment, the specific activity of ALT enzyme noticed was 25.83 IU/mg, 64.99 IU/ mg and 93.63 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 1). Whereas the percentage of enzyme activity with respect to control showed an increase of 51 % to that of control for midgut tissue on 24 h. Later on, at 48 h and 72 h, the enzyme activity was enhanced above 2 folds and 4 folds respectively for *H. suaveolens* treatment (Table VIII. 2 and Figure VIII. 6).

V. negundo treatment caused specific activities of 47.13 IU/mg, 110.4 IU/mg and 154.9 IU/mg for ALT enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). For this treatment, more significant elevation in the ALT activity was noticed for both treatments. The changes in ALT activity caused by this treatment for each time of exposure were found to be much more when compared with ALT activity in the case of *H. suaveolens* treatment (Figure VIII. 3). The percentage of enzyme activity reaches to 175 %, more than 5 folds and 7 folds of increased activities to that of control on exposure to 24 h, 48 h and 72 h of exposures respectively (Table VIII. 2 and Figure VIII. 6).

Figure VIII. 2. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

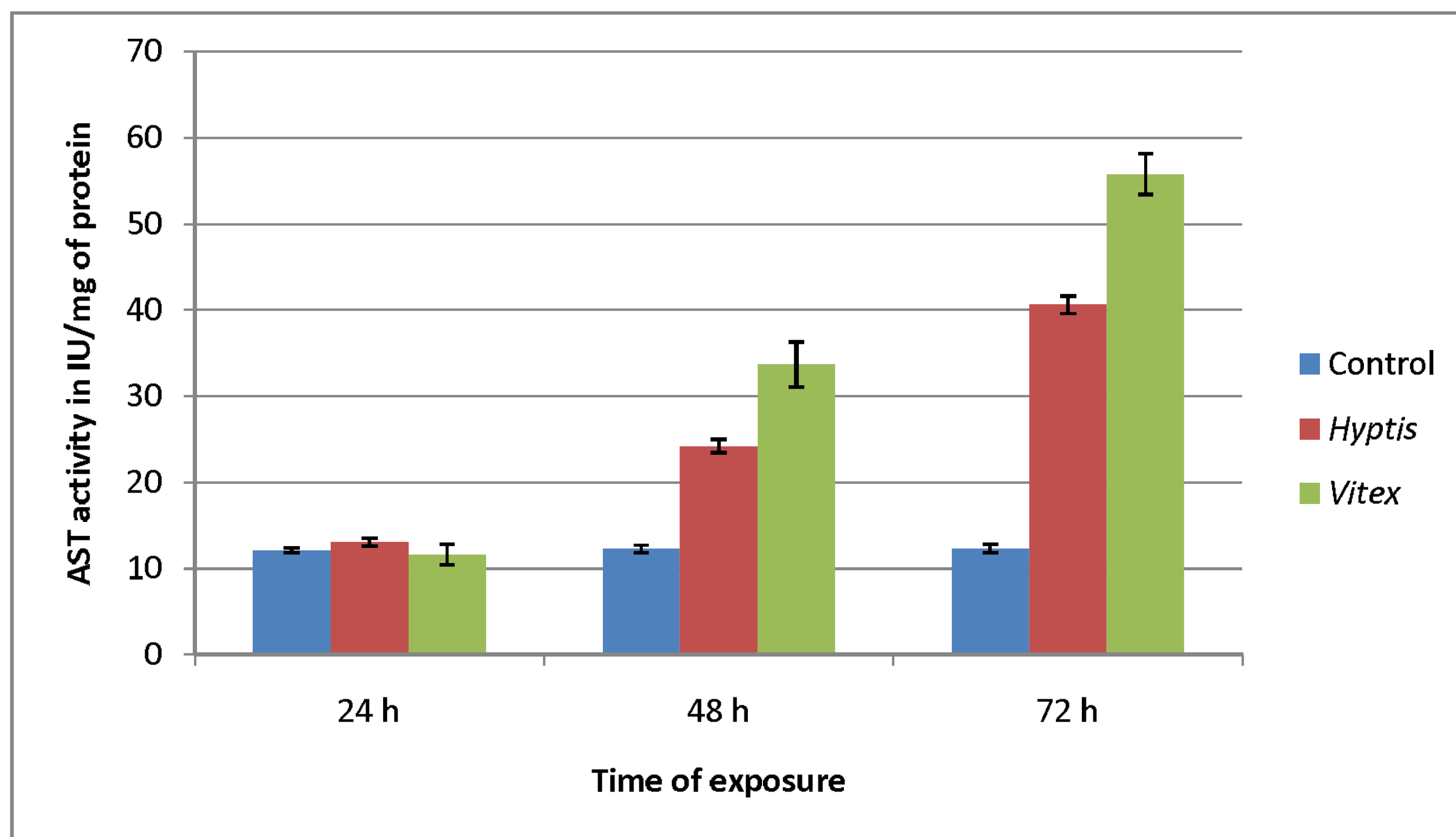
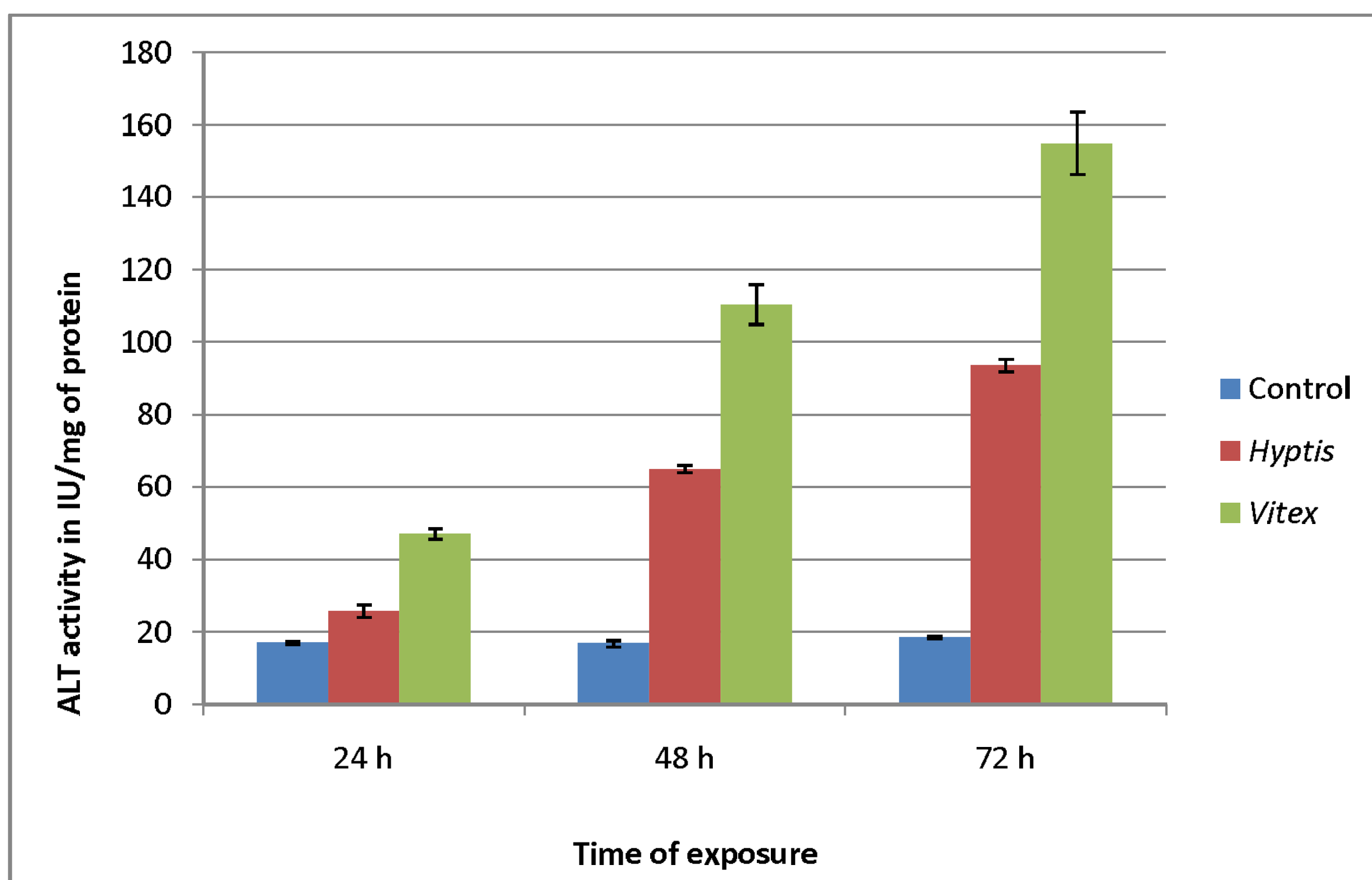


Figure VIII. 3. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.



Phosphatases (ACP/ALP): Generally, ACP and ALP values revealed an increase in activity with time of exposure for both botanical treatments with some exceptions for the *Vitex* treated larva.

ACP: The specific activity of ACP in the control tissue showed slight increase in its activity ranging from 7.27 IU/mg, 8.59 IU/mg and 10.55 IU/mg in accordance with different time of exposure 24 h, 48 h and 72 h respectively (Table VIII.1). The specific activities of both botanical treatments were compared with control and were displayed in figure VIII. 4.

ACP activity in *H. suaveolens* treatment caused a specific activity of 17.26 IU/mg, 27.5 IU/mg and 41.68 IU/mg at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). As the time of exposure added, ACP activity also increased. In this treatment, the percentage of ACP activity noticed were 137 %, 220 % and 295 % to that of control for 24 h, 48 h and 72 h of exposures respectively (Table VIII. 2 and Figure VIII. 6). ACP activity gave the highest percentage of activity in the larva exposed to 72 h with this treatment.

For *V. negundo* treatment, the specific activity of ACP enzyme was found to be 23 IU/mg, 26.2 IU/ mg and 6.08 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 1). The specific activity of this enzyme was found to be decreased after prolonged time of exposure. The percentage of activity was found to be elevated to 216 % that of control on 24 h of exposure itself. On 48 h also there was increase in the ACP activity (205 %) when compared to control, but it showed a slight decrease in its activity when compared to that of 24 h. Low activity of ACP was observed on 72 h of exposure causing a decrease of -42.4 % to that of control (Table VIII. 2 and Figure VIII. 6).

ALP: The ALP activity for the control tissue with different time periods was altered with specific activity of 126.38 IU/mg, 149.95 IU/mg and 158.41 IU/mg at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). Comparison between the specific activities of treatments and control were presented in Figure VIII. 5.

In *H. suaveolens* treatment, the specific activities for ALP noticed were 233.9 IU/mg, 356.5 IU/mg and 697.07 IU/mg for 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). Generally the specific activity in this case was found to be increasing. The percentage of alkaline phosphatase activity in this treatment showed a progressive elevation on 24 h, 48 h and 72 h of exposures from 85 %, 137 % and more than 3 folds to that of control respectively (Table VIII.2 and Figure VIII. 6).

V. negundo treatment caused a specific activity of 377.8 IU/mg, 403.6 IU/mg and 113.8 IU/mg for ALP enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 1). The specific activity in this case was found to be decreasing after 48 h of exposure. On 24 h of exposure itself the percentage of activity was amplified to 198 % with that of control which was found to be much more when compared with ALP activity in the case of *H. suaveolens* treatment (Figure VIII.6). Although there was rise in the activity of ALP on 48 h of exposure (169.1 %) when compared to control, it was found to be declining as the time of exposure increases. On 72 h of exposure, a drastic decrease of about -28.14 % with that of control was observed. Time dependent increase/decrease in ALP activity was observed for both treatments, when compared with the control values.

Statistical analysis of catalase, AST, ALT, ACP and ALP activity in both treatments showed that all variables vary significantly with time of exposure.

Figure VIII. 4. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

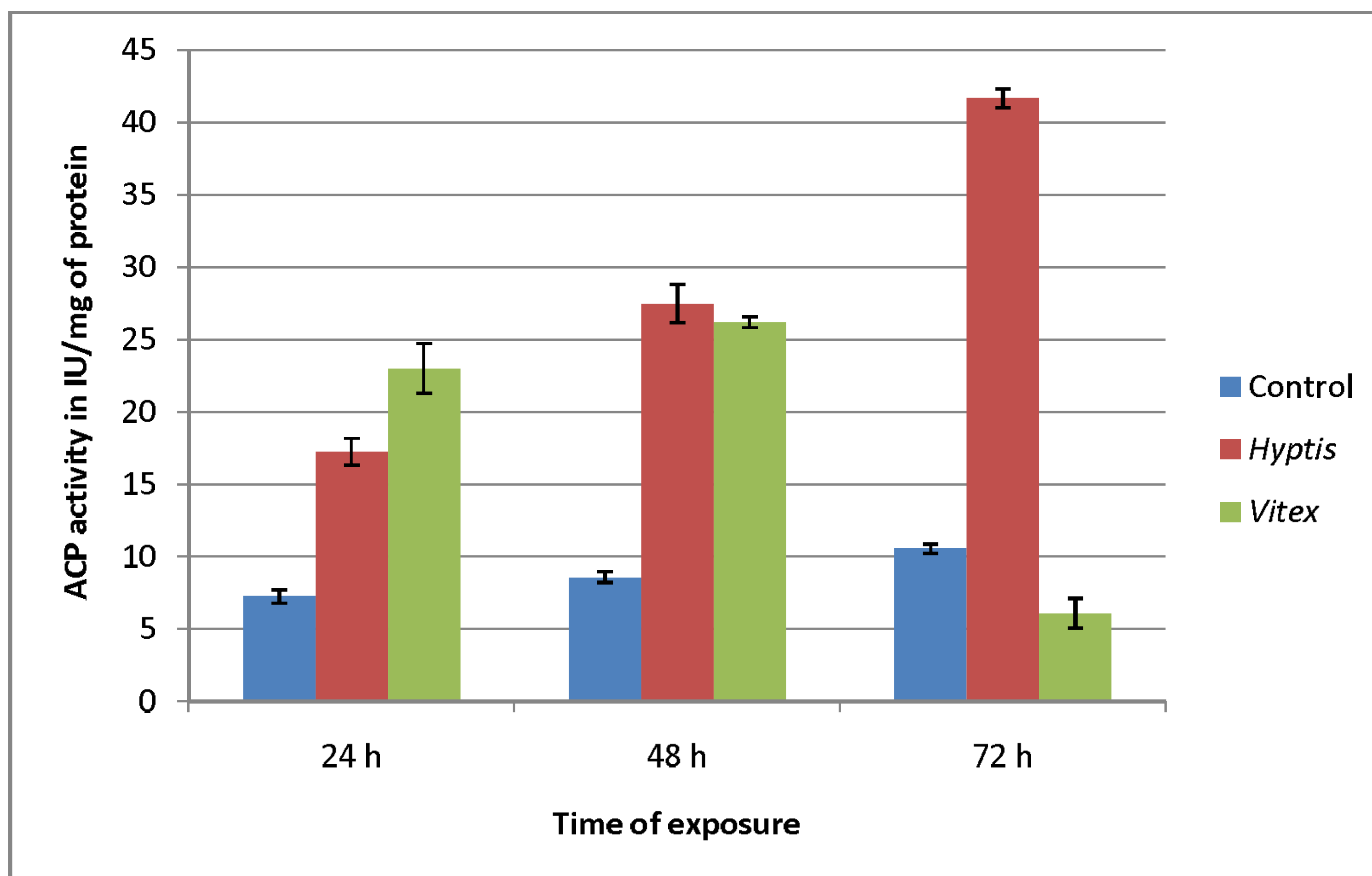


Figure VIII. 5. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

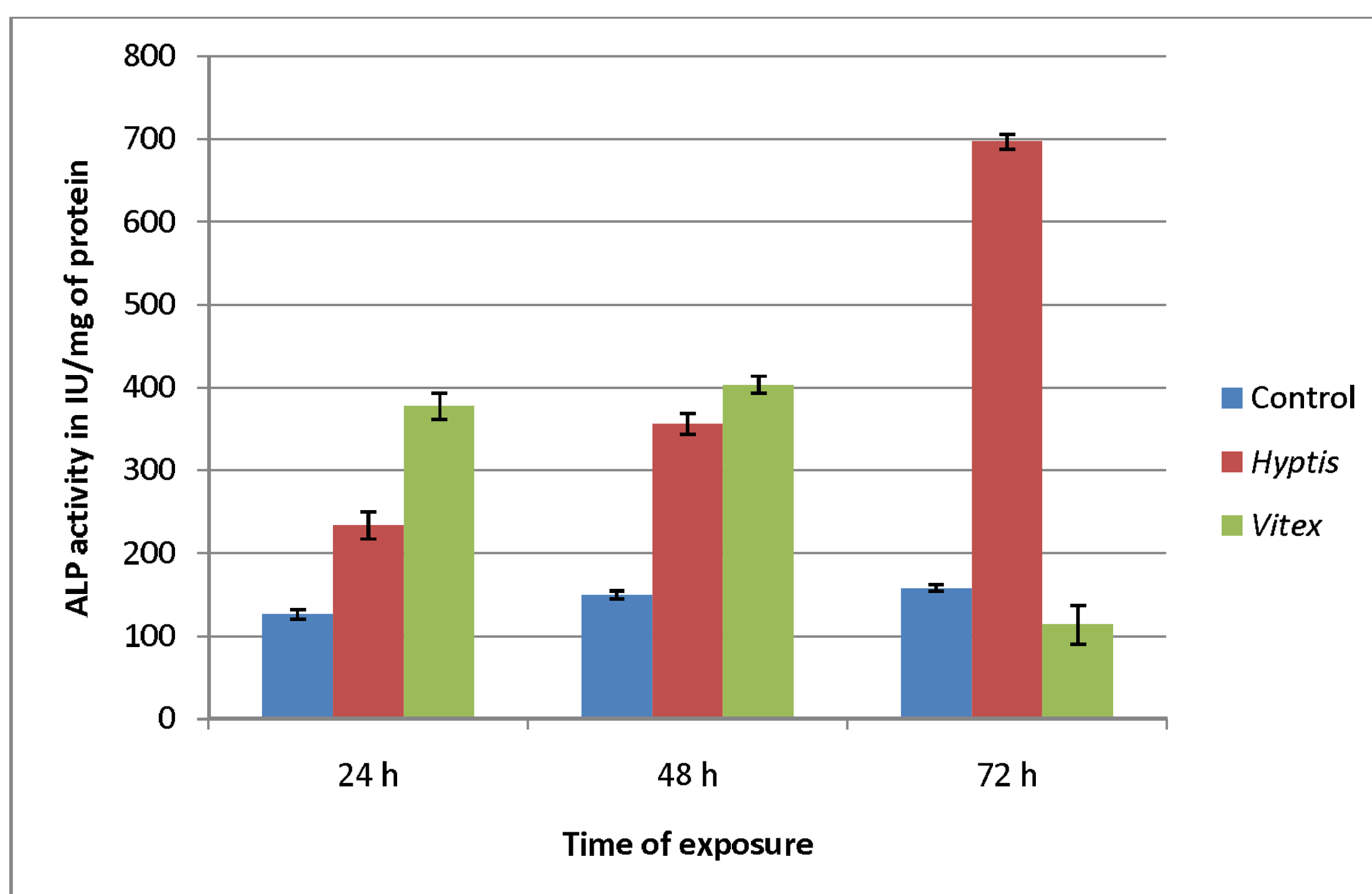


Table VIII. 1. Effect of botanicals on various enzyme activities in midgut tissue exposed to different time of exposure.

Experiments			CAT	AST	ALT	ACP	ALP
Time of exposure	24 h	control	13.72±0.29	12.12±0.15	17.1±0.19	7.27±0.21	126.38±2.6
		Treatment 1	23.87±0.36	13.04±0.21	25.83±0.73	17.26±0.42	233.88±7.24
		Treatment 2	69.12±0.35	11.62±0.53	47.13±0.65	23±0.77	377.78±7.16
		F- value	7656	4.49	716.9	236	432.41
		P- value	0.000	0.035	0.000	0.000	0.000
	48 h	control	15.2±0.15	12.25±0.21	16.85±0.36	8.59±0.17	149.95±2.16
		Treatment 1	50.2±0.39	24.23±0.33	64.99±0.46	27.5±0.59	356.54±5.66
		Treatment 2	663.7±11.13	33.7±1.16	110.4±2.45	26.2±0.16	403.57±4.43
		F- value	3217	230.4	1037	813.6	968.9
		P- value	0.000	0.000	0.000	0.000	0.000
	72 h	control	16.32±0.13	12.3±0.22	18.54±0.18	10.55±0.13	158.41±2
		Treatment 1	90.93±0.92	40.64±0.44	93.63±0.75	41.68±0.3	697.07±4.12
		Treatment 2	131.2±1.45	55.8±1.05	154.9±3.86	6.08±0.46	113.83±10.4
		F- value	3409	1082	904.2	3507	2447
		P- value	0.000	0.000	0.000	0.000	0.000

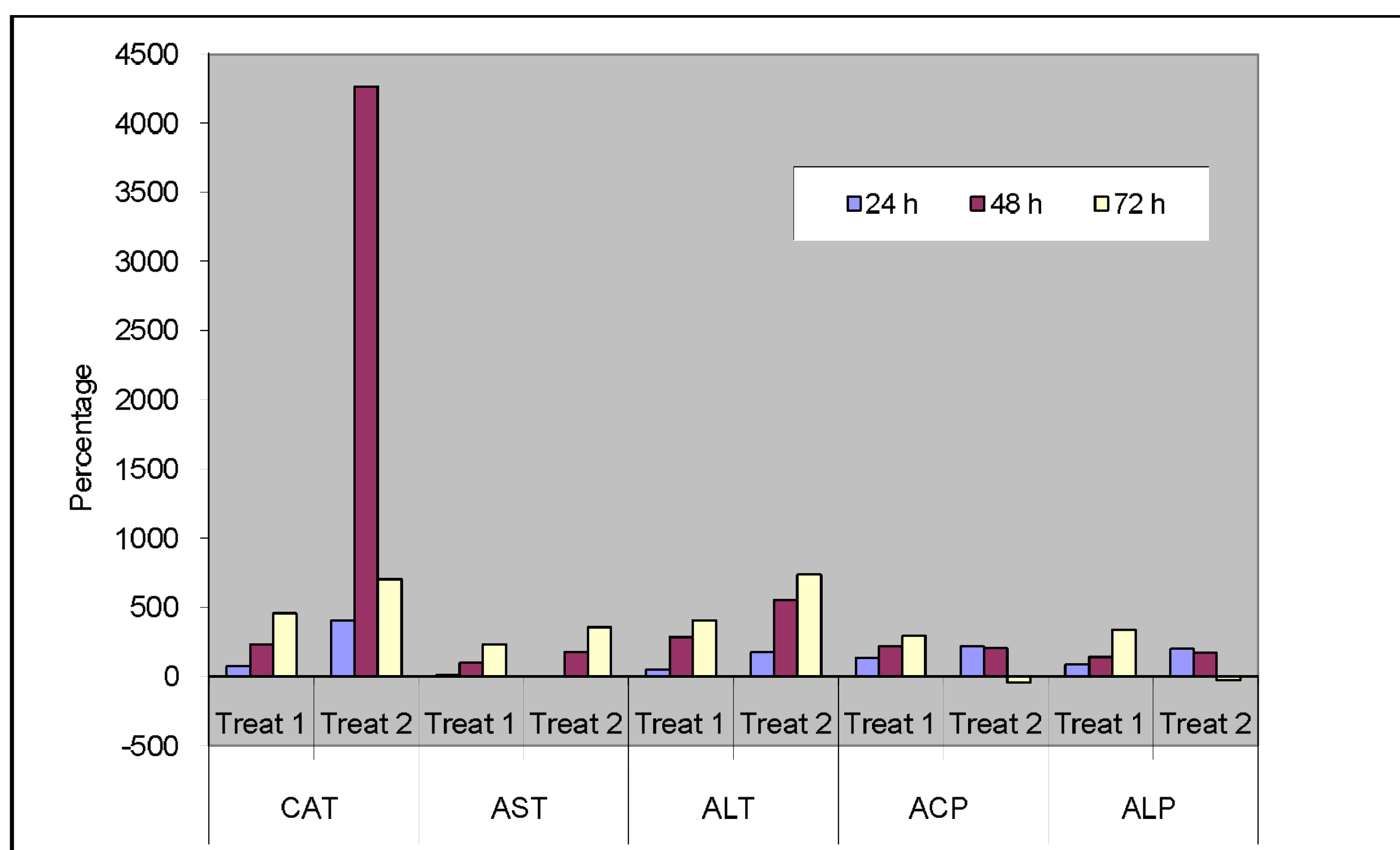
Values are means (± SEM). The activity of enzymes is expressed as units of IU/mg of protein. Treatments were found to be significant at $P < 0.01$.

Table VIII. 2. Percentage of enzyme activity in the larval midgut tissue at different time of exposure for both treatments with respect to control.

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	73.98	7.59	51.05	137.4	85.06
	Treatment 2	403.8	-4.12	175.6	216.4	198.9
48 h	Treatment 1	230.3	97.8	285.7	220.1	137.8
	Treatment 2	4266	175.1	555.2	205	169.1
72 h	Treatment 1	457.2	230.4	405	295	340
	Treatment 2	703.9	353.6	735.5	-42.4	-28.14

Treatment 1- treatment with *H. suaveolens*
Treatment 2- treatment with *V. negundo*.

Figure VIII. 6. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval midgut tissue at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.



8.3.2. Enzyme activity in fat body

The results obtained for the effect of both botanicals on catalase, AST, ALT, ACP and ALP activities in the fat body tissue of *O. exvinacea* at different time intervals are presented in Table VIII. 3 and Figures VIII. 7 to 11. The table shows the mean values for different enzyme activity. The percentage of various enzyme activities in the fat body tissue are presented in Table VIII. 4 and Figure VIII. 12.

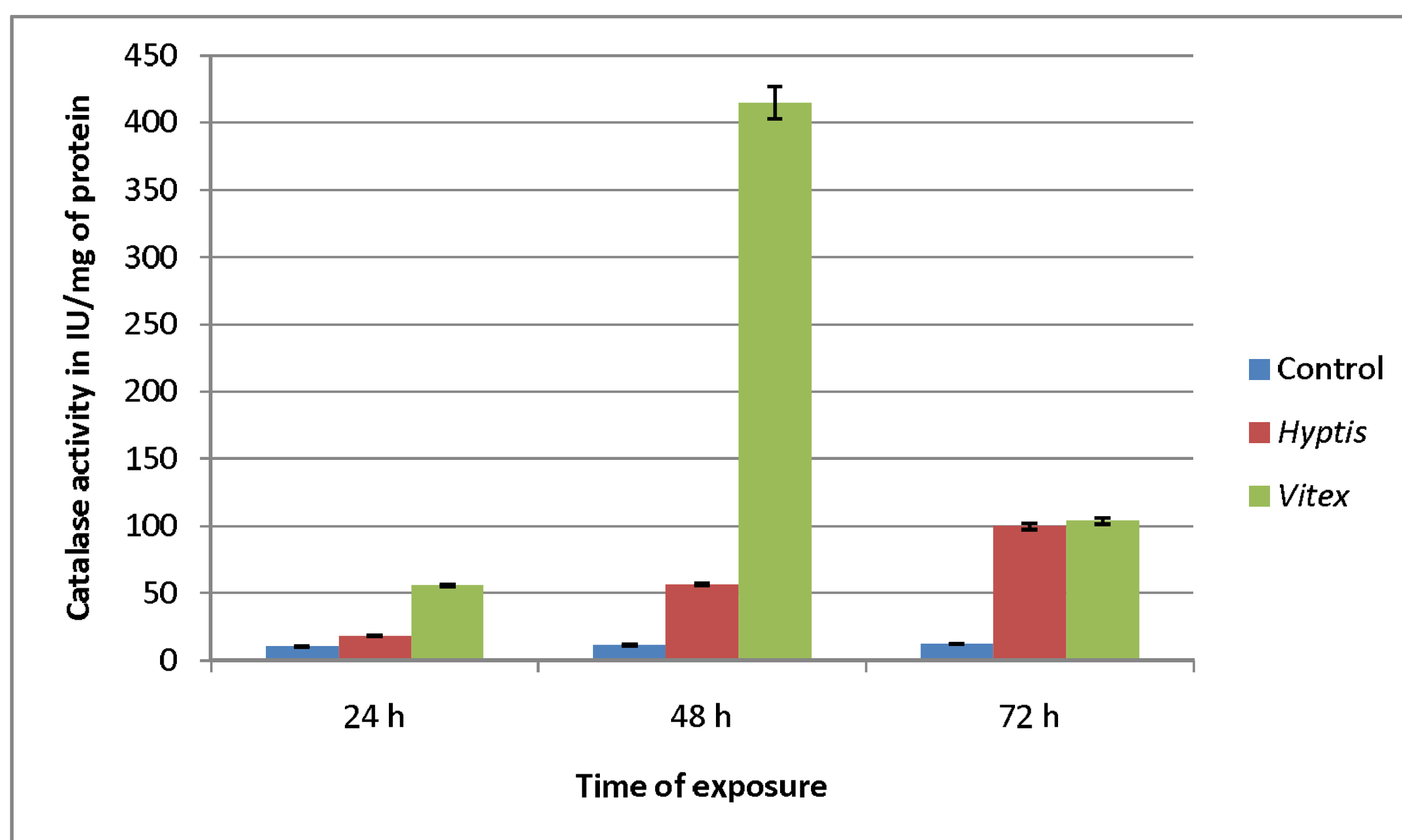
Catalase: The catalase enzyme in the untreated fat body tissue was found to be exhibiting some alterations in its activity ranging from 10.58 IU/mg to 12.8 IU/ mg of protein in accordance with different time of exposures (24 h - 72 h). The specific activity of catalase in the untreated tissue showed slight increase in its activity (Table VIII. 3). Comparison of the specific activities of treatments and control were displayed in Figure VIII. 7.

H. suaveolens treatment caused a significant increase in the specific activity of catalase enzyme on 24 h (18.48 IU/mg), 48 h (56.7 IU/mg) and 72 h (99.94 IU/mg) of exposures (Table VIII. 3). The percentages of activity for catalase with respect to control in both treatments were found to exhibit elevation in its activity at different time of exposures. For *H. suaveolens* treatment an increase of more than 74 % on 24 h followed with more than 3 folds and 6 folds increase to that of control on respective 48 h and 72 h of exposures were also noticed in the fat body tissue (Table VIII. 4 and Figure VIII. 12).

For *V. negundo* treatment, the specific activity of catalase enzyme showed 56.35 IU/mg, 414.9 IU/mg and 103.8 IU/mg on 24 h, 48 h and 72 h of exposure respectively (Table VIII. 3), it exhibited an increasing tendency when compared to the values of control in general (Figure VIII. 7). The specific activity was maximum at 48 h of exposure and thereafter at 72 h of

exposure, a slight declining tendency was noticed in the specific activity of catalase. The percentage of enzyme activity for this treatment, showed above four folds increased activity to that of control on 24 h of treatment. This change in activity was found to be much more increased (above 34 times that of control) on 48 h of exposure and at 72 h, though there was an increase in the activity of catalase when compared to control (above 7 times with that of control), the activity was noticed to be slightly decreased in comparison with the catalase activity for 48 h (Table VIII. 4 and Figure VIII. 12).

Figure VIII. 7. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on CAT activity in fat body of sixth instar larva of *O. exvinacea* at different time of exposures.



Transaminases (AST/ALT): The aspartate and alanine aminotransferase enzymes generally exhibited increasing activity with maximum time of exposure for both botanical treatments with some exceptions on 24 h of exposure for AST (Table VIII. 3).

AST: The AST activity for the control tissue with different time periods was not significantly altered, but showed slight decrease in its activity. i.e., from 11.48 IU/mg to 9.97 IU/mg with increasing time from 24 h - 72 h. Comparison between the specific activities of both treatments and control were presented in Figure VIII. 8

AST activity in *H. suaveolens* treatment caused a specific activity of 11.19 IU/mg, 22.84 IU/mg and 33.07 IU/mg for AST enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 3). Generally the AST activity in this treatment caused a significant increase with different time of exposures. The percentage of activity on 24 h of exposure was 2.53 % decrease for aspartate aminotransferase. Later on 48 h and 72 h of exposure respective increases of about 147 % and more than 2 folds that of control were noticed in the fat body tissue (Table VIII. 4 and Figure VIII.12).

For *V. negundo* treatment, the specific activity of AST enzyme was found to be 10. 37 IU/mg, 26.79 IU/ mg and 43.95 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 3). The specific activity was found to be decreased initially and later enhanced. The percentage of AST activity was found to be decreased to an extent of 9.67 % with that of control on 24 h of exposure and after 48 h and 72 h of exposure, substantial increase of enzyme activity occurred around 190 % and beyond three folds with that of control respectively. More increase in the activity of AST enzyme was found to be detected with this treatment on account of comparison with *H. suaveolens* treatment (Table VIII. 4 and Figure VIII.12).

ALT: The specific activity of ALT in the untreated tissue showed slight increase in its activity from 10.41 IU/mg, 12.66 IU/mg and 14.62 IU/mg with exposure time of 24 h, 48 h and 72 h respectively (Table VIII. 3). Comparison between control and treatments were displayed in Figure VIII. 9.

In the case of *H. suaveolens* treatment, the specific activity of ALT enzyme was found to be 26.73 IU/mg, 45.3 IU/mg and 62.96 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 3). Whereas the percentage of enzyme activity with respect to control for *H. suaveolens* treatment exhibited an increase of 156.8 % that of control on 24 h of exposure. Further exposure to 48 h and 72 h, ALT activity was significantly altered above 2 folds and 3folds respectively (Figure VIII. 12).

V. negundo treatment caused specific activities of 20.74 IU/mg, 59.58 IU/mg and 104.25 IU/mg for ALT enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 3). Maximum increase in the activity was noticed with this treatment in comparison with *H. suaveolens* treatment. Initially, on 24 h, ALT activity was enhanced to 99.2 % to that of control, which have been subsequently elevated beyond 6 folds with that of control, with in 72 h of exposure (Table VIII. 4 and Figure VIII.12).

Figure VIII. 8. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

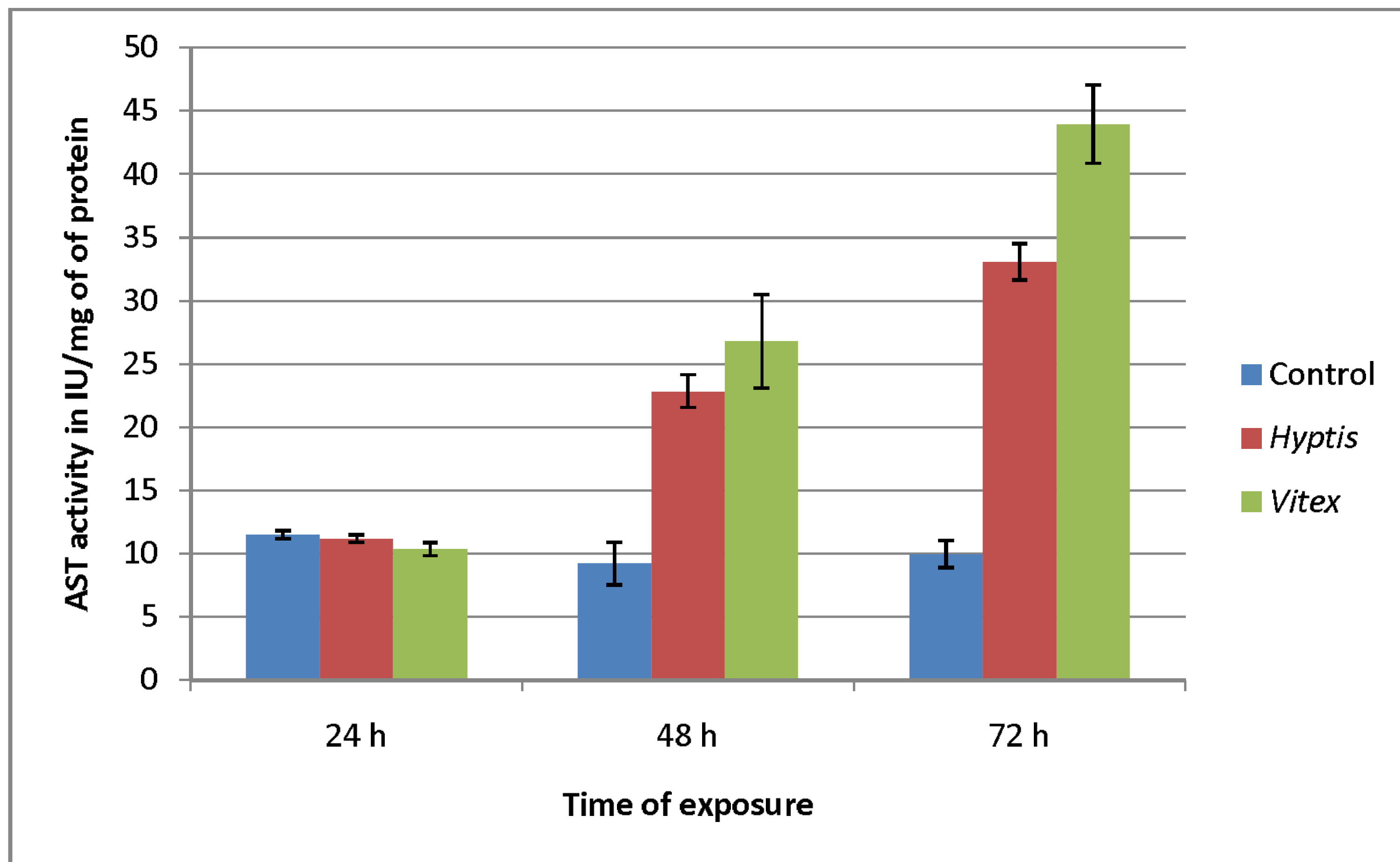
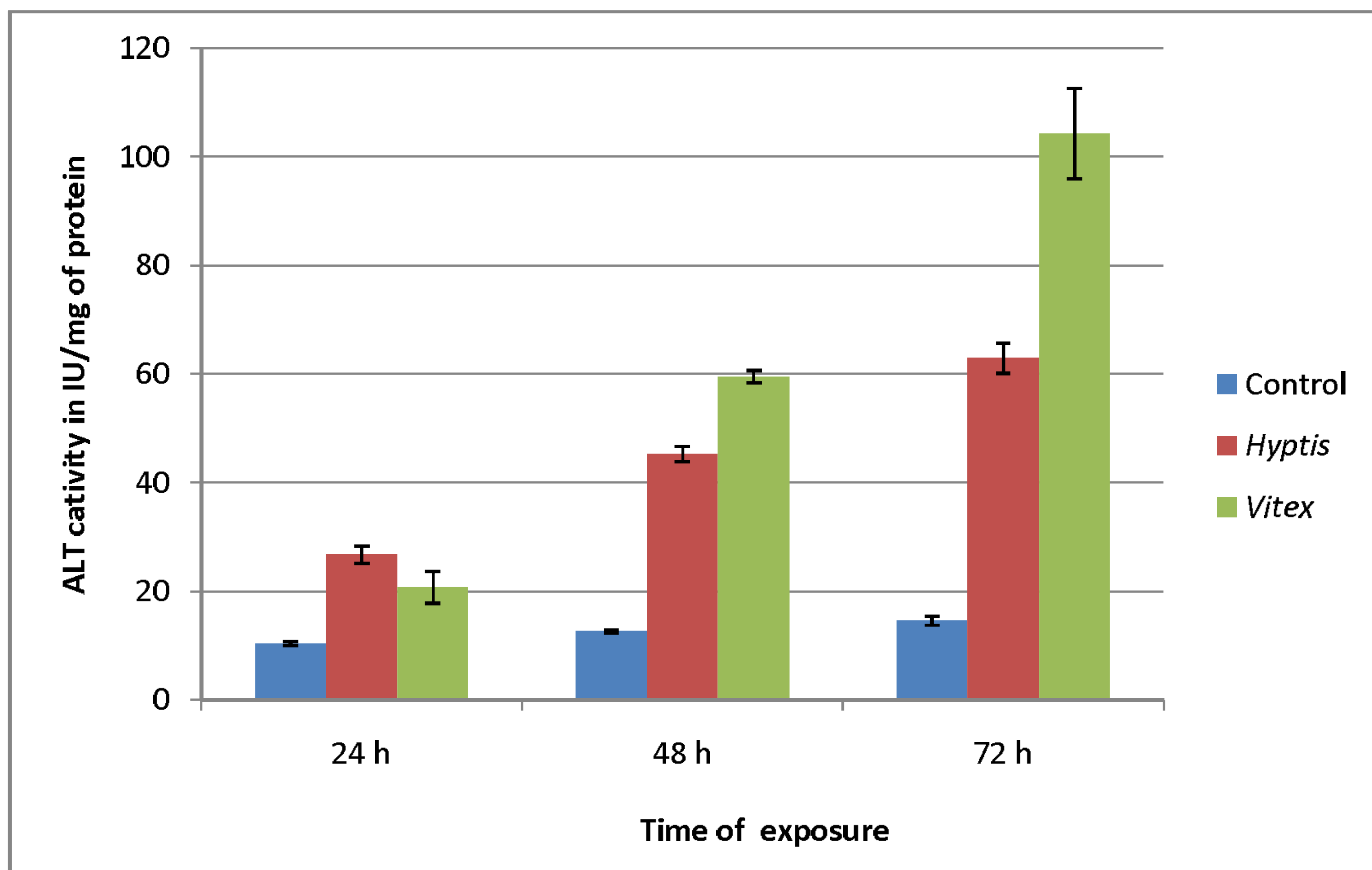


Figure VIII. 9. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.



Phosphatases (ACP/ALP): ACP and ALP activity of fat body taken from both botanical treated individuals was seemed to be enhanced with time of exposure along with slight modifications for the *Vitex* treated larvae. For these two enzymes, control fat body tissue was exhibiting regular increasing changes in their activity when subjected to different time of exposure.

ACP: The specific activity of ACP in the control tissue showed significant increase in its activity ranging from 6.6 IU/mg, 7.8 IU/mg and 15.79 IU/ mg in accordance with different time of exposure 24 h, 48 h and 72 h (Table- VIII.3). Comparative graph between treatments and control with their specific activities were presented in Figure VIII.10.

ACP activity in *H. suaveolens* treatment caused a specific activity of 9.69 IU/mg, 21.84 IU/mg and 33.79 IU/mg at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 3). ACP activity was gradually increasing with time of exposure. The percentage of activity noticed was 46.82 % increase with that of control on 24 h and then respective increases of 180 % and 114 % with 48 h and 72 h of exposures. Even though, increased activity was noticed at every exposure, a slightly lowered activity was detected for 72 h of exposure when compared to that of 48 h (Table VIII. 4 and Figure VIII. 12).

For *V. negundo* treatment, the specific activity of ACP enzyme was found to be 22.98 IU/mg, 37.13 IU/ mg and 84.05 IU/mg upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 3). The specific activity of this enzyme was found to be increased with increasing time of exposure. The percentage of activity was found to be gradually increasing for respective exposure periods. On 24 h of exposure itself, 2 folds increased ACP activity was noticed in the fat body tissue. Furthermore high ACP activity of more than 3 folds and 4 folds increase with that of control was

noticed after 48 h and 72 h of exposure respectively (Table VIII. 4 and Figure VIII. 12).

ALP: The ALP activity for the control tissue with different time periods was slightly altered with specific activity of 52.83 IU/mg, 71.69 IU/mg and 87.77 IU/mg at 24 h, 48 h and 72 h of exposures respectively. Comparison between treatments and control were displayed in Figure VIII. 11.

In *H. suaveolens* treatment, the specific activities for ALP noticed were 139.2 IU/mg, 242.6 IU/mg and 365.45 IU/mg for 24 h, 48 h and 72 h of exposures respectively (Table VIII. 3). The percentage of activity noticed for this treatment to that of control were 163 %, more than 2 folds and 3 folds of increase at exposure periods of 24 h, 48 h and 72 h respectively (Table VIII. 4 and Figure VIII. 12).

V. negundo treatment caused a specific activity of 295.6 IU/mg, 349.1 IU/mg and 167.6 IU/mg for ALP enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 3). The specific activity in this case was found to be decreasing after 48 h of exposure (Figure VIII. 11). Exposure to this treatment caused much elevated percentage of enzyme activity (more than 4 times to that of control) on 24 h of exposure itself. Followed with this increase, there observed a fall in the percentage of ALP activity on account of comparison with enzyme activity on 24 h observation, although *V. negundo* treatment resulted in an overall increase of enzyme activity under varied exposure time (Table VIII. 4 and Figure VIII. 12).

Statistical analysis of catalase, AST, ALT, ACP and ALP activity in both treatments indicated that all variables vary significantly with time of exposure.

Figure VIII. 10. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

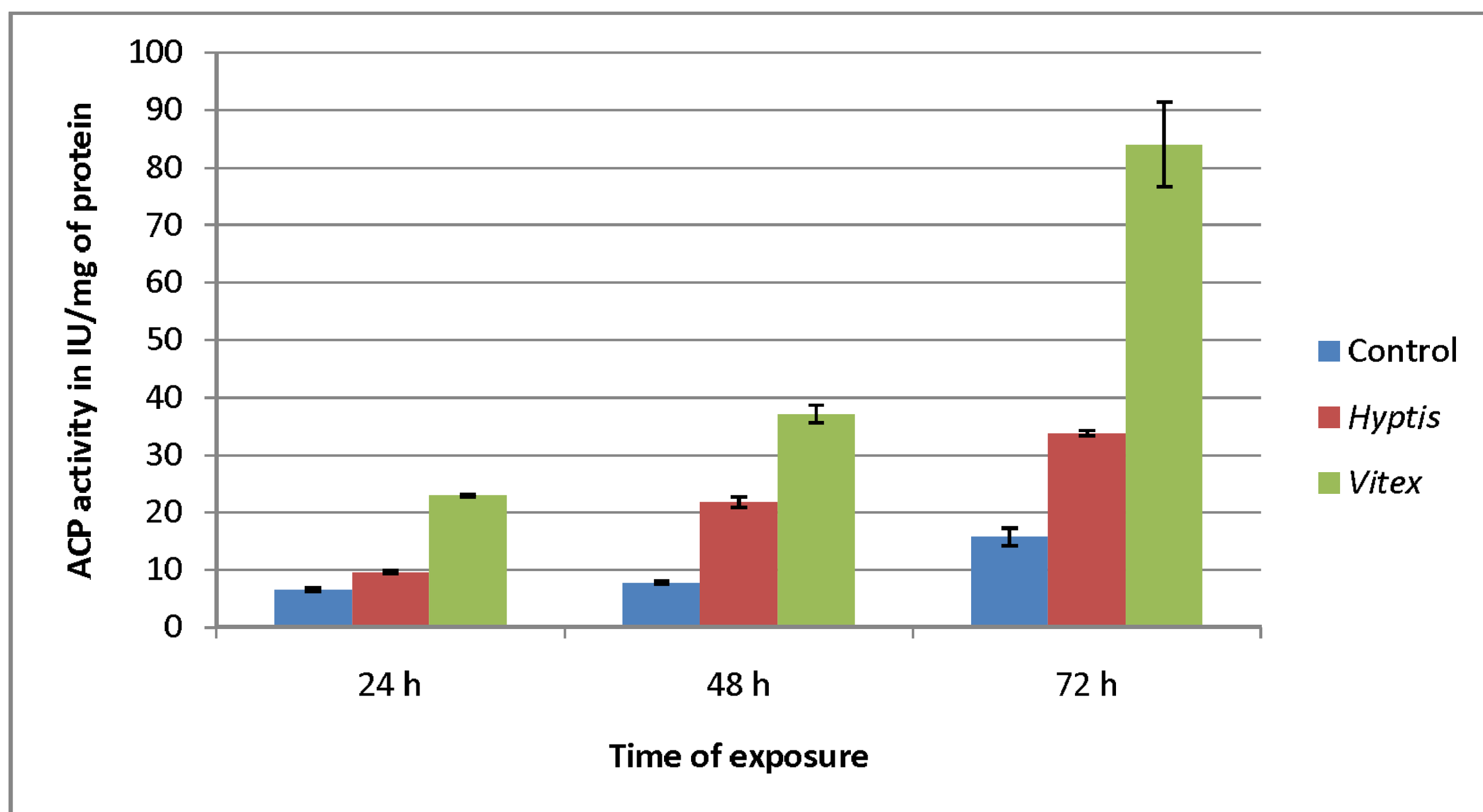


Figure VIII. 11. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

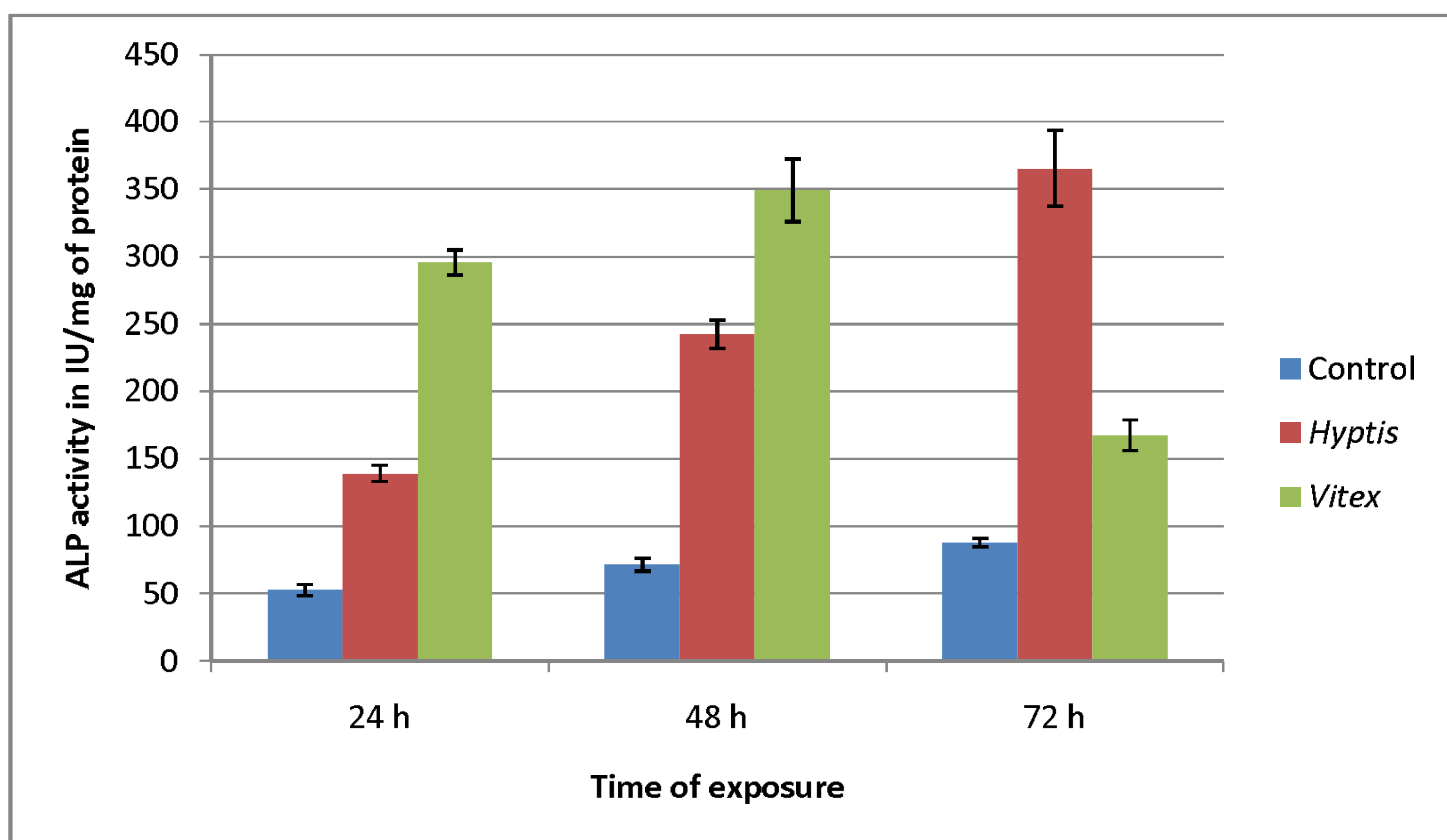


Table VIII. 3. Effect of botanicals on various enzyme activities in fat body exposed to different time of exposure.

Experiments			CAT	AST	ALT	ACP	ALP
Time of exposure	24 h	control	10.58±0.3	11.48±0.14	10.41±0.18	6.6±0.14	52.83±1.79
		Treatment 1	18.48±0.25	11.19±0.13	26.73±0.7	9.69±0.11	139.2±2.66
		Treatment 2	56.35±0.37	10.37±0.23	20.74±1.33	22.98±0.68	295.6±4.18
		F- value	6226	11.03	89.06	457.8	1637
		P- value	0.000	0.002	0.000	0.000	0.000
	48 h	control	11.77±0.2	9.24±0.76	12.66±0.12	7.8±0.13	71.69±2.2
		Treatment 1	56.7±0.48	22.84±0.58	45.3±0.65	21.84±0.42	242.6±4.65
		Treatment 2	414.9±5.26	26.79±1.66	59.58±0.51	37.13±0.21	349.1±10.46
		F- value	5238	69.4	2467	2714	432.5
		P- value	0.000	0.000	0.000	0.000	0.000
	72 h	control	12.8±0.21	9.97±0.49	14.62±0.38	15.79±0.11	87.77±1.38
		Treatment 1	99.94±1.05	33.07±0.64	62.96±1.26	33.79±0.69	365.45±12.5
		Treatment 2	103.8±1.1	43.95±1.38	104.25±3.7	84.05±3.3	167.6±5.02
		F- value	3368	351.6	389.6	331.44	333.02
		P- value	0.000	0.000	0.000	0.000	0.000

Values are means (± SEM). The activity of enzymes is expressed as units of IU/mg of protein. Treatments were found to be significant at $P<0.01$.

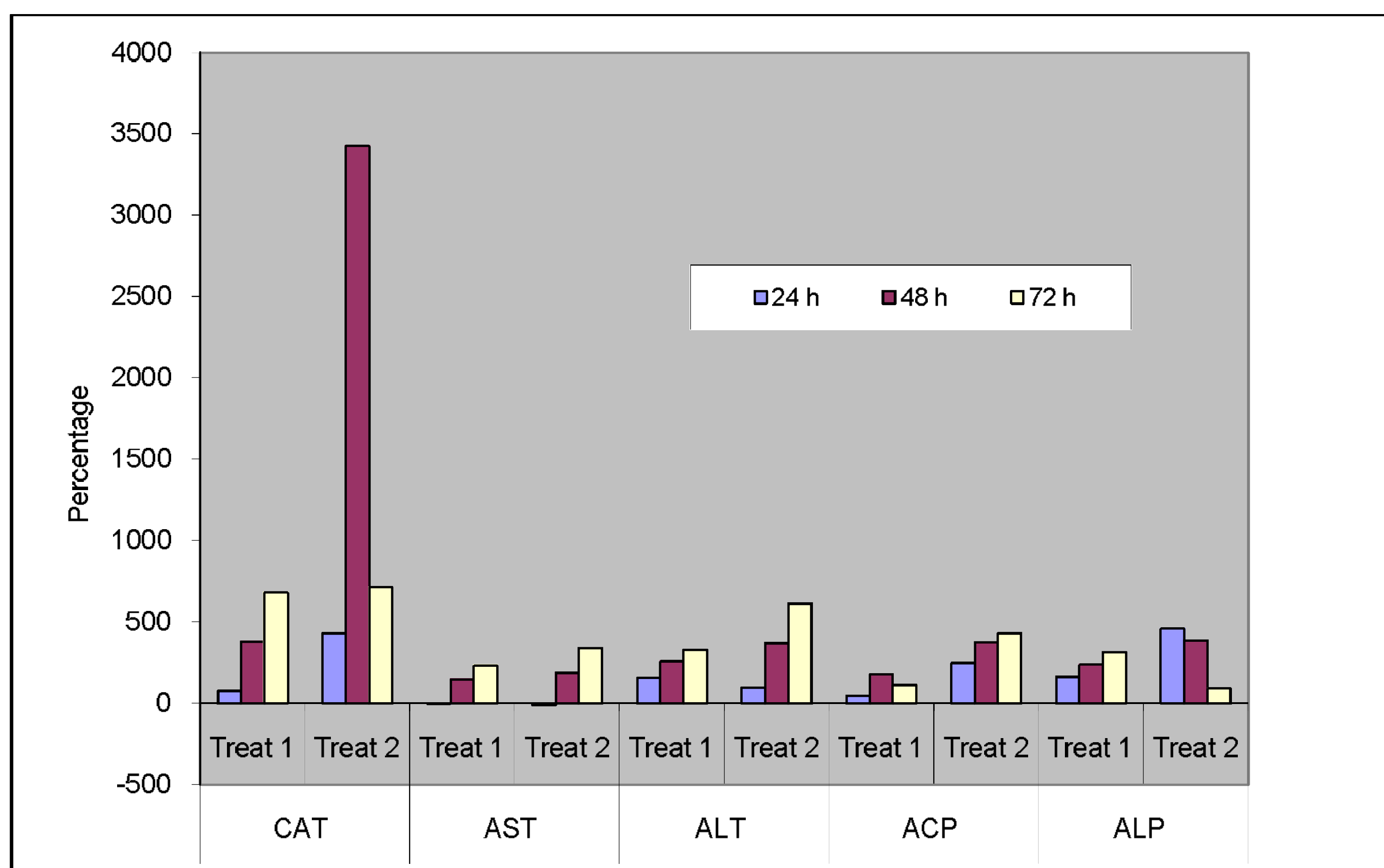
Table VIII. 4. Percentage of enzyme activity in the larval fat body at different time of exposure for both treatments with respect to control.

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	74.67	-2.53	156.8	46.82	163.5
	Treatment 2	432.6	-9.67	99.2	248.2	459.5
48 h	Treatment 1	381.7	147.2	257.8	180	238.4
	Treatment 2	3425	189.9	370.6	376	386.9
72 h	Treatment 1	680.8	231.7	330.6	114	316.4
	Treatment 2	710.9	340.8	613	432.3	90.95

Treatment 1- treatment with *Hyptis suaveolens*

Treatment 2- treatment with *Vitex negundo*.

Figure VIII. 12. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval fat body at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.



8.3.3. Enzyme activity in haemolymph

The results obtained for the effect of both botanicals on catalase, AST, ALT, ACP and ALP activities in the haemolymph of *O. exvinacea* at different time intervals were presented in Table VIII. 5 and Figures VIII. 13 to VIII. 17. The table shows the mean values for different enzyme activity. The percentage of various enzyme activities in the haemolymph was presented in Table VIII. 6 and Figure VIII. 18.

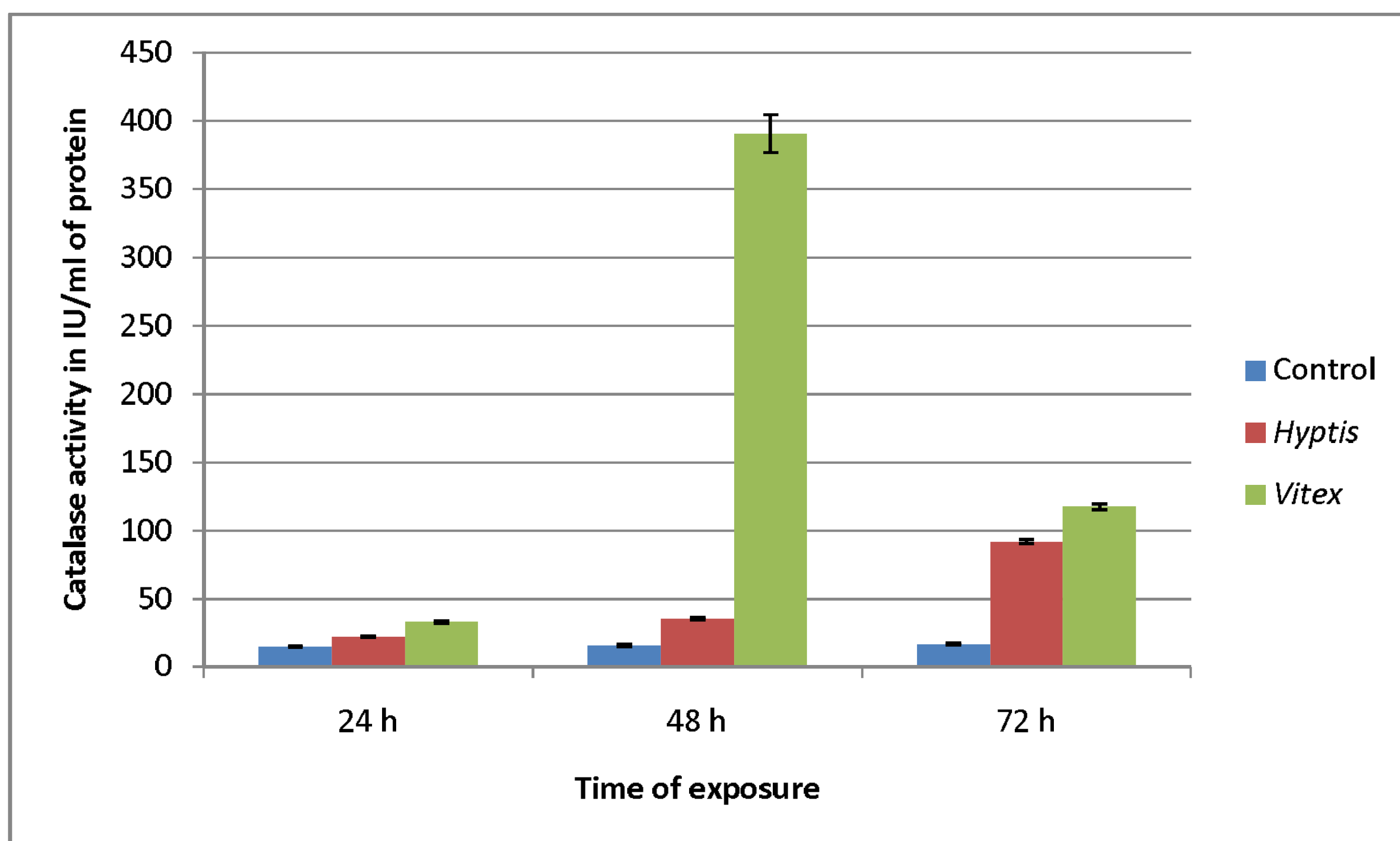
Catalase: Haemolymph of control larva was exhibiting a normal increase of catalase activity upon different exposure time. The specific activity of catalase in the untreated tissue showed slight increase in its activity from 14.84 IU/ml, 15.67 IU/ml and 16.69 IU/ml on 24 h, 48 h and 72 h of exposure respectively (Table VIII. 5). Comparative graph between treatments and control larva were shown in Figure VIII. 13.

H. suaveolens treatment caused a significant increase in the specific activity of catalase enzyme on 24 h (21.95 IU/ml), 48 h (35.48 IU/ml) and 72 h (92.02 IU/ml) of exposures (Table VIII. 5). Catalase activity in this treatment was found to be gradually increasing when compared to control. The percentage of enzyme activity with respect to control also showed significant increase in the activity of catalase with increasing time of exposure from 24 h (47.9 %), 48 h (126.4 %) to 72 h (451.3 %) (Table VIII. 6).

In the case of *V. negundo* treatment, the specific activity of catalase enzyme showed 32.98 IU/ml, 390.4 IU/ml and 117.4 IU/ml on 24 h, 48 h and 72 h of exposure respectively (Table VIII. 5), generally found to be increasing when compared to the values of control. The specific activity was maximum at 48 h of exposure and thereafter at 72 h of exposure, a slight declining tendency was noticed in the specific activity of catalase (Figure VIII. 13). The percentage of enzyme activity on 24 h showed an increase of 122.2 % to that

of control catalase activity. Thereafter an increased activity of more than 23 folds with that of control was demarcated for 48 h exposure period. Even though, increase in the activity of catalase was recognized for 72 h of exposure (6 times to that of control) the activity was found to be decreased to some extent when compared with the catalase activity for 48 h (Table VIII. 6 and Figure VIII. 18).

Figure VIII. 13. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on CAT activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.



Transaminases (AST/ALT): In the case of haemolymph samples, both botanical treatments caused significant effects on AST and ALT activities with time of exposure and were displayed in Table VIII. 5.

AST: The AST activity for the haemolymph of control individuals for different time periods was seemed to be slightly altered from 5.38 IU/ml to 6.87 IU/ml with 24 h to 72 h of exposure. Comparison between the specific activities of treatments and control were given in Figure VIII. 14.

AST activity in *H. suaveolens* treatment caused a specific activity of 22.54 IU/ml, 26.53 IU/ml and 41.84 IU/ml for AST enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). AST activity in this treatment resulted in an increased percentage of AST activity with time of exposure for 24 h (318.9 %), 48 h (347.4 %) and 72 h (509 %) (Table VIII. 6 and Figure VIII. 18).

In the case of *V. negundo* treatment, the specific activity of AST enzyme was found to be increasing from 28.26 IU/ml, 127.04 IU/ml and 161.3 IU/ml upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 5). The percentage of AST activity was detected to be increased to four folds to that of control on 24 h of exposure. Furthermore a more significant upturn (20 folds that of control) in the AST activity was noticed for 48 h of exposure, which again pronouncedly increased to an extent of 22 folds with that of control as the exposure time increased to 72 h (Table VIII. 6 and Figure VIII. 18).

ALT: Generally ALT activity was noticed to be slightly increased along with the duration of exposure in the haemolymph of control larvae. The specific activity of ALT in the untreated tissue showed some increase in its activity from 7.98 IU/ml, 10.08 IU/ml and 12.12 IU/ml with exposure time of 24 h, 48

h and 72 h respectively (Table VIII. 5). Comparison between treatments and control were given in Figure- VIII. 15.

In the case of *H. suaveolens* treatment, the specific activity of ALT enzyme noticed was 26.89 IU/ml, 50.83 IU/ ml and 71.89 IU/ml upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 5). While the percentage of enzyme activity with respect to control for the same treatment increased from 2 folds to more than 4 folds upon exposure of 24 h - 72 h (Table VIII. 6 and Figure VIII. 18).

Whereas *V. negundo* treatment caused specific activities of 41.58 IU/ml, 185.3 IU/ml and 345.1 IU/ml for ALT enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). In this case the specific activity was found to be increasing with time of exposure. 4 times of enhancement in enzyme activity occurred on 24 h of exposure itself. Further exposure to 48 h and 72 h, respective increased activities of more than 17 times and 27 times with respect to control were noticed. The associated changes in ALT activity with this treatment for every duration of exposure was found to be much more when compared with the changes of ALT activity for *H. suaveolens* treatment. Maximum activity was noticed on 72 h of exposure when compared to control larva (Table VIII. 6 and Figure VIII. 18).

Figure VIII. 14. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

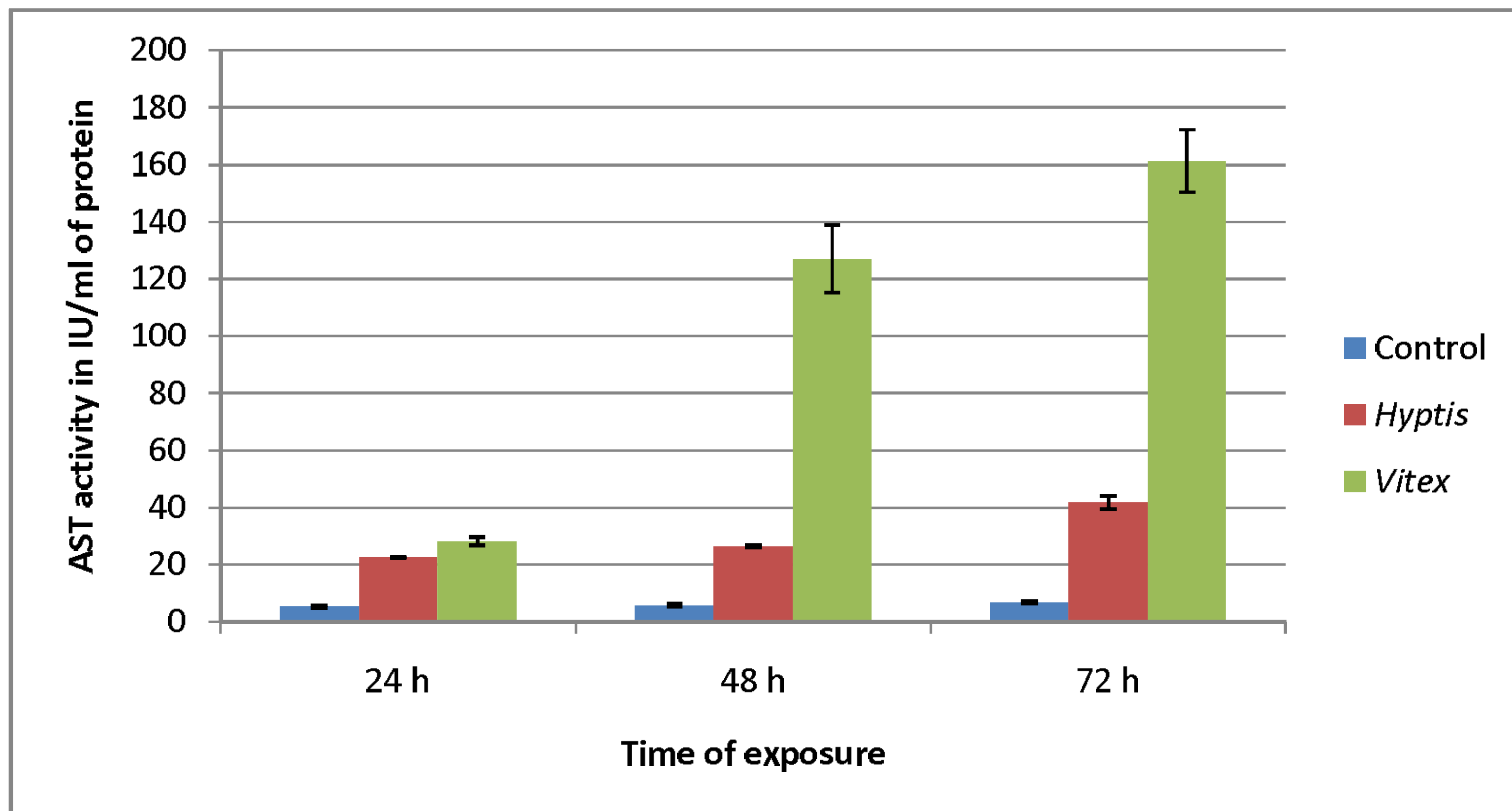
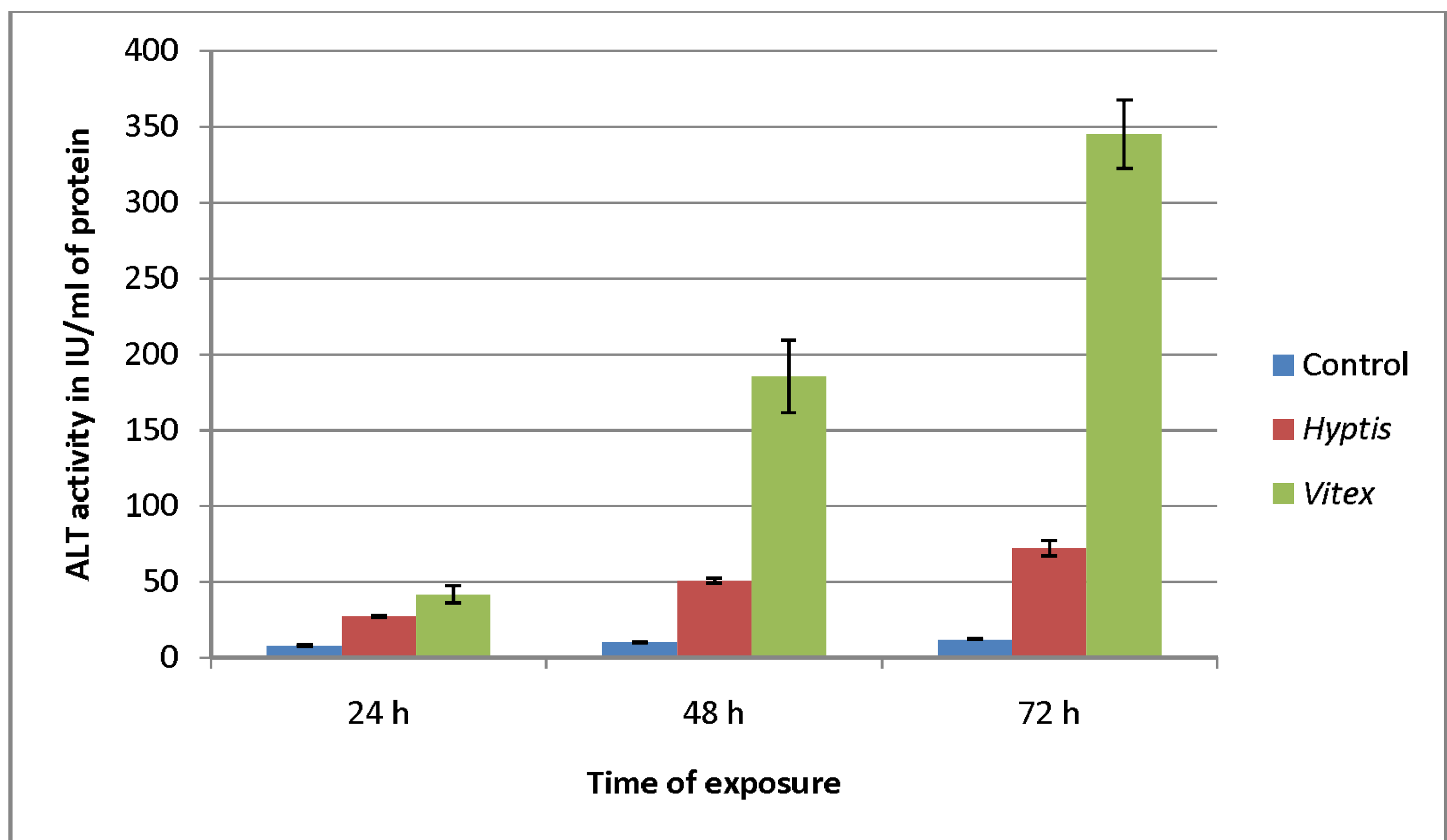


Figure VIII. 15. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.



Phosphatases ACP/ALP: ACP and ALP activities in haemolymph were seemed to be altered significantly upon exposure to botanical treatments.

ACP: The specific activity of ACP in the control tissue showed significant increase in its activity ranging from 6.81 IU/ml, 9.97 IU/ml and 11.93 IU/ ml in accordance with different time of exposure 24 h, 48 h and 72 h (Table VIII. 5). Comparison between treatments and control were given in Figure VIII. 14.

ACP activity in *H. suaveolens* treatment caused a specific activity of 22.27 IU/ml, 30.38 IU/ml and 47.73 IU/ml at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). In this case the specific activity was found to be increasing with each period of exposure (Figure VIII. 14). More than two fold elevation (227 %) in the percentage of enzyme activity was noticed on 24 h of exposure. Although the ACP activity was increased (204.7 % that of control) for 48 h of exposure, a slight decrease was noticed when compared with that of 24 h of observation. Further, an increase of 254.5 % of ACP activity was noticed on 72 h of exposure (Table VIII.6 and Figure VIII.18).

For *V. negundo* Treatment, the specific activity of ACP enzyme was found to be 69.7 IU/ml, 91.5 IU/ ml and 42.29 IU/ml upon different time of exposures of 24 h, 48 h and 72 h respectively (Table VIII. 5). Although the specific activity of this enzyme was found to be increased with increasing time of exposure, after 48 h it was found to be slightly decreased. In this treatment, the percentage of activity was found to be considerably increased to an extent of more than 9 folds to that of control on 24 h of exposure itself. For 48 h of exposure, more than 8 folds increased ACP activity with respect to control was noticed, its activity was found to be slightly decreased when compared to that of 24 h. On 72 h of exposure, an increase of above two folds to that of control ACP activity was observed in the haemolymph which was found to be low to that for 24 h (Table VIII. 6 and Figure VIII. 18).

ALP: The ALP activity for the control tissue with different time periods was altered with specific activity of 116.7 IU/ml, 124.5 IU/ml and 151.5 IU/ml at

24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). Comparison between the specific activities of treated and control were displayed in Figure VIII.17.

In *H. suaveolens* treatment, the specific activities for ALP noticed were 216 IU/ml, 379.2 IU/ml and 669 IU/ml for 24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). In this case, the specific activity of alkaline phosphatase activity was detected as progressively enhanced in the haemolymph of the larvae under different time of exposures. The percentage of activity when compared with control also showed much increased activity on exposure for 24 h (85 %), 48 h (204.6 %) and 72 h (341.6 %), in which highest activity (more than 3 folds that of control) was noticed in the larvae subjected to 72 h of exposure (Table VIII. 6 and Figure VIII. 18).

V. negundo treatment caused specific activity of 656.3 IU/ml, 1316.2 IU/ml and 728 IU/ml for ALP enzyme at 24 h, 48 h and 72 h of exposures respectively (Table VIII. 5). Though the specific activity of this enzyme was found to be increased with respect to control, a decrease was noticed after 48 h of exposure. While the percentage of ALP activity was found to be amplified in the range of 4 folds to that of control on 24 h of exposure. Further exposure to 48 h caused a more significant increase in the ALP activity to an extent of more than 9 folds with that of control. Though there was promotion in the activity of ALP under 48 h of exposure when compared to control, further exposure for 72 h caused an increase of only above 3 folds to that of control which was noticed to be slightly declining in its activity (Table VIII. 6 and Figure VIII.18).

Statistical analysis of catalase, AST, ALT, ACP and ALP activity of haemolymph in both treatments exhibited that all variables vary significantly with time of exposure.

Figure VIII. 16. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

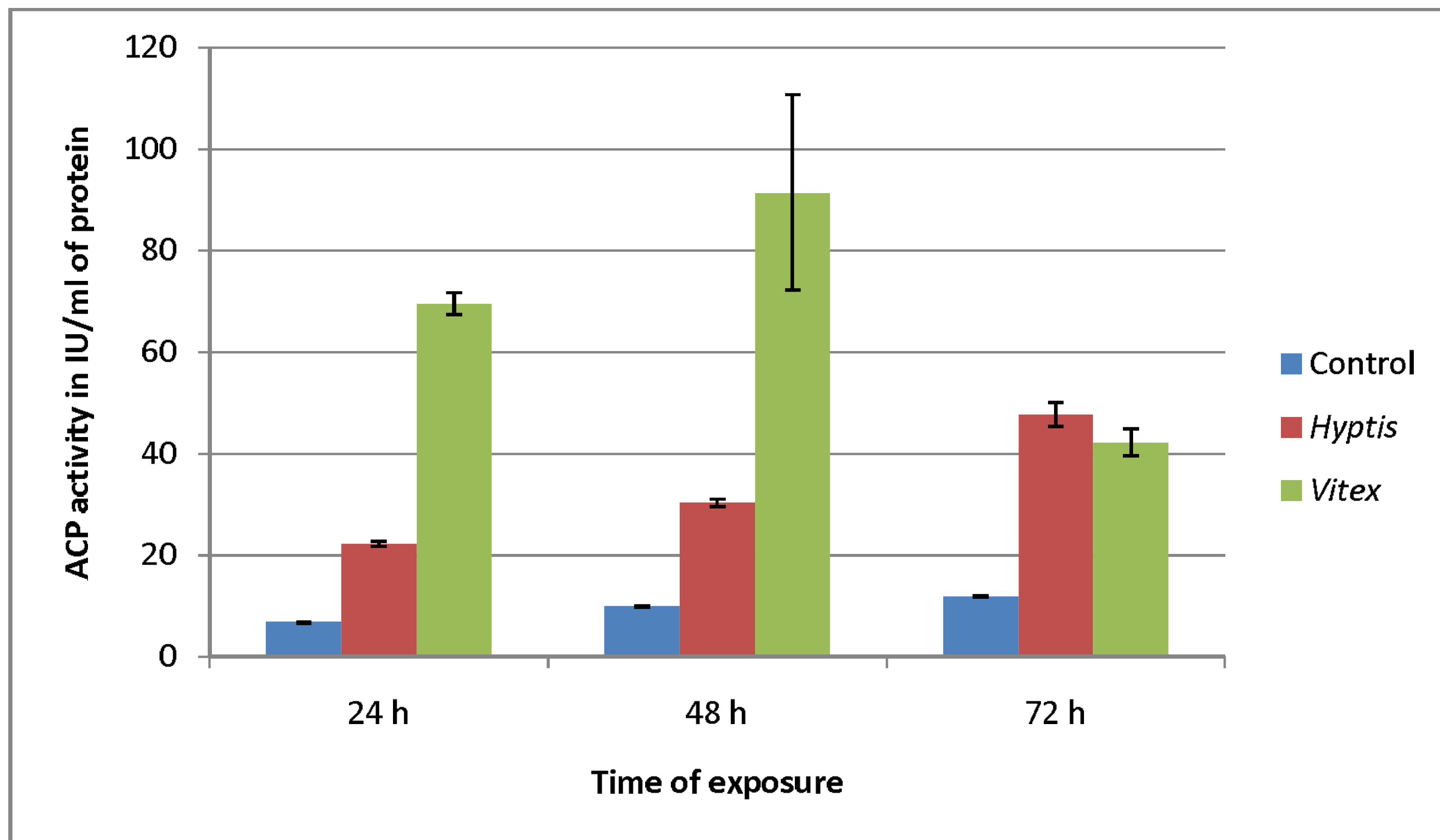


Figure VIII. 17. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

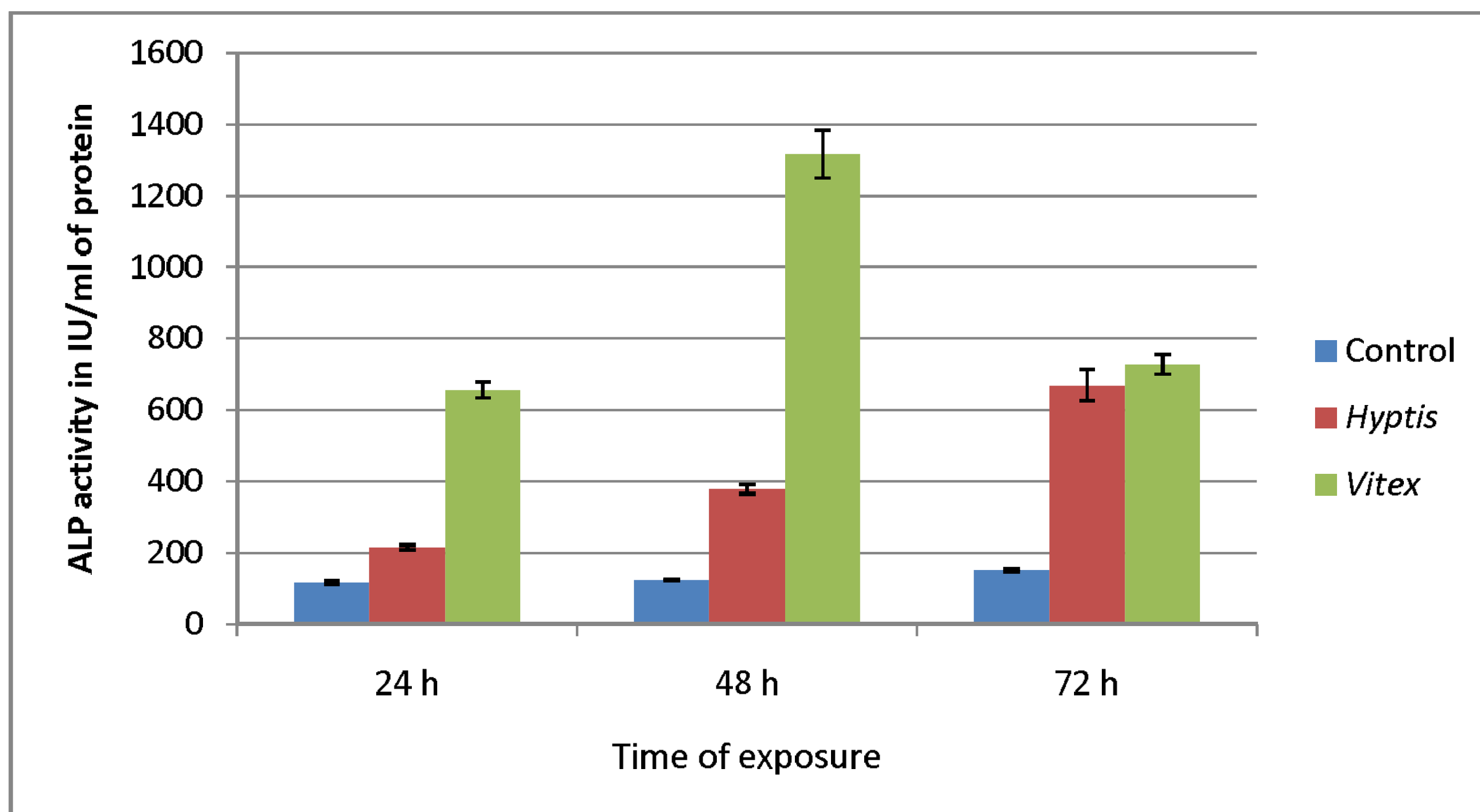


Table VIII. 5. Effect of botanicals on various enzyme activities of haemolymph exposed to different time of exposure.

Experiments			CAT	AST	ALT	ACP	ALP
Time of exposure	24 h	control	14.84±0.29	5.38±0.24	7.98±0.25	6.81±0.07	116.7±2.04
		Treatment 1	21.95±0.25	22.54±0.09	26.89±0.23	22.27±0.23	216±3.02
		Treatment 2	32.98±0.39	28.26±0.63	41.58±2.52	69.7±0.97	656.3±10.09
		F- value	843.01	924.06	131.73	3181	2152
		P- value	0.000	0.000	0.000	0.000	0.000
	48 h	control	15.67±0.39	5.93±0.27	10.08±0.07	9.97±0.09	124.5±1.32
		Treatment 1	35.48±0.3	26.53±0.21	50.83±0.78	30.38±0.34	379.2±5.84
		Treatment 2	390.4±6.2	127.04±5.33	185.3±10.66	91.5±8.6	1316.2±30.1
		F- value	3452	441.42	220.52	72.87	1253
		P- value	0.000	0.000	0.000	0.000	0.000
	72 h	control	16.69±0.25	6.87±0.17	12.12±0.15	11.93±0.07	151.5±2.06
		Treatment 1	92.02±0.64	41.84±0.99	71.89±2.28	47.73±1.04	669.1±19.3
		Treatment 2	117.4±00.88	161.3±4.87	345.1±10.12	42.29±1.19	728±12.44
		F- value	6647	794.54	878.27	445.11	566.88
		P- value	0.000	0.000	0.000	0.000	0.000

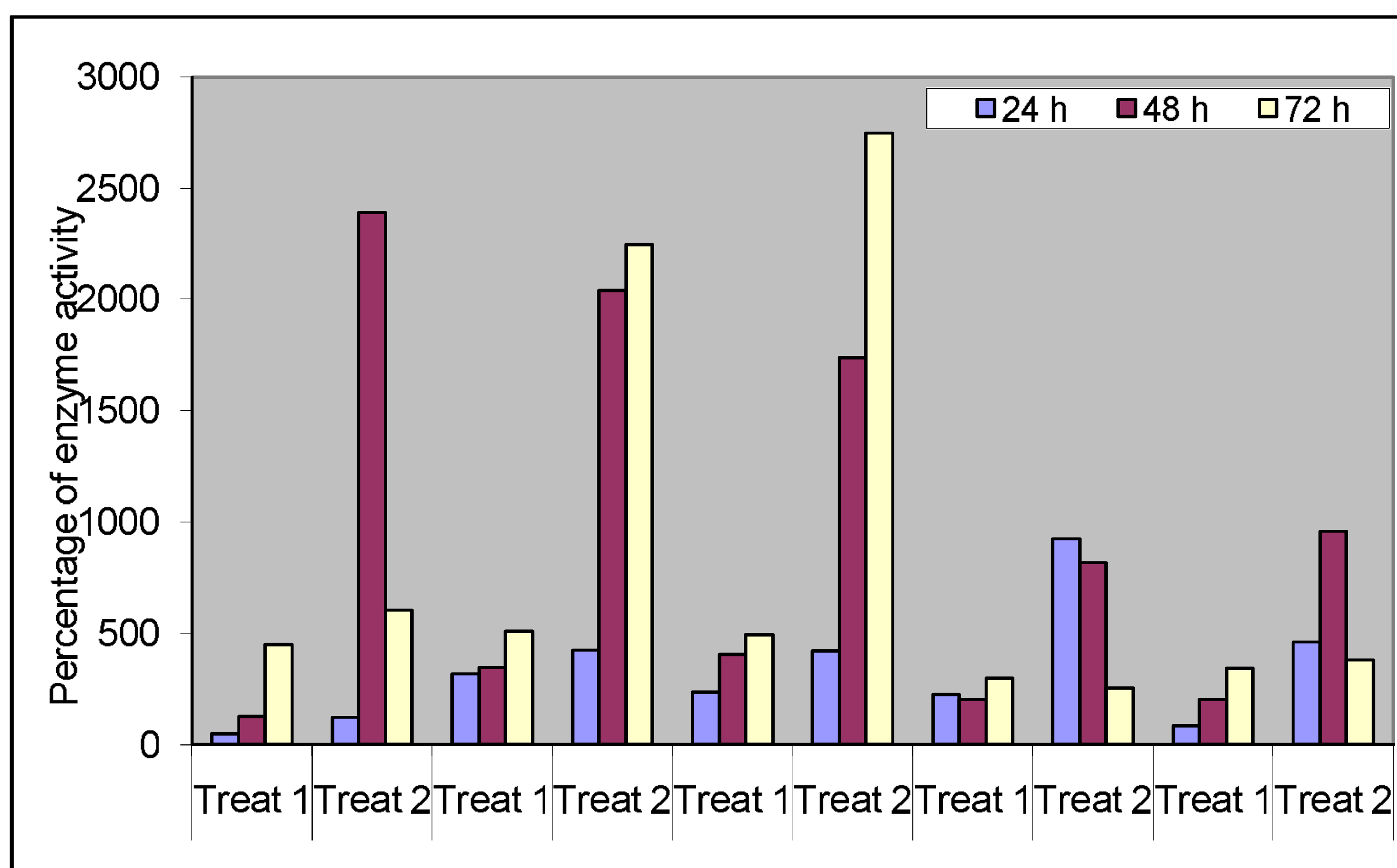
Values are means (± SEM). The activity of enzymes is expressed as units of IU/ml of protein. Treatments were found to be significant at $P<0.01$.

Table VIII. 6. Percentage of enzyme activity in the larval haemolymph at different time of exposure for both treatments with respect to control

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	47.9	318.9	237	227	85
	Treatment 2	122.2	425.3	421	923.5	462
48 h	Treatment 1	126.4	347.4	404	204.7	204.6
	Treatment 2	2391	2042	1738	817.7	957.2
72 h	Treatment 1	451.3	509	493.1	300	341.6
	Treatment 2	603.4	2248	2747	254.5	380.5

Treatment 1- treatment with *Hyptis suaveolens*
Treatment 2- treatment with *Vitex negundo*.

Figure VIII. 18. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval haemolymph at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.



8.4. Discussion

The studies on the effect of both botanicals on the enzymatic activities in different tissues indicated that the normal physiological processes of sixth instar larvae of *O. exvinacea* can be influenced drastically.

In the present study, catalase activity was found to be increasing generally with time of exposure for all the tissues. For each treatment interval the activity of the enzyme was gradually increasing in the case of *H. suaveolens*. Whereas for *V. negundo* maximal increase was noticed at 48 h of exposure and later it was found to be decreased when compared with the activity at 48 h of post treatment.

In the present study, an increase in catalase activity was found to be noticed for *H. suaveolens* treatment at all-time intervals in the midgut tissue, fat body and haemolymph. Whereas in the case of *V. negundo* treatment, significantly elevated catalase activity was observed from 24 h - 48 h of exposure. Thereafter at 72 h, its activity was decreased to some extent, although it shows an increasing trend when compared to control. The present study results were in agreement with the findings of Keywanlee and Berenbaum (1990) who observed increased catalase activity in the fruit fly, *Drosophila melanogaster* upon feeding plants which have abundant furanocoumarins. Similarly, increased catalase activity was reported with the treatment of kinetin on the larvae of vinegar fruit fly, *Zaprionus indianus* (Sharma *et al*., 1997) and nymphs of *Lipaphis erysimi* when fed with kinetin treated food (Rup *et al*., 2006). Likewise, increased CAT activity was noticed in *Bactrocera cucurbitae* larvae with the treatment of partially purified soyabean protease inhibitors (Kaur and Sohal, 2012) and certain plant extracts (Kaur *et al*., 2014). Moreover, parallel results were reported by Patel *et al*. (2014) with the treatment of biopesticide formulation (9 botanicals: 2

chemicals: cow urine) for the control of mealy bug borer activity in brinjal which caused an increased catalase activity when compared with control.

In the present study, the treatment of *V. negundo* caused a decrease in enzymatic activity at 72 h when compared to 48 h exposure time although it shows an increase with respect to control. Complementary results were reported by Vasudev *et al.* (2015) in which they observed low catalase activity during initial treatment interval but was significantly increased after 48 h and 72 h (less than 48h of catalase activity) durations of treatment with methanolic bark extract of *Acacia nilotica.* The same study reported that catalase activity was found to be decreased for 24 h and 48 h whereas it was increased for 72 h of feeding with the treatment of acetone bark extract of *A. nilotica.*

Generally, variation in the catalase activity in an organism is associated with stress causing environmental conditions. When an organism is subjected to chemicals or insecticides, irradiation or sometimes infection, it may encounter oxidative stress which inturn lead to the increased production of reactive oxygen species (ROS) (Knopowski *et al*., 2002). Moreover, the formation of ROS collapses biomembranes through lipid peroxidation, noticed to be a common process during stress provoked reactions in living systems (Panda *et al*., 2003). Generally, the initial response towards oxidative stress involves the formation of reactive oxygen species (ROS) which in turn instigate multiple auto oxidative sequential reactions on membrane unsaturated fatty acids and proteins, yielding lipid peroxides and protein carbonyls correspondingly, giving rise to a series of reactions eventually causing destruction of organelles and macromolecules (Jamieson, 1989). Even under normal circumstances, minute amounts of ROS are produced naturally, as byproducts of redox reactions such as oxidative phosphorylation in the mitochondria and β- oxidation of fatty acids. But its excessive formation

impairs cellular lipids, nucleic acids, proteins which ultimately result in lipid peroxidation, genome instability or gene mutation; protein carbonyl formation and enzymatic lethargy, which inturn bring about various degenerative processes (Finkel and Holbrook, 2000). Normally animal cells possess an antioxidant defense system consisting of three enzymes; superoxide dismutase, catalase and glutathione peroxidase to defend against the excessively formed ROS to maintain minimum balanced levels of ROS and other radicals in the cell, a process concerning correct management for their location and amount (Meng *et al*., 2009). SOD acts upon the superoxide anions directly while there is formation of potentially toxic hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969). Catalases and glutathione peroxidases are responsible for the degradation of this toxic hydrogen peroxide into water and oxygen (Dringen, 2000).

Many acceptable views rationalize the reasons for altered catalase activity under stress conditions. The reduction in enzyme activity with increasing time of exposure in the case of *V. negundo* treatment indicates reduced metabolism in the insect and it may perhaps attributed to the toxic effects of the plant compounds on membrane permeability, especially on the gut epithelium (Senthil-Nathan *et al*., 2005). According to Kono and Fridovich (1982) the decrease of catalase activity with increasing time of exposure, may be due to the fact that inhibition of CAT activity could be triggered with the buildup of superoxide anion during annihilation processes, which may be caused by toxic ingredients of botanicals. Later, the enhanced assemblage of free radicals may lead to depletion or inactivation of CAT enzyme. Perić-Mataruga *et al*. (2006) reported similar results in the midgut of *L. dispar* larvae fed on an unfavorable host plant.

The increase observed in the catalase activity in the present findings in *O. exvinacea* larva could be linked to its substantial function in the

detoxification of free radicals generated by botanical treatment. An alteration in the activity of CAT observed in *O. exvinacea* after botanical treatment suggest that the toxic components of both plant extracts may cause oxidative damage in the larva, probably by generating reactive-oxygen stress in their bodies, similar findings for CAT have also been observed by Fornazier *et al.* (2002). In the present study an increased level of CAT activity has been observed in the midgut, fat body and haemolymph of *O. exvinacea* which might be an effect of the high resistance of CAT to overcome the generation of free radicals.

The increase in catalase activity in all the three tissues for different time periods might be due to increased generation of ROS. The increased CAT levels induced by botanicals in the larva of *O. exvinacea* suggest elevated antioxidant level in order to nullify the impact of ROS. It was supported with the statement, enhancing CAT activities were most likely a response towards increased ROS formation in pesticide toxicity (John *et al.*, 2001).

The botanical treatments in the present study significantly transformed the activity of the two assayed aminotransferases at different time of exposures. The effect on these two enzymes was found to be quite similar, i.e., an increasing trend for both botanicals. Although, activity of these enzymes was found to increase with exposure time, the maximum activity was noticed at 72 h of treatment.

ALT, a transaminase enzyme which catalyzes two parts of the alanine cycle in transamination process, was found in various tissues especially in liver (vertebrates) and fat bodies (insects) (Thomas, 1998). Any impairment in the cells of these tissues affected enzyme activity with the corresponding discharge of these enzymes accompanied by other intracellular proteins/enzymes into the haemolymph causing boosted activities of these

enzymes in the blood. Elevation of ALT activity occurs under altered physiological state of the organism which was brought about by various circumstances such as infection by microorganisms, tissue damage or entry of toxic materials (Giboney, 2005).

AST is similar to ALT, which is also found to be catalyzing transamination process. It accelerated the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate as a crucial step during transamination in insects (Klowden, 2007). The results of the present study were correlated with the findings of many previous studies on the effect of plant extracts on ALT and AST activity. It was reported that botanical extracts have the capacity to alter the enzyme pattern in the insect body for several causes (Naqvi *et al*., 1991). Moreover, comparable results of increasing activity for AST and ALT was reported with the treatment of different concentrations of *A. annua* on *Xanthogaleruca luteola* at different time intervals (Shekari *et al*., 2008) and with the toxic effects of *Tephrosia purpurea* and *Acalypha indica* aqueous extracts on *Dysdercus cingulatus* (Sahayaraj and Sobha, 2012). Similarly, disturbed AST activities were reported on *S. gregaria* with the application of botanicals such as *Nigella sativa* extracts (Hamadah, 2009) and *Fagonia bruguieri* extracts (Tanani *et al*., 2009). Moreover, complementary results of inducing effects on ALT and AST activity were reported in the haemolymph and fat bodies of *S. gregaria* after treatment with different solvent extracts of *Punica granatum* peel (Ghoneim *et al*., 2014).

Several possible reasons have been suggested for increasing activity of aspartate aminotransferase and alanine-aminotransferase enzymes. Since these enzymes function as a strategic link between the carbohydrate and protein metabolism, thereby undergo changes during various physiological and pathological conditions, which is strongly supported by Hasheminia *et al.*

(2011) who reported alteration in aminotransferases upon treatment with botanicals to Lepidopteran insects. Moreover, Smirle *et al*. (1996) described that metabolic changes and reduction in the protein content also affected enzyme titers of *C. rosaceana* upon treatment with neem. Enhanced levels of AST and ALT in different tissues of *O. exvinacea* might be due to the stress response induced by the toxic principles of the treated botanicals in order to produce keto acids like α- ketoglutarate and oxaloacetate for energy production through gluconeogenesis to encounter the high energy demands.

The increasing activity of ALT and AST level in the tissues of *O. exvinacea*, in response to the tested plant extracts in the present study might be due to the direct or indirect effect of toxic ingredients on synthesis or functional aspects of the enzymes through cytomorphological changes or by the toxic effect of active components of the tested extracts on the neurosecretory hormonal pattern (Nath, 2000). According to Rawi *et al*. (2011) the excessive immediate discharge of AST and ALT may perhaps be attributed to the increasing demand of aspartate or alanine for gluconeogenesis particularly during impaired carbohydrate metabolism or under stimulated tissue damage, which provoke secretion of more amount of both AST and ALT to nullify the effect of secondary metabolites present in the botanicals.

Apart from botanicals, chemical insecticides also may alter the transaminase levels, for instance, Ender *et al.* (2005) noticed similar increased activities of ALT and AST in greater wax moth, *Galleria mellonella* larvae with the treatment of methyl parathion.

In the present study, *H. suaveolens* and *V. negundo* leaf extracts significantly altered the activities of two assayed phosphatases (ALP and ACP) in the midgut tissue, fat body and haemolymph of *O. exvinacea* upon different time of exposure. Although, alteration was noticed for both

phosphatase enzymes in the untreated tissue, both treatments showed significant differences in the variation of enzyme activities when compared with that of control. Comparison between treatments in terms of efficacy revealed that *H. suaveolens* at different time of exposures possess an enhancing effect upon the two phosphatases in midgut tissue, fatbody and haemolymph whereas *V. negundo* in general resulted in a declining activity except haemolymph ALP activity in which it caused much progressive increase initially and later followed a decreasing pattern of phosphatase activity in accordance with increasing time of exposure.

Conclusively, on the grounds of change in percentage of enzyme activity in comparison with that of the control the effects on midgut tissue were inducing in the case of *Hyptis* treatment for both ACP and ALP activity whereas, the same tissue trailed an inhibiting trend for *Vitex* treatment. In the case of fat body, treatment with *H. suaveolens* resulted in an increasing trend for both ACP and ALP while that for *V. negundo* brought about enhanced activity for ACP and declined activity for ALP. The activity in haemolymph displayed an increasing trend with the treatment of *H. suaveolens* for both phosphatases and for *V. negundo*, ACP enzyme activity was found to be decreasing while that of ALP activity was noticed to be maximally increased upto 48 h and then found decreased at 72 h, though there was an increase with respect to that of control exist.

The inducing effect on the ACP and ALP activity of the methanolic extracts of *H. suaveolens* in midgut tissue, fat body and haemolymph of *O. exvinacea* was in concurrence with that of the results reporting elevated effects of a number of botanicals on ACP and ALP enzymes in various insects such as, treatment of azadirachtin on *H. armigera* (Babu *et al.*, 1996); Margosan-O (a neem preparation) and Jojoba oil on *Musca domestica* (Ghoneim *et al.*, 2008); Neemazal on *S. gregaria* (Hamadah, 2009); *Ammi visnaga* extracts on *S. gregaria* (Ghoneim *et al*., 2014) and treatment of garlic oil on *T. castaneum* larvae (Beltagy and Omar, 2016).

Consistent results with the present study were again reported for the inhibitory ACP and ALP activity with the treatment of neem fraction on *S. litura* (Ayyangar and Rao, 1990). Reducing activity level of ACP and ALP for *V. negundo* treatment in the tissues of present study corroborate with similar reports in various insects such as declining tendency with the treatment of plant extracts and insect growth regulators on *S. littoralis* (El-Sheikh, 2002; Hassan, 2002); azadirachtin and nuclear polyhedrosis virus individually and in combination against *S. litura* (Senthil-Nathan *et al*., 2005) and *Melia azedarach* seed extract treatment on rice leaves fed by *C. medinalis* (Senthil-Nathan, 2006).

The present study results obtained for haemolymph ALP enzyme activity in the case of *V. negundo* treatment showed a preliminary upturn in its activity reaching its maximum at 48 h and later declined even though the activity was higher than that of control at 72 h. This report correlated with the findings of many researchers. For instance, Upadhyay *et al*. (2011), in which they noticed toxic effects of synthetic termiticides, fibronil, thiomethoxam and malathion on Indian white termite, *Odontotermes obesus* caused a significant increase in phosphatases activity after 4 h of treatment in comparison to that of control and later on a decrease was recorded at 16 h of treatment. Moreover parallel results with the present study were reported by Zibaee and Bandani (2010) with Sunn pest, *Eurygaster integriceps* after exposure to methanolic extract of *Artemisia annua*, the phosphatase activity maximally increased at 12 and 24 h of exposure but it found decreased at 48 h and 72 h of treatment. Similarly, Zibaee *et al*. (2011) reported elevation of the same kind of enzyme activity after treatment of methanolic extract of *A. annua* on fall webworm, *Hyphantria cunea* larvae under different exposure periods (24 h, 48 h and 72 h).

The increased ACP activity with the treatment of *H. suaveolens* in the present study on *O. exvinacea*, may be due to the toxicity of secondary metabolites present in the botanicals which enhances lysosome number,

lysosomal mobilization and cell necrosis increases which in turn provokes the supply of phosphate group for energy metabolism pointing to the breakdown of lysosomes and bring about discharge of lysosomal enzymes into the surroundings or else the disturbance of ACP activity might be caused by the interruptions due to botanicals used in the study, since the ACP activity may directly or indirectly linked with digestion, absorption and positive transport of nutrients in the midgut (Senthil-Nathan *et al*., 2004).

Several factors like unusually stressed or diseased conditions and administration of pesticides may instigate disturbance in the activity of ALP such as diminution (Miao, 2002). In mid gut tissue, fat body and haemolymph a general promoting effect of the botanicals on ALP activity could be detected, with some exceptions. On the other hand, increasing ALP activity in tissues of *O. exvinacea* in the present study may indicate the involvement of this enzyme in detoxification process against the toxicants contained in botanical extracts, as suggested by Shekari *et al.* (2008) for other plant extracts as insecticides. Sometimes the inhibited activity of both phosphatases might be due to the imbalance in enzyme substrate complex and inhibition of peristaltic movement of the gut in the treated insects (Senthil-Nathan *et. al.,* 2005). On the whole, the increase in ALP/ACP may be possibly due to the abrupt effort of *O. exvinacea* larva to reimburse the energy loss owing to the stress induced by the pesticides with the enhancement of metabolic activity. Under acute stress, the normal physiological response of an organism ensues with the boosted secretory activity. In the present study, the resulted enzymatic changes probably brought about with the alteration in metabolic pathways are symptomatic of the cellular toxicity and tissue damage provoked by the botanical treatments in *O. exvinacea*.

CHAPTER IX
GENERAL DISCUSSION

The indiscriminate application of chemical insecticides, has led to many unforeseen problems such as development of resistance in pests, pest outbreak, ground water contamination, environmental and food chain contamination, suppression of natural enemies or predators/ parasitoids and side effects on non-target organisms. Moreover, several public health problems are also reported. This scenario initiated renovated attention towards the emergence of biodegradable pesticides as secure route for use in integrated pest management strategies of crops, in which, the application of botanical pesticides were reported to be the most definitely biodegradable, ecofriendly and target specific control measure (Ignacimuthu, 2004).

Plants liberate broad collection of chemical compounds as defensive arsenals termed as secondary metabolites which are far superior to the synthetic insecticides. These secondary metabolites present in plants were reported to be promising against agricultural pest attack (Brito *et al*., 2006). Though the use of plant extracts by human is an ancient practice that continues today also. The isolation and purification of these defensive chemicals extracted from plants and their identification and determination of chemical structures also would make available some beneficial hints for the elaboration of noval pesticides and innovative methods of pest control.

Among the pests of mango orchards, the mango leaf webber, *Orthaga exvinacea* recently have got a serious pest status causing severe damage to the mango plantation leading to loss of crop productivity. Since it is an important fruit crop with great economic values in India, heavy infestation of this pest causes immense economic loss. Literature survey showed that there is no biopesticidal control measure reported yet for the effective management of

O. exvinacea with the treatment of botanicals. In the present study, *Hyptis suaveolens* and *Vitex negundo* were used for testing the lethal toxicity and biopesticidal effects on the sixth instar larva of *O. exvinacea*. Since these two plants were having insecticidal properties towards larval stages of many insects that have been already discussed in chapter IV, these botanicals were selected for the present study.

During the present investigation, toxicity studies were performed with the most toxic chloroform fraction separated from the methanolic leaf extracts of both plants, *H. suaveolens* and *V. negundo*. Chloroform fractions of both plants were found to be toxic against the mango pest, *O. exvinacea*. Results obtained in the present study indicated that both plants contained several toxic components with varying toxic effect on the insect.

The chloroform fractions were tested on the sixth instar larva of *O. exvinacea* to find out the LD_{50} value for both plant materials within minimum requirement of time. For *H. suaveolens* 60 µg, 65 µg, 70 µg, 75 µg and 80 µg concentrations were used to find out the lethal dosage. The LD_{50} value for this treatment was found to be 70.16 µg within 60 h post treatment. For *V. negundo* 20 µg, 25 µg, 30 µg, 35 µg and 40 µg concentrations were used for treatment and the LD_{50} value noticed was 33.16 µg for 48 h post treatment. When the toxicity results were compared for the botanicals, 50 % mortality was obtained for *V. negundo* within 48 h while that of *H. suaveolens* was after 60 h of post treatment.

Among the two plants tested, *V. negundo* exhibited the highest toxic effect than *H. suaveolens* since *V. negundo* need only 48 h of treatment with its minimum concentration. However, both plant fractions were found to possess larvicidal effect on *O. exvinacea*. Earlier, various workers have reported the insecticidal effect of *V. negundo* on some crop pests (Arivoli and Tennyson, 2013; Yankanchi *et al*., 2014; Ranilalitha *et al*., 2015) and in the

case of *H. suaveolens,* many insects were reported to be susceptible resulting in mortality (Okigbo *et al*., 2010; Arivoli and Tennyson, 2011; Conti *et al*., 2012; Ohimain *et al*., 2015). Anyhow, mortality response was realized to be dose and time-dependent with all the concentrations tested. To ascertain the efficacy of extracts, more important factor to be considered is the period of exposure rather than dosages (El-Nahal *et al*., 1989). According to this aspect, *V. negundo* was found to be more effective in causing mortality within short period of time and also with minimum concentration.

Generally plants possess many toxic ingredients with varying toxic properties. Identification of these active toxic ingredients is very important in toxicity studies. For this HPTLC analysis was done and some of the compounds were identified using specific visualizing reagents. HPTLC analysis showed that both plant fractions were showing the presence of alkaloids, phenolics and terpenoids. From these observations, it was summarised that the lethal toxicity of these plant fractions were attributed to the cumulative effect of these compounds. Presence of these active ingredients was confirmed by GC-MS analysis of both plant fractions.

With GC-MS analysis, an array of chemical components were separated and identified. In the toxic fraction of *H. suaveolens*, a total of 73 components were identified. Among the constituents detected, the most pronounced constituent in *H. suaveolens* fraction is a diterpenoid compound. GC-MS analysis of the toxic fraction of *H. suaveolens* revealed the presence of many other constituents like phenolics, fatty acid, steroid and other esters, ketones, sesquiterpenoids, heterocyclic compounds, flavones, alcoholic compounds, pyran and furan derivatives. In the toxic fraction of *V. negundo,* 49 constituents were identified. In this case, the prominent component identified is phthalate esters. GC-MS analysis of the toxic fraction of *V. negundo* revealed the presence of constituents like phenolics, fatty acid esters

and some other esters, ketones, alkenes, sesquiterpenoids, nitrogen heterocyclic compounds, alcoholic compounds, azulene and morphinone derivatives in varying quantities.

In the present investigation, methanolic leaf extracts prepared by soxhlet extraction of *H. suaveolens* and *V. negundo* were used for testing the biopesticidal property against the sixth instar larva of *O. exvinacea.* The observations for the histopathological changes on midgut tissue of treated larva showed degenerative effects such as disruption of peritrophic membrane prominently elongated columnar cells with their nuclei, cytoplasmic vacuolization, upward migration of nuclei of columnar cells, sloughing off epithelial apical part, congested apical border of columnar cell with their nuclei for both botanical treatments in common. Unique changes for treatment with *H. suaveolens* include presence of numerous secretory vesicles inside the lumen and also blebbing of the plasma membrane of columnar cells apically and that for *V. negundo* include complete detachment of epithelial layer from basement membrane and diverse pattern of columnar epithelial sloughing off forming large vacuoles inside the lumen and loss of identity of the columnar cells. The ultrastructural changes noticed for both botanicals include some common changes such as epithelial vacuolization, detachment of epithelial layer from basement membrane, elongation of epithelial cell along with its nucleus, detachment of longitudinal muscle layer from circular muscle layer and totally disrupted peritrophic membrane. Noticeable changes in the case of *H. suaveolens* treatment include numerous cell organelles in the epithelial cell with abundant secretory vesicles, goblet cells with rich supply of RER, mitochondria, secretory vesicles and abundant microvilli, blebbed apical plasma membrane of epithelial cell with rich supply of SER and few mitochondria and RER and that for *V. negundo* include reduction in cell organelles including RER and secretory vesicles at the apical and basal part of columnar cells, thinning of circular muscle layer with very few amount of

mitochondria and RER, completely degenerated goblet cell with disrupted microvilli and shrunken mitochondria.

These observations were concomitant with the results observed by Ranjini and Nambiar (2015) on the histopathological changes in the larval midgut epithelium of *O. exvinacea* treated with botanicals, *C. infortunatum* and *E. odoratum*. Moreover, parallel histopathological changes reported by Nambiar and Ranjini (2016) noticed significant ultrastructural changes in the same larvae upon treatment with *C. infortunatum* and *E. odoratum.*

In addition, the present study results were parallel to the cytotoxic changes observed in the midgut of *S. littoralis* larva when treated with the extracts of *A. monosperma* (Adel *et al.*, 2010). Similarly, these histopathological changes noticed in the present study resemble with the previous reports in many lepidopteran species which may denote some common degenerative responses in the gut against toxins. The results of the morphometric studies along with histopatholgical changes discussed in chapter VI were in agreement with the reports of Prasad and Roy (2011) in which they noticed, reduced cell dimensions as well as disrupted cell architecture of midgut tissue with the treatment of leaf extract of *L. camara* in fourth instar larvae of *H. armigera.*

Many secondary metabolites present in the plant extracts might be invoved in the degeneration of midgut tissue. For instance, the properties of peritrophic membrane made up of chitin was found to be altered with the binding of lectin (Saxena *et al.*, 1992). This membrane acts as a protective sheath for the inner epithelial layer of midgut tissue. If any rupture happens once to the peritrophic membrane, the food along with the toxic ingredients may pass simply into the midgut epithelial architechture. The degenerative changes occurred for epithelial layer with the treatment of these botanicals were found to be quite reasonable because columnar epithelial layer are the

first victims of the entered toxic principles. In both leaf extract treatments, columnar epithelial layer presented maximum histopathological changes. Generally, plant extracts upon treatment with food, principally start their functions on the cell layers of midgut tissue.

In general, midgut is the most important part of alimentary canal in an organism, where physiological digestion, absorption and assimilation of food occurs (Cruz-Landim *et al*., 1996). The digestive system serves as the main physiochemical barrier in an insect for the insecticides or pathogens entering through food and crossing of this barrier would impair the normal cellular architecture (Prasad and Roy, 2011).

In the present study, peritrophic membrane found to be completely disappeared with the application of both botanical treatments. The excessive cell elongation of epithelial cells and blebbing of materials towards the luminal area from the apical part of the columnar cells cause the rupturing of peritrophic membrane which is an acellular sheath for the protection of midgut epithelial layer (Raikhel *et al*., 1993).

Furthermore, the occurrence of numerous cellular components in the apical part of columnar cells is involved in the elaboration of digestive enzymes and those in the basal region are meant for secretion and absorption of fluids and electrolytes (Kasper, 1978). In the case of *H. suaveolens* treatment, the enhanced activities of enzymes in different tissues might be due to the presence of numerous cell organelles.

The observations like detachment of epithelial layer from the basement membrane with the treatment of *V. negundo* in the present work also revealed that it might create space in between epithelial layer and basement membrane which led to the formation of vacuoles in the cell as well, which were

supported by the findings of Richards and Richards (1977) and Raikhel *et al.* (1993) .

The effect of both *H. suaveolens* and *V. negundo* on the total protein and free amino acid contents in the larva exhibited significant reduction potential in all the three kinds of tissues. Both plant extracts differ in their potential to decrease the protein and amino acid concentration of the treated larva. Among the two botanicals, *V. negundo* was found to be more effective in reducing the concentration of the biochemical molecules. The results concurrent with the present study reports were noticed in *S. littoralis* larvae when treated with *Eucalyptus* oil and its combination with gamma radiation (El-Naggar and Abdel- Fattah, 1999).

The present study results corroborate with the findings of Ulrichs *et al.* (2008) who reported reduction of the total protein content in the midgut tissue of *S. litura* after treatment with *P. coarctata* leaf extracts. Similarly, reduced protein content in *S. litura* was reported by (Renuga and Sahayaraj, 2009) with the treatment of *A. conyzoides* and *A. vulgaris* extracts.

Moreover, these observations were in conformity with the findings of Padmaja and Rao (2000) in which they reported reduced haemolymph protein content in *H. armigera* with the treatment of *A. annua* and *A. conyzoides* oil. Similar records of depleted total protein content which agree with the present study results include reduced total proteins in *S. litura* larva due to the effect of methanolic extracts of *L. wightiana* (Rathi and Gopalakrishnan, 2010).

Anyhow, there are many probable explanations for the reduced concentration of biomolecules in the treated larva. Generally, proteins are considered as the most important structural organic constituents of animal tissues with a key role in energy production. During stress conditions, normally, tissue proteins are responsible for the activation of compensatory

mechanism (Wigglesworth, 1972). Usually, high energy demands are needed under adverse conditions and the energy requirement may have led to the protein catabolism. Or else, the decrease of larval protein and amino acid content might be due to the reduced synthesis of proteins or increased breakdown of proteins to detoxify the active ingredients present in the plant extracts (Vijayaraghavan *et al*., 2010). The decrease of protein content might also be due to breakdown of protein into amino acids to provide intermediates for energy synthesis to counter the excess energy needed under stress conditions. This was best explained by Nath *et al*. (1997) with the statement that insecticides bring about proteolysis, releasing amino acids so as to enter in toTCA cycle as a keto acid, for the immediate supply of energy for the insect. In other words, increased rate of gluconeogenesis and glycogenesis results in significant decrease in the level of free amino acid content also (Sancho *et al*., 1998). Moreover, the feeding rate of an insect also possesses an influence on the level of amino acids. Likewise, alteration in protein and amino acid contents in the insect body is seemed to be associated with the ingestion of botanicals which may induce pathological changes in the midgut epithelial cells, thereby leading to severe turbulence in insect metabolism especially for proteins due to changes in activities of various midgut enzymes (Jing *et al*., 2005).

Qualitative analysis of protein content of the treated larva showed polymorphic variations with regard to the number and intensity of protein bands in the midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea* with different treatments. These observations were in conformity with the findings of Boreddy *et al*. (2000) in which treatment of *Annona* seed extract on *S. litura* larva caused significant variations such as appearance of new bands and disappearance of existing bands in the protein profiling studies. Furthermore, Padmaja and Rao (2000) found similar results in *H. armigera* with neem oil that showed alterations in the prominence and

number of protein bands present in the haemolymph protein content. Much complementary results were reported by Mariselvi and Manimegalai (2016) on the qualitative changes in the haemolymph protein pattern of the larvae and pupae of *Sylepta derogata* due to the effect of Econeem, *A. calamus* and *Piper longum* plant extracts.

According to El-Bermawy (1994) the disappearance of the electrophoretic banding patterns in some individuals or the presence of extra bands in others may be due to the activation of related genes to produce these types of protein or because of the depression or mutation of the regulating genes responsible for biosynthesis of polypeptide chains for assembling more detoxification enzymes.

Another finding of this investigation is that the variations in the activities of different enzymes present in the midgut tissue, fat body and haemolymph of the treated larva. It is obvious from these studies that the activities of both aminotransferases at different time of exposures exhibited an enhancing effect in all the three tissues for both *H. suaveolens* and *V. negundo* treatment. Similarly, for the phosphatase enzymes, *H. suaveolens* at different time of exposures exhibited an elevating effect for the two phosphatases in all the tissues whereas *V. negundo* in general resulted in a decreasing activity except in haemolymph ALP activity in which it caused an initial increase followed with a decreasing pattern of phosphatase activity in accordance with increasing time of exposure. In the case of catalase activity an inducing effect of *H. suaveolens* treatment at all time intervals in the midgut tissue, fat body and haemolymph was noticed. But for *V. negundo* treatment, elevated catalase activity was noticed from 24 h to 48 h of exposure followed with decreased activity at 72 h post treatment although it showed an increasing trend when compared to control.

Generally, metabolic processes of organisms are regulated with biochemical macromolecules, the enzymes and any kind of minute variation in enzyme activities would interrupt the normal functions in an organism. The development of stress associated with pesticidal exposure may induce biochemical changes which in turn bring about a series of responses like metabolic disturbance, vital enzymes inhibition, retardation of growth, reduction of fecundity and longevity of organisms (Lissandra *et al*., 2006). Normally, the disruption of cell integrity under stress conditions will result in the leakage of enzymes into the plasma/serum. In this case, the activity of enzymes can be considered as a valuable indicator of cell integrity (Coppo *et al*., 2002). Consequently, the assessment of enzyme activities in an organism reflects metabolic disturbances.

Much complementary results were reported by Rawi *et al*. (2011) on *S. littoralis* larvae when exposed to *A. indica and C. colocynthis* extracts that caused progressive increase in the activity of ALT with increasing time of exposure. Similarly, the present work reports were in concurrence with the results of Manjula *et al*. (2010) regarding the increased activities of AST and ALT enzymes in the haemolymph of silkworm supplemented with *Dolichos lablab* treated mulberry leaves. Correspondingly influenced AST and ALT activities were reported by a number of researchers with the treatment of botanicals like annonain treatment on *S. litura*; Margosan-o (a neem preparation) and *M. azedarach* on *S. littoralis* (Hassan, 2002).

Accordingly, the disturbance in AST and ALT levels will be closely related to metabolism of proteins and amino acids. Thus it will disrupt many physiological functions and ultimately lead to death or in other words control the pest (Ezz and Fahmy, 2009). The decline in protein level with increase in transaminase activities suggests the mobilization of amino acids during insecticide stress to meet the energy demands.

In the present study, both phosphatases were also found to be influenced with the treatment of plant extracts. Similar inducing effects noticed in the present work were found to be complementary with the reports of inducing action of methanolic extract of *Silybium marianum* on *P. rapae* larva (Hasheminia *et al*., 2013) and treatment of nimbecidine and neemazal at varying exposure time on *E. vitella* (Bhardwaj *et al*., 2016). Reducing activity level of ACP and ALP for *V. negundo* treatment was supported with the report of Senthil-Nathan and Kalaivani (2005) on *S. litura* fed with azadirachtin treated *R. communis* leaves.

The increased phosphatase activity noticed in the present study might be due to phosphatase release from the tissues as a result of the rupture in the cellular and lysosomal membranes (Abou-Donia and Ashry, 1978). They also noticed that the alteration in the activity of phosphatases due to botanical treatment may result in the significant functional impairment of lysosomal metabolism in the tissues possibly with the action of toxic principles on the enzyme system. Or in otherwords, these toxic ingredients may directly affect the enzyme synthesizing sites for the lysis of damaged tissue and their corresponding release of enzymes with the destruction of cellular and lysosomal membranes. Since the phosphatases are chiefly distributed at cell membrane and so even slight impairments in the cells mark changes in phosphatases activity. Many authors suggested that the rise in acid phosphatase activity might be due to pesticide intoxication which indicated an enhanced protein catabolism and probable tissue damage in the organism (Sharma, 1999). Increased ACP activity suggested that this may be due to the strong toxic effect of the toxicant on the cellular and lysosomal membranes resulting in their rupture (Dalela *et al*., 1978). Hence it can be assumed that the increased phosphatases activity may be due to stress induced protein catabolism and damage in the midgut architecture.

It has been earlier reported that insecticides may interfere with the production of certain types of proteins (Smirle *et al*., 1996) and digestive enzyme activity (Huang *et al*., 2008). An increase in ACP content can also be attributed to the tissue damage recorded in the present study. As this enzyme is associated with lysosomal activity, its increase may be due to decrease in the stability or rupture of lysosomal membranes resulting in the release of this enzyme into surrounding tissues. As the tissue damage progressed, the activities of acid phosphatase also increased progressively. It is suggested that this may be due to the leakage of lysosomal enzymes in to the haemolymph.

Enhancing activities of this enzyme accompanied by a reduction in protein content in the different tissues indicated augmented protein catabolism to meet energy demands under stress condition (Kumar and Saradhamani, 2004).

The decrease in ALP and ACP activities by botanicals may be due to the direct binding of the toxic component present in plant extracts with enzyme protein (Passow *et al*., 1961) or the toxic effects produced by them on tissues leading to decreased synthesis of enzymes. The decrease could be attributed to the destruction of cell membrane and lysosomes which in turn lead to tissue damage (Mir *et al*., 2016).

ALP enzyme, found in the intestinal epithelium of animals is having significant roles in the digestion of phospholipids present in food particles. So, these phosphatases are primarily involved in the transphosphorylation reaction and hence, midgut must have the highest ALP and ACP activity as compared to other tissues (Sakharov *et al*., 1989).

The fall in the activity of ALP in the haemolymph can be associated with the tissue damage and this develops an imbalance between degradation and synthesis of the enzyme. Since midgut tissue form a major site of its

synthesis, severe damage at later stages of exposure to pesticides might have reduced the concentration of this enzyme in the tissues due to the destruction of enzyme synthesizing sites. This drop in the activity of the enzyme may be interrelated with its function in the contribution of inorganic phosphates for DNA synthesis or may probably due to disruption of the cell plasma membrane, resulting in the leakage of this enzyme into extracellular fluids which may perhaps completely affect the rate of hydrolysis of phosphoric group of esters and consequently production of energy in the form of ATP (Ajayi *et al.*, 2005).

Detoxification enzymes possess significant functions in insect physiology, deactivating ingested foreign toxic materials and protect the growth and development of the organism. These enzymes found to be stimulated by plant allelochemicals in a number of insects (Caballero *et al.,* 2008). Hence it is imperative to know how methanolic extracts having lot of plant phytochemicals causing the biochemical changes in the insect while an insect feed on botanical treated plant parts. Casillas *et al.* (1983) described that toxicological studies of acute exposure caused variations in concentrations and activities of enzymes, which was considered to be a direct measure for cell damage in specific organs. The severe drop of protein under insecticide stress conditions might be due to the increased amount of acid phosphatase in tissues (Shaikila *et al.*, 1993). The increased level of transaminases and phosphatases indicated enhanced metabolic activity perhaps to meet the energy demand during stress induced by the pesticides.

It has been demonstrated that pesticide exposure will result in the generation of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radical, hydrogen peroxide and hydroperoxides (Mittapalli *et al.*, 2007). To combat the toxic effects of ROS insects developed a complex antioxidant mechanism, which consists of superoxide dismutase (SOD),

glutathione peroxidase (GPx), catalase (CAT), reduced glutathione (GSH) and glutathione reductase enzyme actions (GR) (Meng *et al*., 2009). Reactive oxygen species induces oxidative stress by inducing cellular damage, which are acted upon by the enzymes of the antioxidant system. The present result agree with the statement of Alves *et al*. (2012) that the exposure to pesticides elicit pro-oxidant conditions that trigger adaptive responses such as increase in the activity of the antioxidant enzymes.

In the present study, catalase activity was found to be increasing with increasing time of exposure for both botanical treatments in all the three kinds of tissues in the treated larva when compared to the control set of larva. But in the case of *V. negundo* treatment, catalase was exhibiting much elevated activity at 48 h of exposure. Later, upon increasing time of exposure upto 72 h, when compared to activity at 48 h post treatment, it was declining in nature although it shows an increased activity to that of control.

The observations of decreasing CAT activity for *V. negundo* in the present study were concurrent with the findings of Pristos *et al*. (1991) in which they noticed reducing CAT activity in *P. polyxenes* when fed with a flavonoid, quercetin treated parsley leaves. Similar reducing effect of plant allelochemicals on *S. eridania* was again reported by Pristos *et al*. (1991). Similar induced CAT activity was reported in *S. littoralis* fed with polyphenolic compounds containing food (Krishnan and Kodrick, 2006) and tannic acid incorporated food (Krishnan and Sehnal, 2006)

Generally catalase enzyme is responsible for the conversion of hydrogen peroxide to water and oxygen (Fridovich, 1976). Therefore, the enhancement in CAT activity after botanical treatments in *O. exvinacea* larva could be due to the increased CAT level with time of exposure indicating the presence of more hydrogen peroxide under extreme stress condition. The decreasing CAT activity at increasing time of exposures may be due to the

inhibition of CAT with the accumulation of intracellular concentration of hydrogen peroxide during destruction processes, which may be caused by botanical treatment (Kono and Fridovich, 1982). This is found to be quite suggestive of the excessive production of free radicals and it may result in the reduction or inactivation of CAT enzyme. Moreover it might be due to the inability of cells to increase the production of catalase with increasing time of exposure because of cell damage and the activity of it is limited as a result of their failure to neutralize the effect of toxic ingredients present in botanical treatments.

In the case of *V. negundo*, the whole columnar epithelial cell layer showed more damage than *H. suaveolens* treatment and it is quite apparent that regenerative cells and the secretory activity of goblet cells might have lost their function and the disrupted goblet with atrophied microvilli inside were no longer capable of any kind of secretory activities. This might be the reason for decreased enzyme activity noticed at 72 h of post treatment.

From the present investigation, it may be assumed that the phytochemicals present in both plant extracts can cause alterations in cellular machinery leading to histopathological and biochemical changes in the tissues of sixth instar larva of *O. exvinacea.* Moreover, both plant extracts were showing larvicidal properties also. Anyhow, the chloroform fractions separated from the methanolic extract of these plants are thus more appropriate for the formulation of insecticide and for the effective management of *O. exvinacea.*

Plants own a mysterious wealth of enormous chemical ingredients, which can be very easily exploited from nature and used effectively for pest management of crops. This study also implies that complete information of the various constituents of these plants will absolutely offer new methods of insect pest control in the natural sense itself. So, further investigations can be

carried out for the isolation and characterization of other toxic phytochemical constituents present in these plants by techniques such as flame ionization detector (FID), mass spectroscopy (MS), NMR, IR and UV spectroscopy. Identification and elucidation of these innovative biologically active metabolites and their structures provide a solid base for the production of improved marketable analogues or commercial products for IPM. This study therefore, initiates the development of innovative methods for the management of lepidopteran pests using the reliable native plant resources, with which we can avoid the vulnerabilities associated with the application of synthetic insecticides. These active ingredients may also be tried against a number of crop pests and insect vectors other than lepidopterans for their effective control.

In spite of the large scale production of these active principles, common farmers usually go for locally set up decoctions of fresh plants for pest control. But they always encounter some limitations with the use of these plant preparations such as fast degradation, thermal and photolabile nature, slow action, limited availability of plant materials and also their standardization etc. To overcome these problems, systematic studies to find out the active principles and their effective dosage should be carried out and the formulations of operative and safe pesticides could be made to improve their efficacy in the IPM framework.

CHAPTER X
CONCLUSIONS

The present investigation was to study the effect of *Hyptis suaveolens* and *Vitex negundo* leaf extracts on midgut tissue, fat body and haemolymph of mango leaf webber, *Orthaga exvinacea* and to detect the biopesticidal properties of these plants for the effective management of mango pest infestation.

To achieve this goal, the research work was carried out to study the phytochemical extraction and separation of fractions, toxicity effect of the most toxic fraction, HPTLC and GC-MS studies to identify the toxic components of the fractions, histopathological studies of treated larval midgut tissue with both light microscopy and electron microscopy, quantitative and qualitative changes of protein along with quantitative changes of amino acids in midgut tissue, fat body and haemolymph of treated insects and biochemical changes in the enzyme activities in these tissues under treatment.

The leaf extracts of both plants were prepared using methanol as solvent by soxhlet extraction. In order to carryout toxicity studies with the most toxic fraction on the larva, the methanolic extract of both botanicals were subjected to fractionation using immiscible solvents such as n- hexane, chloroform, ethyl acetate and methanol. A preliminary screening study was conducted with all the four fractions to find out the most toxic fraction. In which, chloroform fraction of both treatments (*H. suaveolens* and *V. negundo*) were found to be causing 100 % mortality of *O. exvinacea* sixth instar larva. Toxicity studies were performed through topical application method. Toxic effects resulted on *O. exvinacea* larva were noticed to find out the lethal

dosage to cause 50 % mortality for both treatments. The percentage mortality and LD $_{50}$ values were calculated for both treatments and the probit analysis showed an LD_{50} value of 70.16 µg for *H. suaveolens* treatment at 60 h duration and that noticed for *V. negundo* was 33.16 µg for 48 h duration. On comparison of the botanicals in causing mortality, *V. negundo* was exhibiting strong toxicity with low dosage and minimum time of exposure. Whereas for *H. suaveolens,* it requires increased concentration and more time of exposure to cause 50 % mortality.

Chloroform fraction of both *H. suaveolens* and *V. negundo* was subjected to high performance thin layer chromatographic studies to identify the type of toxic components. HPTLC studies indicated the presence of various chemical classes of compounds like alkaloids, phenolics and terpenes. Moreover, GC-MS studies with the toxic fractions were also performed to identify the volatile chemical constituents present in these fractions of both *H. suaveolens* and *V. negundo*. The spectral data of *H. suaveolens* and *V. negundo* revealed the presence of an array of chemical components. A total of 73 components were identified in the case of *H. suaveolens* whereas *V. negundo* showed the presence of 49 constituents with varying chemical nature. The presence of these components in both botanicals may induce larvicidal activity due to the synergistic or independent action of these compounds against *O. exvinacea* larva suggesting that these plants can also be incorporated in pest management programmes against *O. exvinacea*.

The effects of different concentrations (1-5 %) of both *H. suaveolens* and *V. negundo* upon midgut histology and various biochemical parameters were studied. General histopathological changes of the midgut tissue of treated larva with different concentrations of *H. suaveolens* as observed in the light microscopic study include disruption of peritrophic membrane, elongation of columnar epithelial cells with its nuclei, upward displacement

of nuclei of columnar cells, sloughing off brush border, cytoplasmic vacuolization, increased number and size of goblet cells and highly overlapped and congested apical border of columnar cell with their nuclei. Similarly, the histopathological changes noticed after treatment with *V. negundo* consists of distorted peritrophic membrane, extreme elongation of epithelial cells with more prominent vacuolization in accordance with increasing botanical concentration, detachment of epithelial layer from basement membrane and at the 5 % concentration of *V. negundo*, varied pattern of epithelial sloughing off in columnar cells was noticed forming large vacuoles with in the lumen and loss of columnar cell integrity.

Morphometric studies were also conducted to detect the changes occurred in the columnar and goblet cells with botanical treatments. Both columnar and goblet cells with their nuclei experienced alteration in their height and width. Columnar cells and their nuclei showed increased height and decreased width while the size of goblet cells with their nuclei was enlarged with the treatment of *H. suaveolens*. *V. negundo* treatment caused maximum elongation of columnar cells with increasing botanical concentration. Moreover, it was observed that columnar nuclear length increased but width decreased. The size of the goblet cell and its nucleus was found to be increased with the treatment of *V. negundo*.

The effect of botanicals on ultrastructural changes in the midgut tissue of *O. exvinacea* larva was observed after treatment of the larva with 5 % concentration of botanicals using transmission electron microscopy. In the case of *H. suaveolens* treatment the changes observed were epithelial vacuolization, detachment of epithelial layer from basement membrane, elongation of epithelial cell along with its nucleus, appearance of numerous cell organelles in the epithelial cell cytoplasm with abundant secretory vesicles, detachment of longitudinal muscle layer from circular muscle layer,

goblet cells with rich supply of RER, mitochondria, secretory vesicles and abundant microvilli, blebbed apical plasma membrane of epithelial cell with rich supply of SER and few mitochondria and the totally disrupted peritrophic membrane etc. Whereas for *V. negundo* the changes include extensive vacuolization inside the cytoplasmic area of columnar cells and secretory cells, no peritrophic membrane, completely detached columnar layer from the basement membrane, extensive elongation of epithelial cell along with displacement of its nucleus, very few cell organelles including RER and secretory vesicles in the cytoplasm of columnar cells, thinning of circular muscle layer with very few mitochondria, detachment of longitudinal muscle layer from the circular layer, completely degenerated goblet cell with disrupted microvilli and shrunken mitochondria.

To realize the effect of botanicals upon biochemical changes of total protein and free amino acid content in the midgut tissue, fat body and haemolymph of treated larva both quantitative and qualitative changes in protein content along with quantitative biochemical alteration of free amino acid concentration of treated larva were estimated. The results indicated significant decrease in protein and amino acid content at the higher (5 %) concentration of both plant extracts. Generally the reduction of these factors was found to be more with the treatment of *V. negundo* than *H. suaveolens*. The reduction in the concentration of protein and amino acid content of treated larva was noticed to be dose dependent i.e., directly proportional to the increasing concentration of botanicals.

Qualitative changes in the protein content of different tissues in the treated larva were performed with poly acrylamide gel electrophoresis. In this study, polymorphic variations were detected in the number and intensity of protein bands in the midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea* with different percentage treatments of both botanicals.

The fluctuation in the normal banding pattern consists of appearance or disappearance of protein bands which may be resulted with the altered physiological activities of experimental larva upon botanical treatment.

Moreover, modified enzyme activities in the midgut tissue, fat body and haemolymph of *O. exvinacea* sixth instar larva with the treatment of botanicals were also analysed quantitatively for catalase, AST, ALT, ACP and ALP enzymes at different time durations (24 h, 48 h and 72 h). Since high treatment concentration was found to be much effective for bringing about significant biochemical changes, determination of alterations in enzyme activities were restricted for treatment with 5 % concentration of both botanicals. In the case of catalase enzyme activity, all the three tissues were showing an increased level of CAT activity with the treatment of *H. suaveolens* for different time of exposures. While treatment with *V. negundo* resulted in decreased CAT activity at 72 h when compared to activity at 48 h exposure time eventhough there was an increase with respect to control exists. Both *H. suaveolens* and *V. negundo* treatments caused an increase in AST and ALT activities in all the three tissues with increasing time of exposure from 24 h to 72 h with maximum increased activity at 72 h of post treatment. In the case of phosphatases (ALP and ACP) of midgut tissue, fat body and haemolymph of treated larva, both treatments exhibited significant differences in the variation of enzyme activities when compared with control. The treatment of *H. suaveolens* at different time of exposures showed an enhancing effect upon the two phosphatases in all the three tissues whereas, *V. negundo* exhibited a declining activity in general except haemolymph ALP activity which exhibited an initial increase followed by decreased phosphatase activity with increasing time of exposure.

In the light of the present findings it can be concluded that both plants can be used as effective larvicidal agents against *O. exvinacea* larva or in

other words efficient in bringing about various toxic effects like histopathological and biochemical changes in various tissues of *O. exvinacea* larva. From these studies it becomes evident that both *H. suaveolens* and *V. negundo* contains some toxic ingredients which owned good insecticidal properties on *O. exvinacea* larva. Moreover, these plant materials could be recommended for use as effective control measures against *O. exvinacea* larva as an environment friendly contact poison. Anyway, more future investigations are desirable to confirm the efficacy of these botanicals for the management of *O. exvinacea* larva in field conditions and also to recognize its impact on non-target organisms.

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PLATE VI. 2.

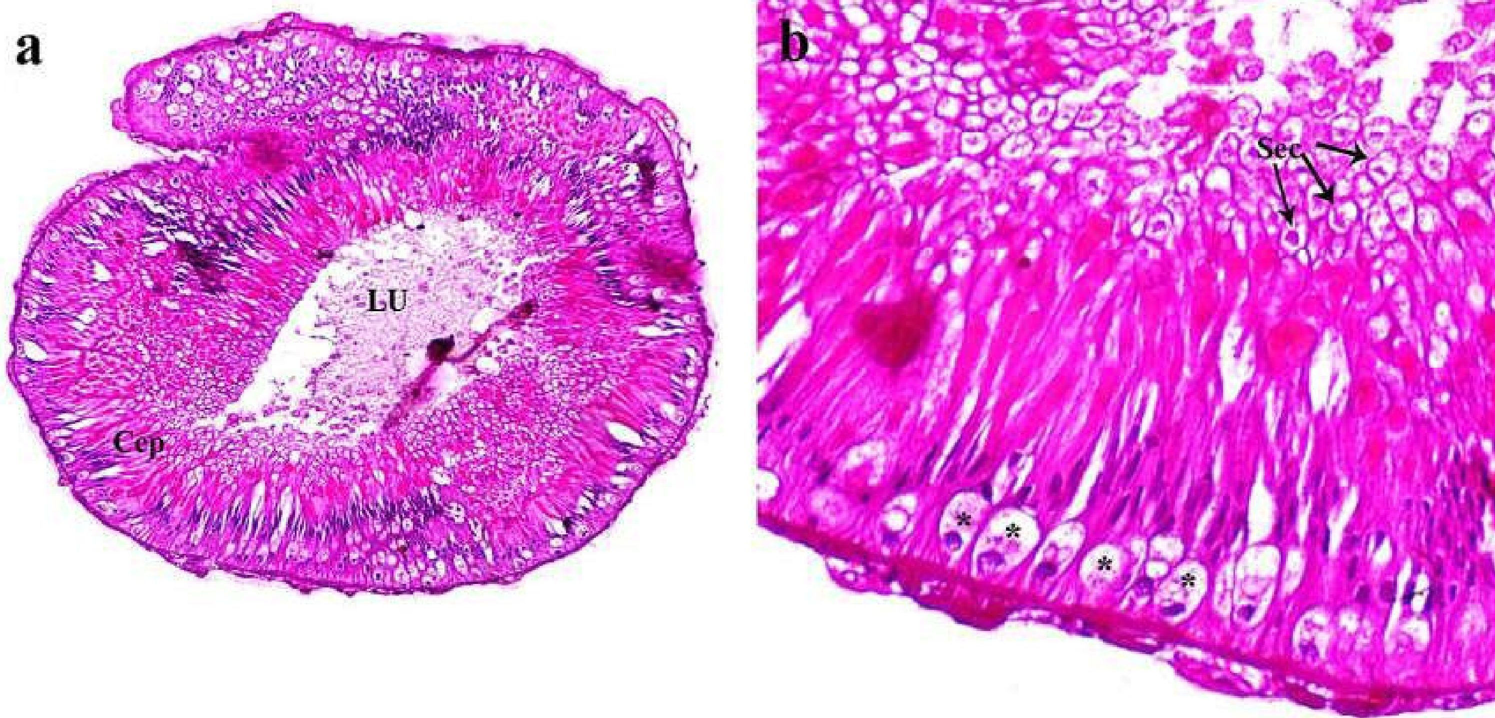
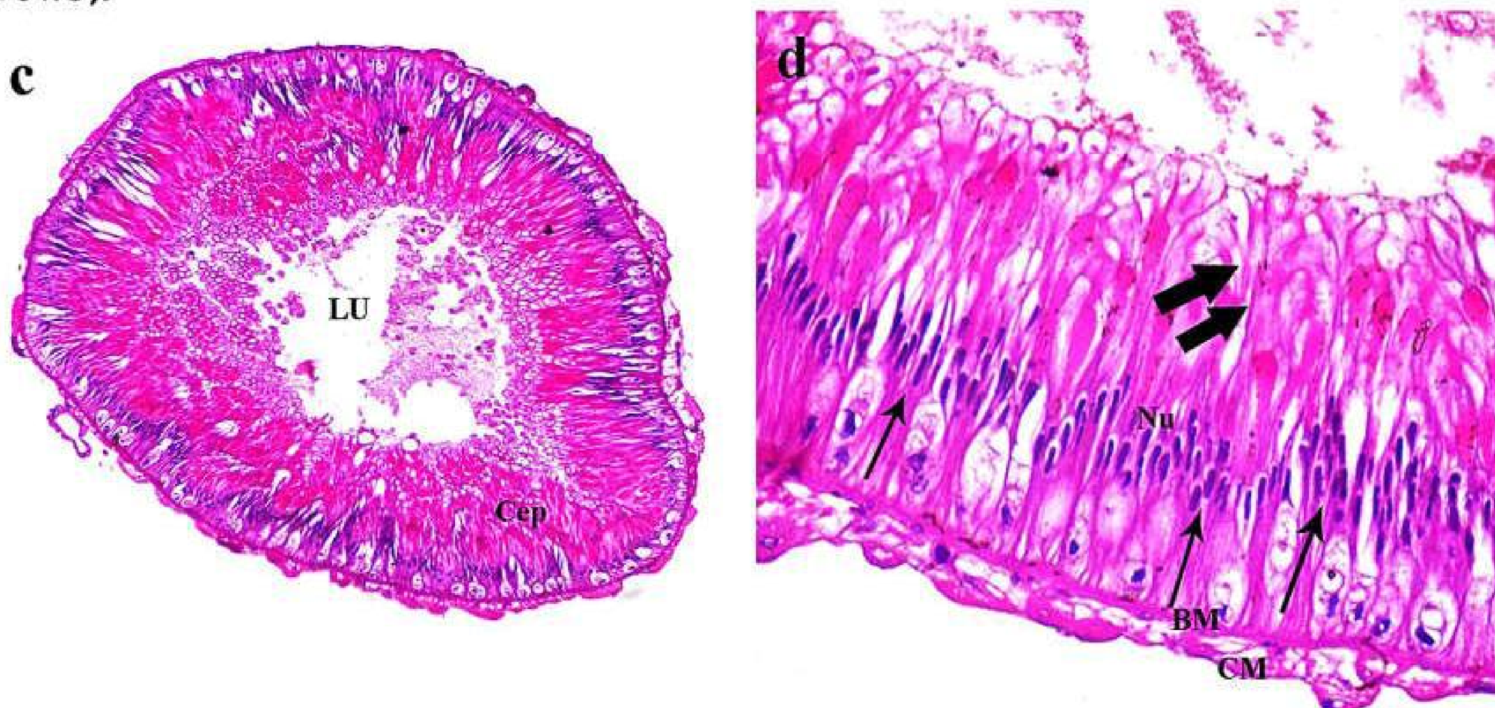
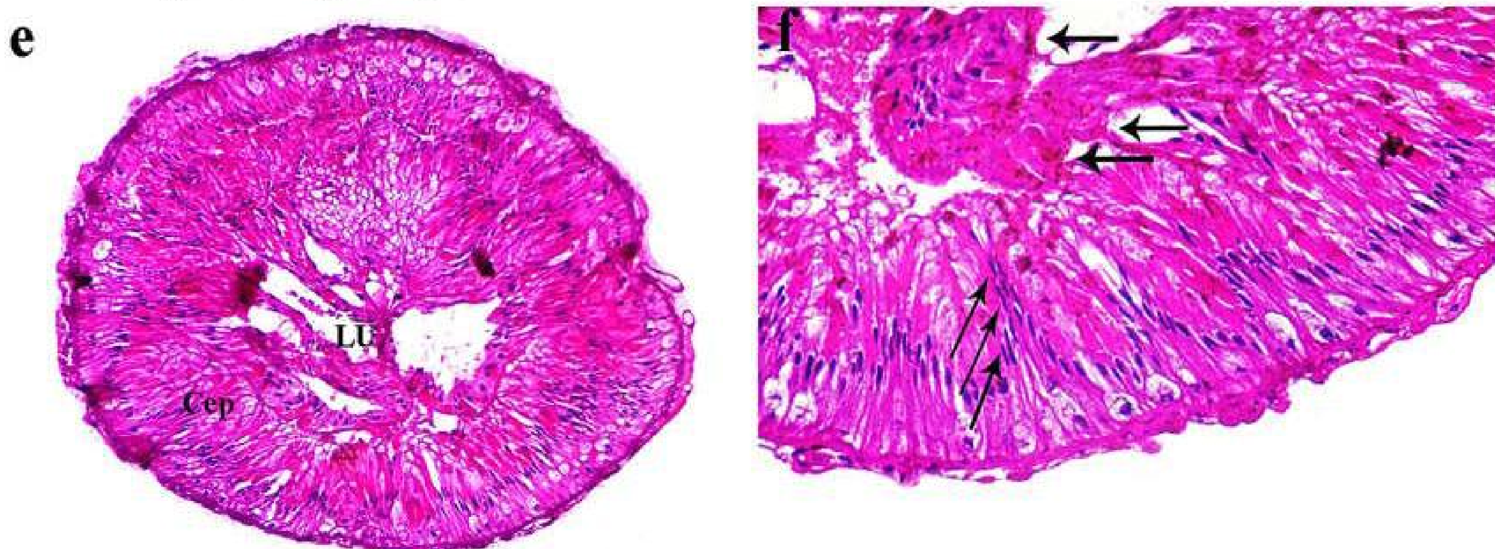


Fig. 2. a. T.S of the larval midgut treated with 1% of *H. suaveolens* (100X); b. A portion enlarged (400 X). Numerous GC (asterik), secretory vesicles (Sec) (arrows).



c. T.S of the larval midgut treated with 2% of *H. suaveolens* (100X); d. A portion enlarged (400X). Thin arrows-elongation of columnar cells and their nuclei; thick arrows- congested apical part.



e. T.S of midgut epithelium treated with 3% of *H. suaveolens* (100X); f. A portion enlarged (400X). Thick arrows-sloughing off; thin arrows-elongation, over lapping and congestion of columnar cells and nuclei. Basement membrane (BM), Columnar epithelium (Cep), Lumen (LU), Secretory vesicle (Sec).

PLATE VI. 9

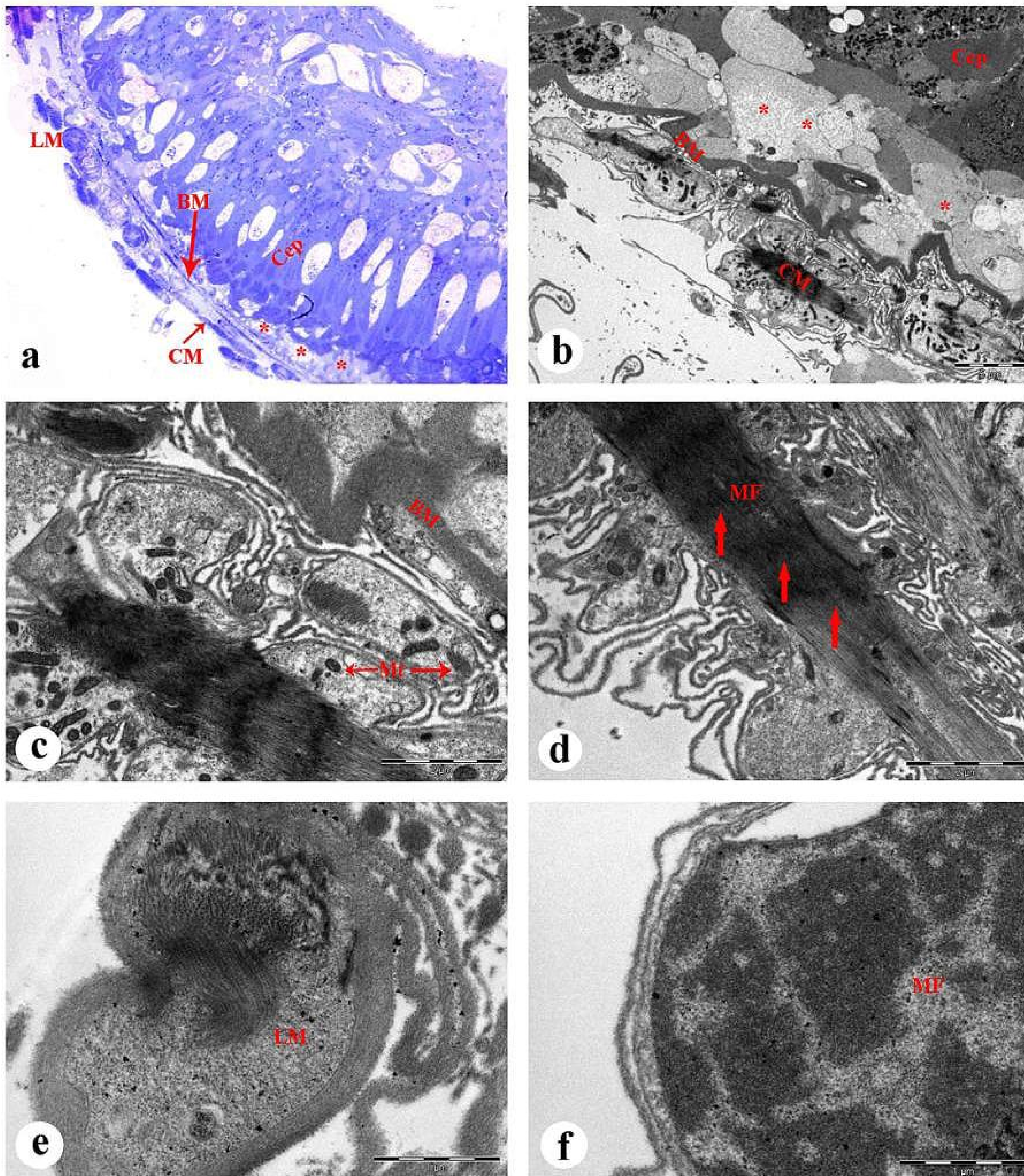
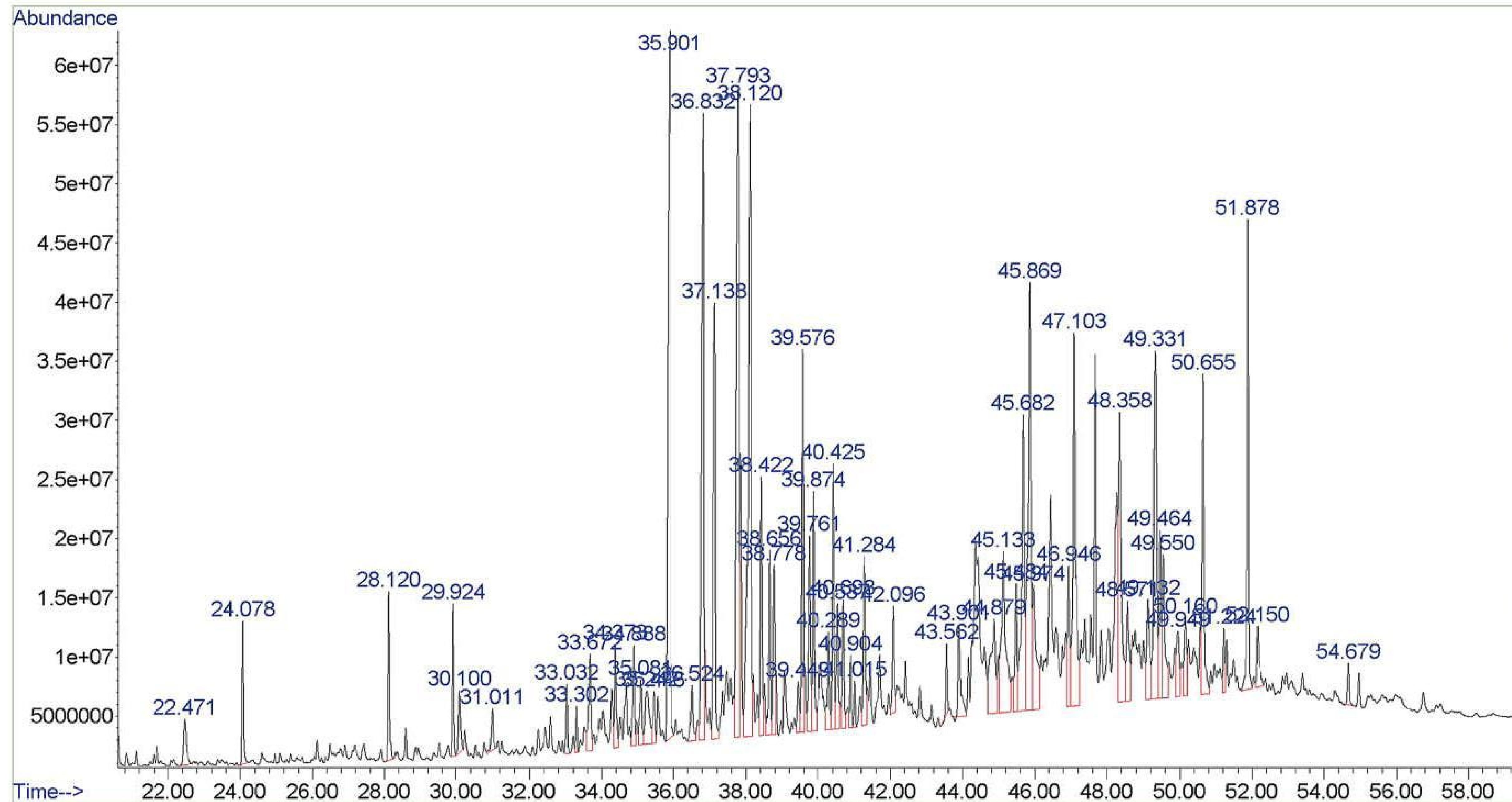


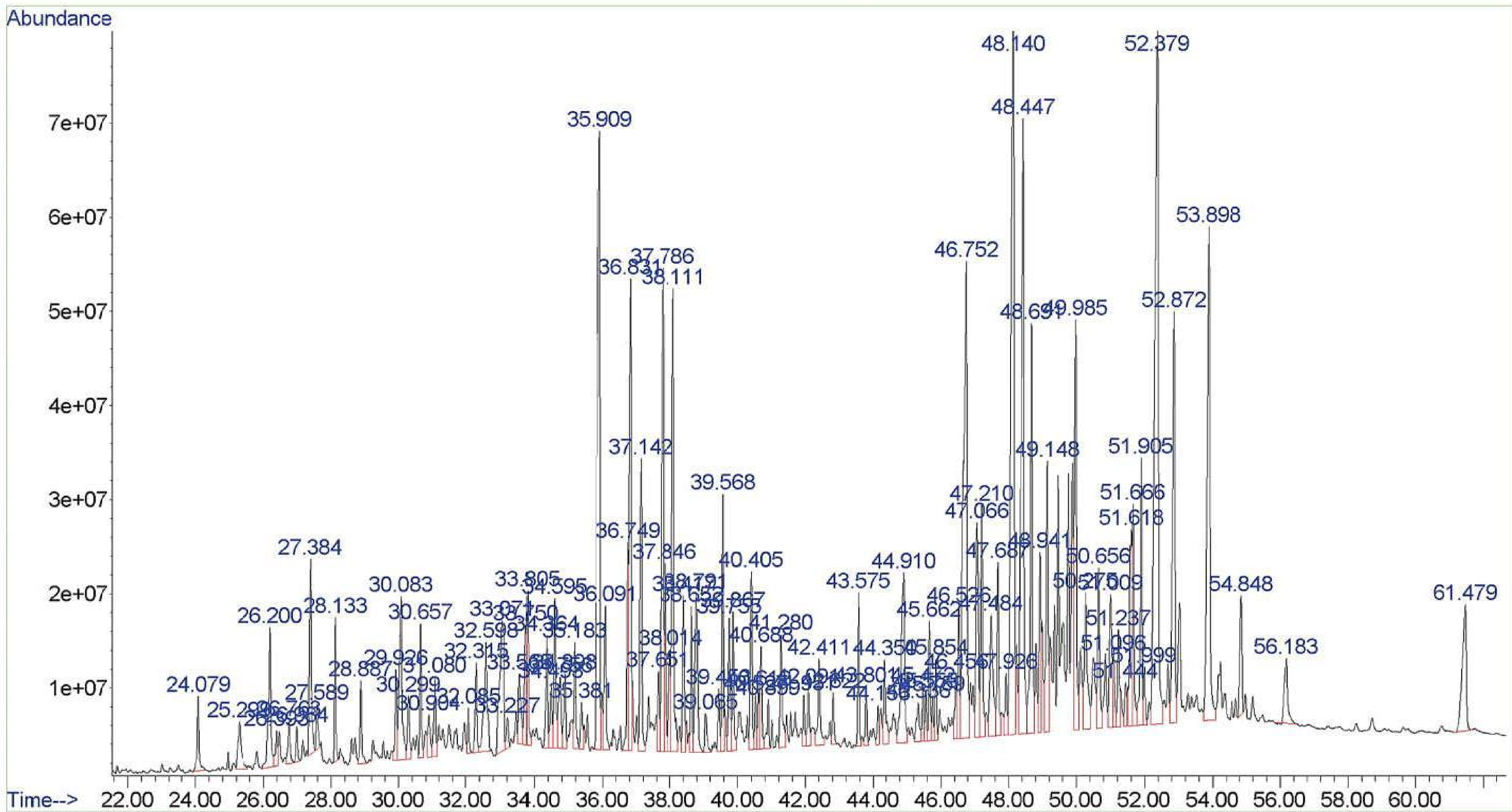
Fig. 4. a. Semithin section of larval midgut tissue treated with 5% of *H. suaveolens*-prominent epithelial vacuolization in between the Basement Membrane (BM) and Colmnar epithelium (Cep) (asterik)-showing the detached epithelial layer from the BM (1000X); b. Ultrathin section showing large vacuoles in between BM and epithelial layer (asterik) (2900X); c. Magnified view of circular muscle (CM) and BM (11000X): d. Enlarged view of CM showing compact nature of myofibrils (MF) (arrow) (11000X); e. Longitudinal muscle layer (23000X); f. Enlarged view of a part of LM with myofibrils (23000X).

PLATE V. 5



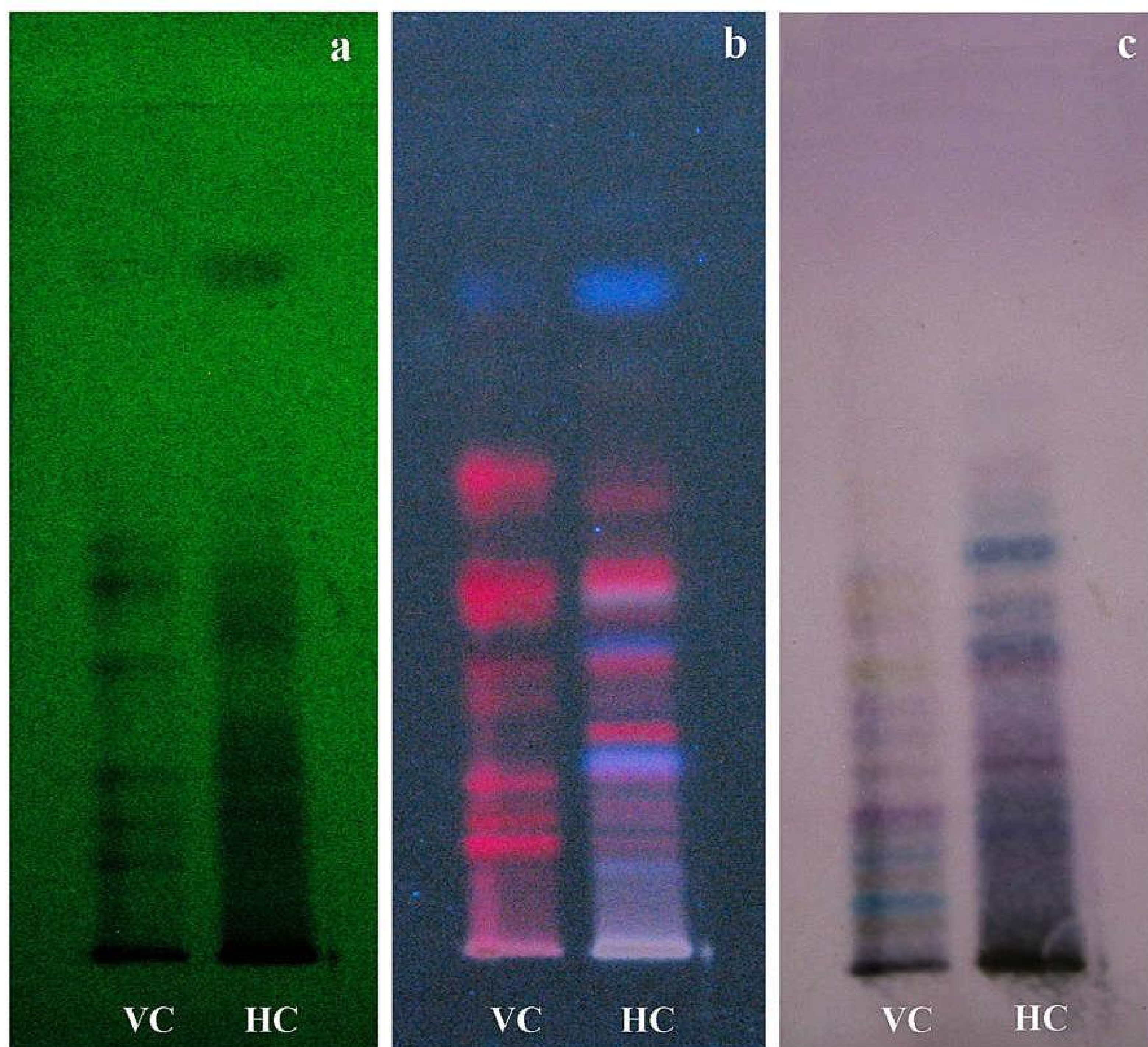
GC- MS analysis of toxic fractions of *Vitex negundo*

PLATE V. 4



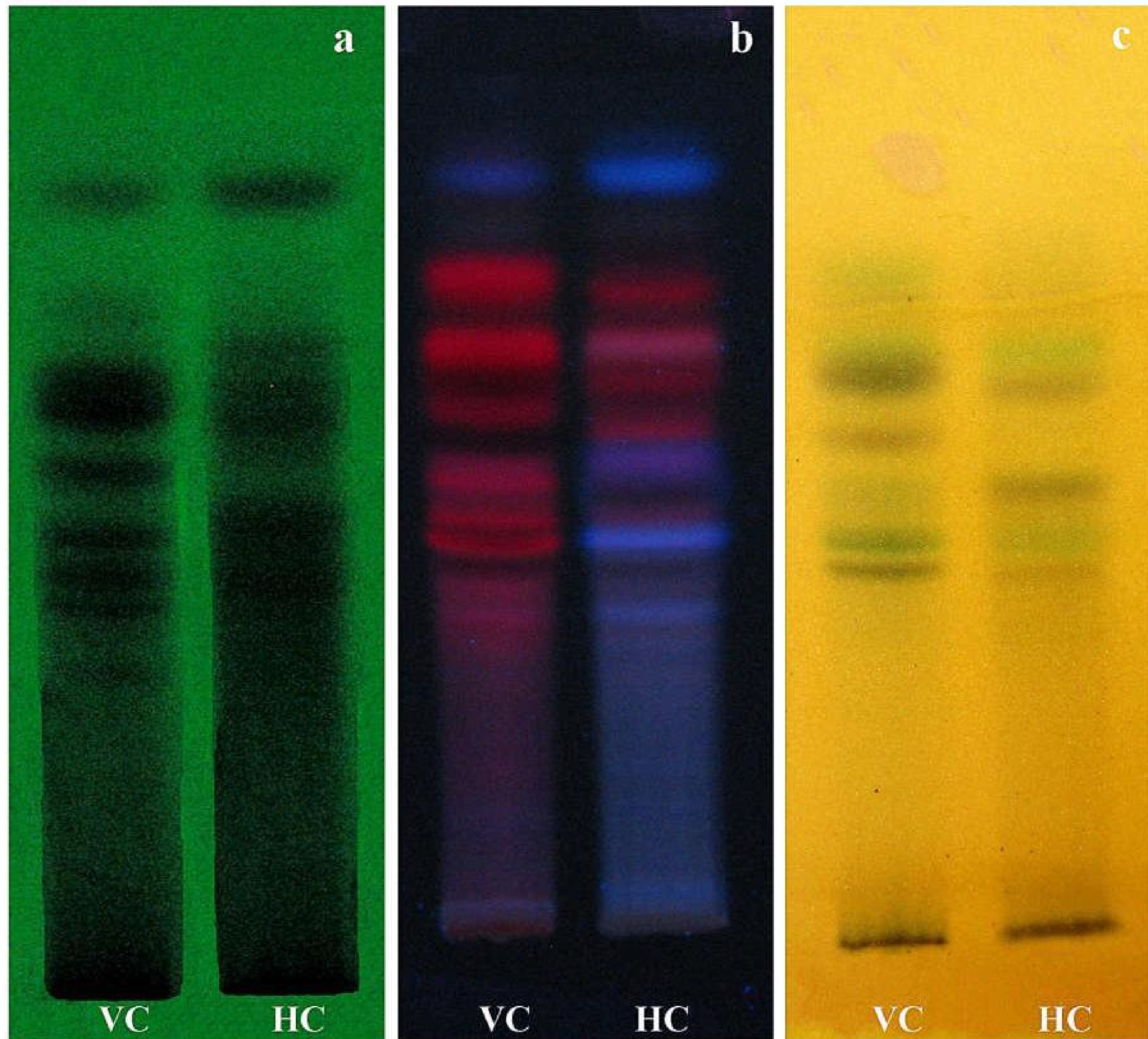
GC-MS analysis of toxic fractions of *Hyptis suaveolens*

PLATE V. 3



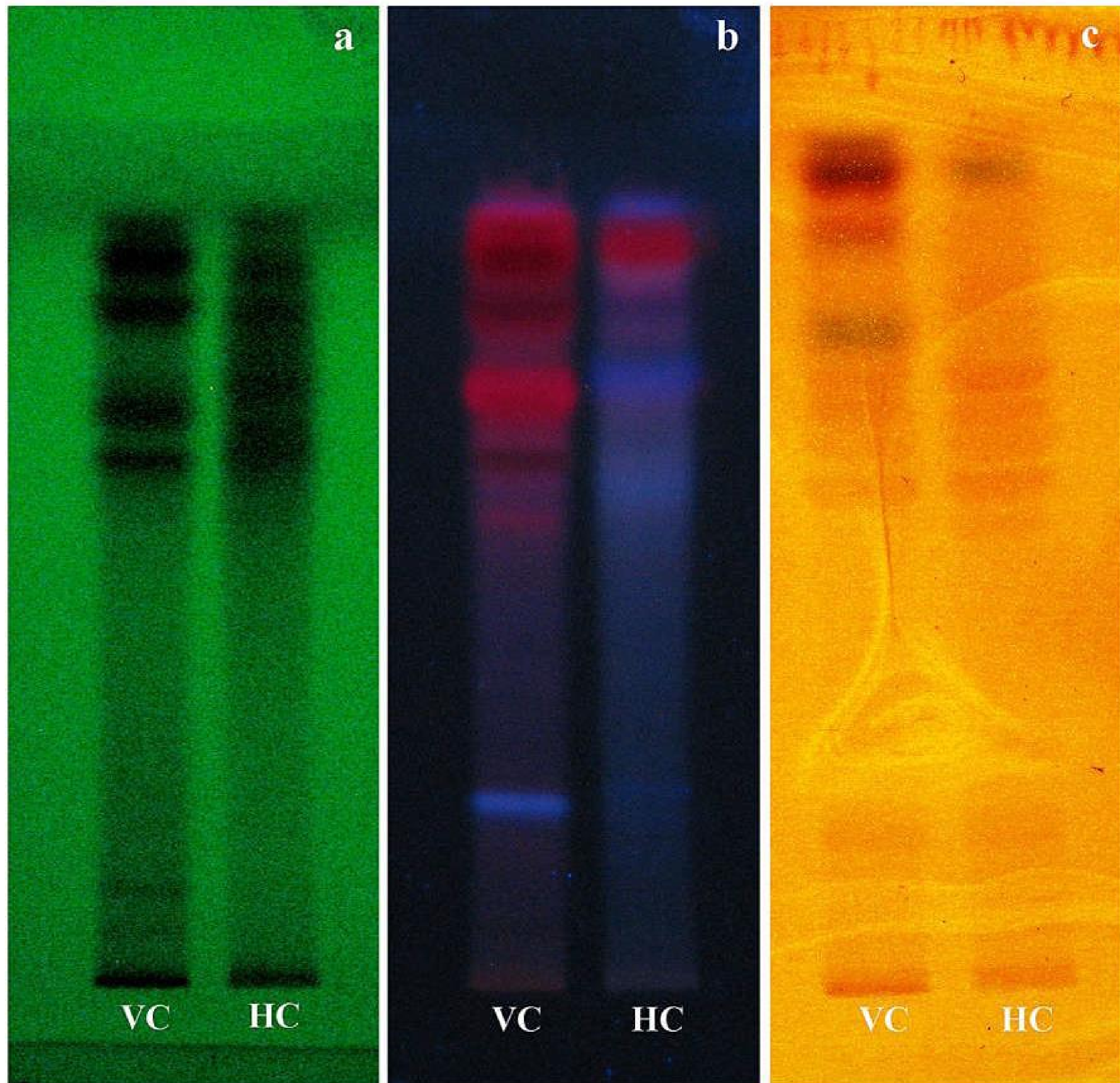
HPTLC profile images of toxic fractions of *V. negundo* and *H. suaveolens* showing the presence of terpenoids observed under short UV (254 nm), long UV (366 nm) and at 550 nm after derivatization. VC-*Vitex* Chloroform, HC-*Hyptis* Chloroform

PLATE V. 2



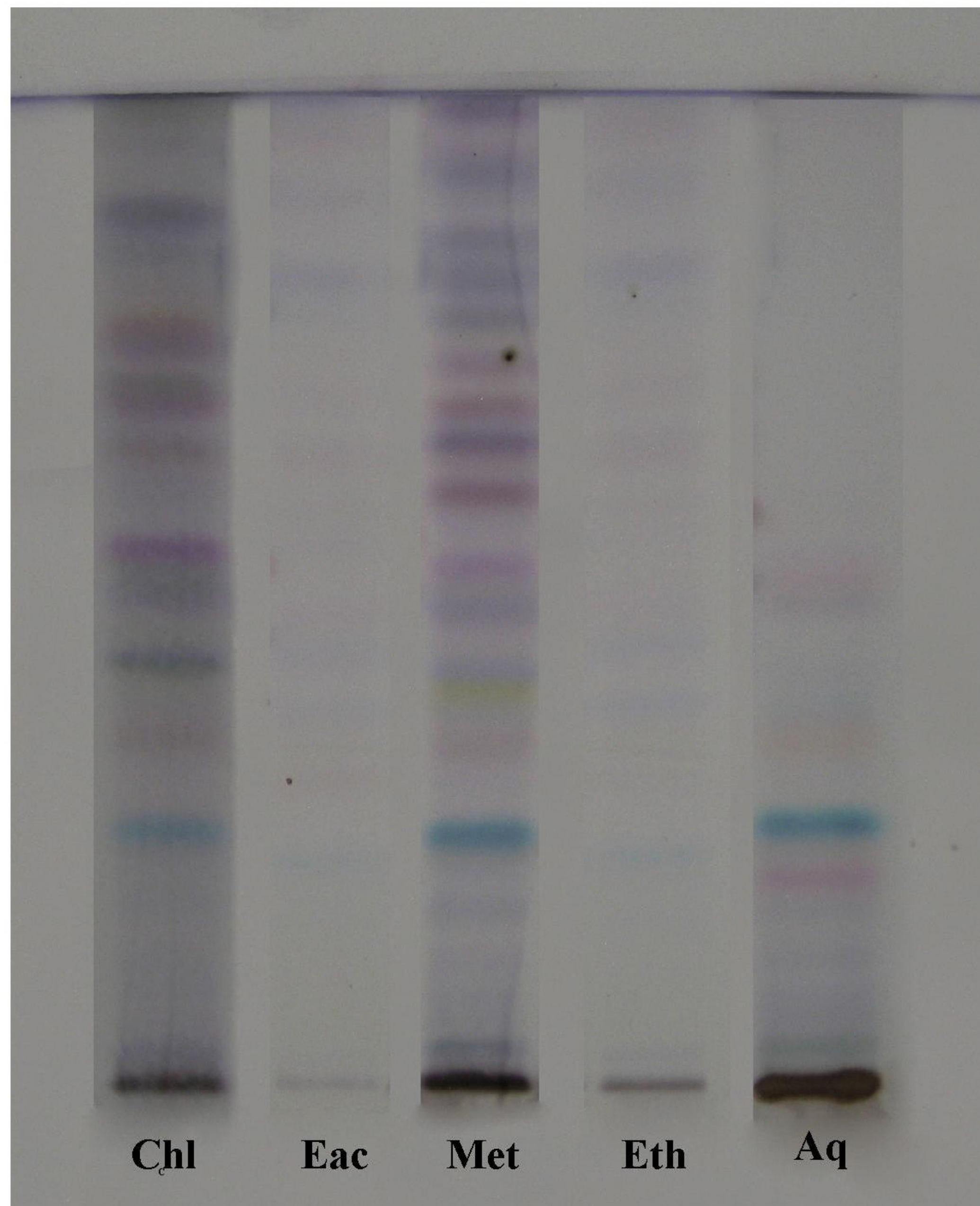
HPTLC profile images of toxic fractions of *V. negundo* and *H. suaveolens* showing the presence of phenolics observed under short UV (254 nm), long UV (366 nm) and at 550 nm after derivatization. VC-*Vitex* Chloroform, HC-*Hyptis* Chloroform

PLATE V. 1



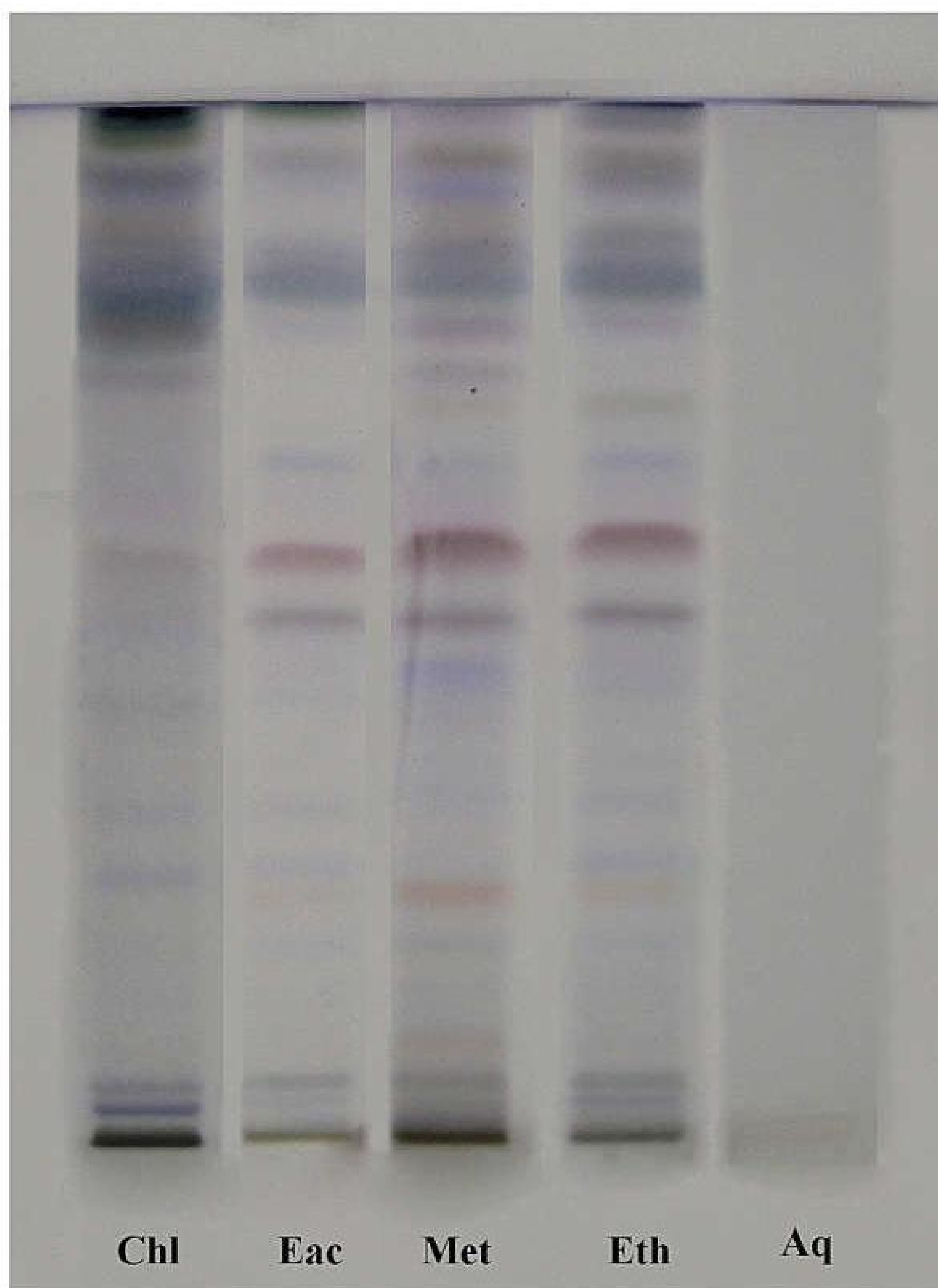
HPTLC profile images of toxic fractions of *V. negundo* and *H. suaveolens* showing the presence of alkaloids observed under short UV (254 nm), long UV (366 nm) and at 550 nm after derivatization. VC-*Vitex* Chloroform, HC-*Hyptis* Chloroform

PLATE IV. 2



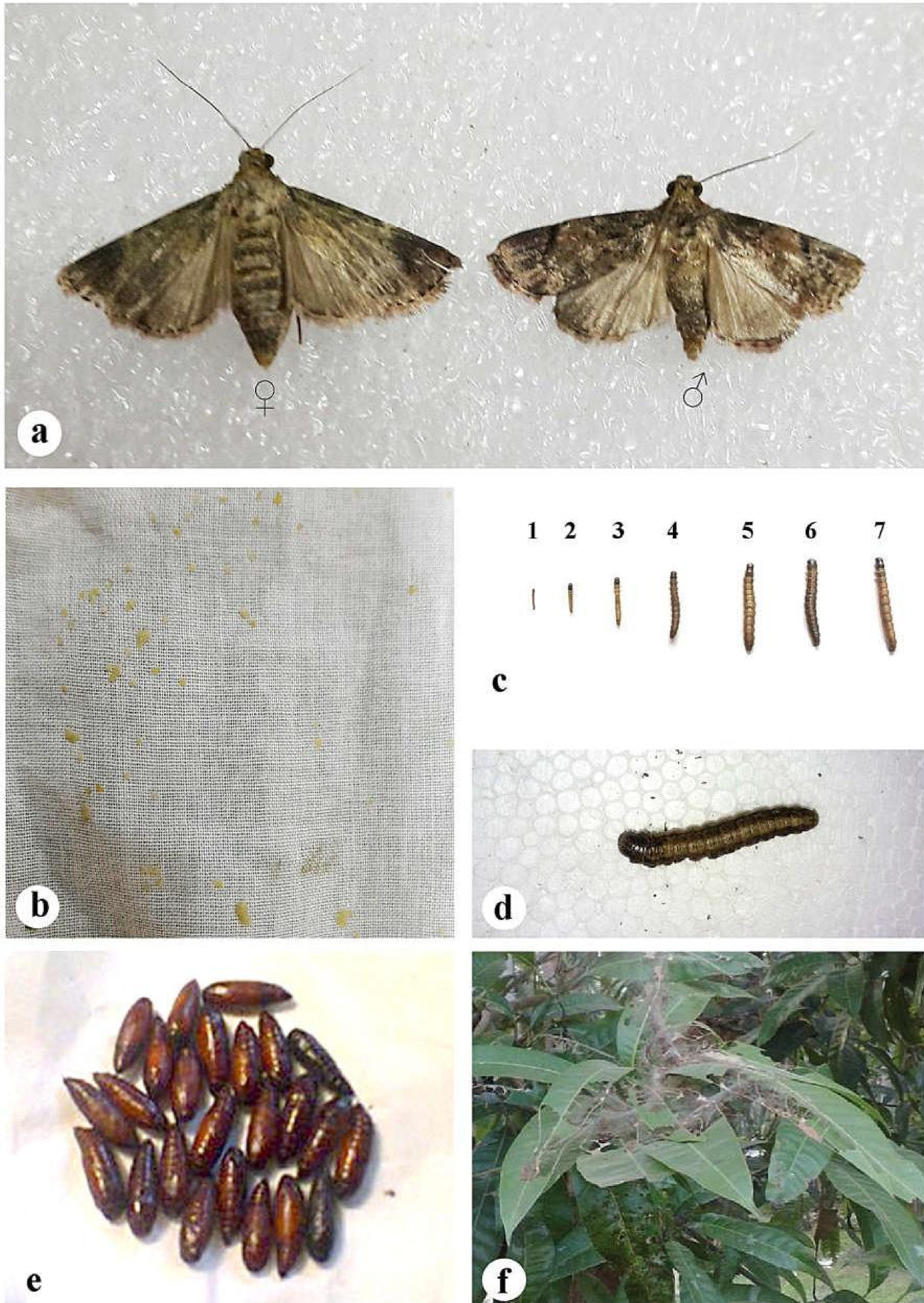
TLC image of different solvent extracts of *Vitex negundo* (550 nm). Abbreviations: Chl-Chloroform, Eac-Ethyl acetate, Met-Methanol, Eth-Ethanol, Aq-Aqueous.

PLATE IV. 1



TLC image of different solvent extracts of *Hyptis suaveolens* (550 nm). Abbreviations: Chl-Chloroform, Eac-Ethyl acetate, Met-Methanol, Eth-Ethanol, Aq-Aqueous.

PLATE III. 1

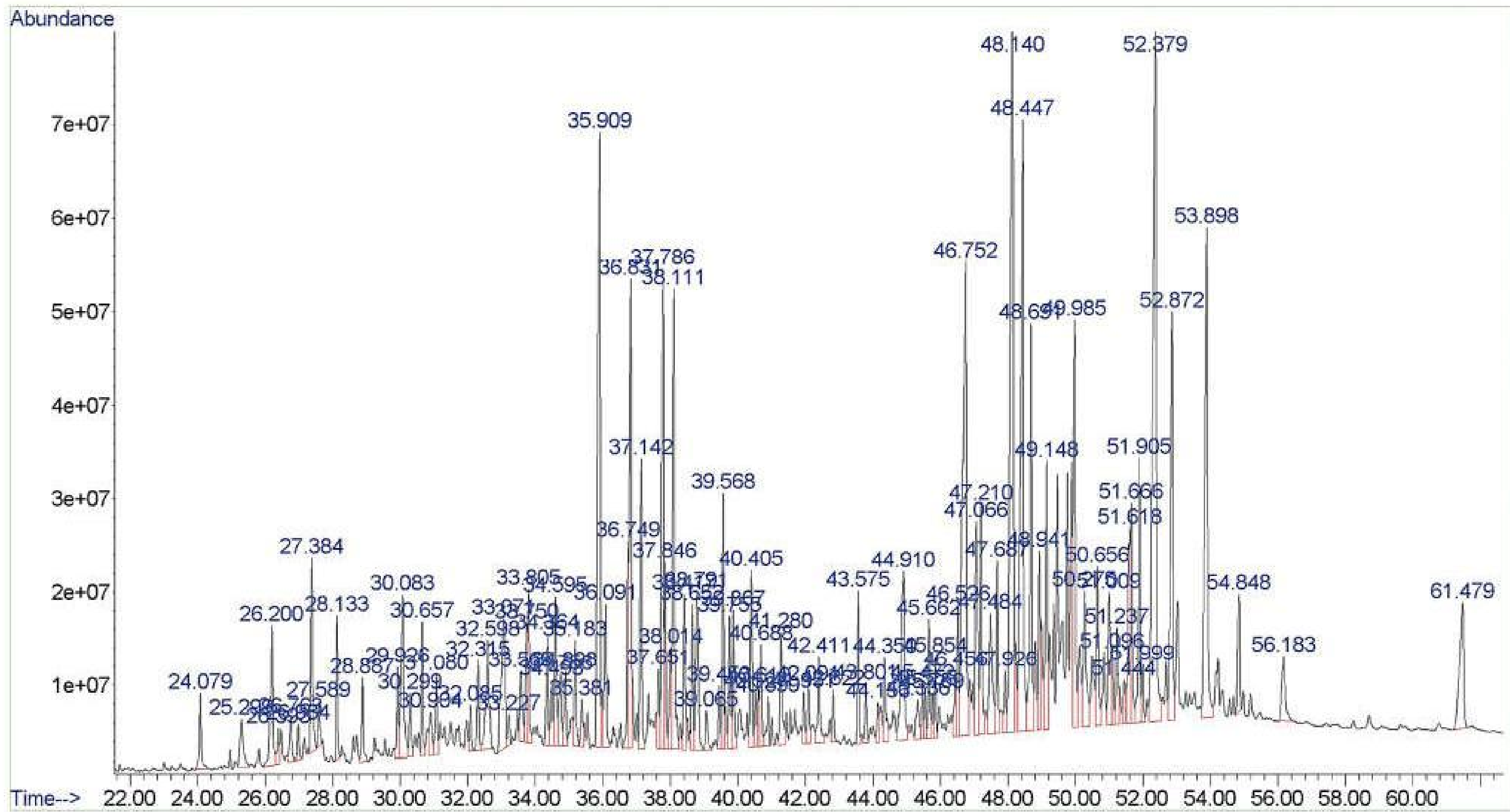


***Orthaga exvinacea*, a. Adult- ♀ ♂, b. Eggs, c. Larval instars- 1-7, d. Pre-pupa, e. Pupa, f-Infestation**

PLATE III. 2

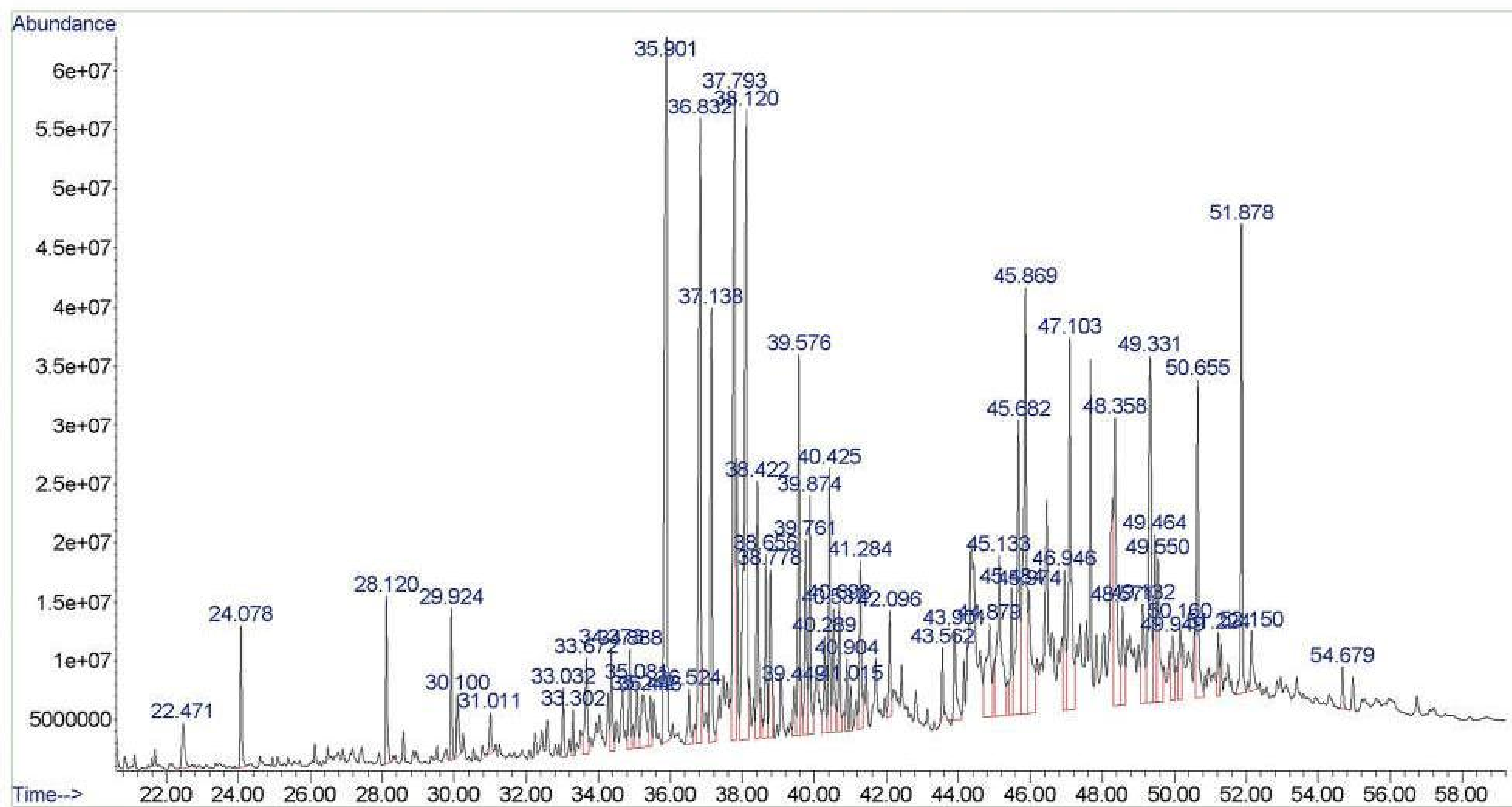
a. *Hyptis suaveolens*; b. *Vitex negundo*

PLATE V. 4



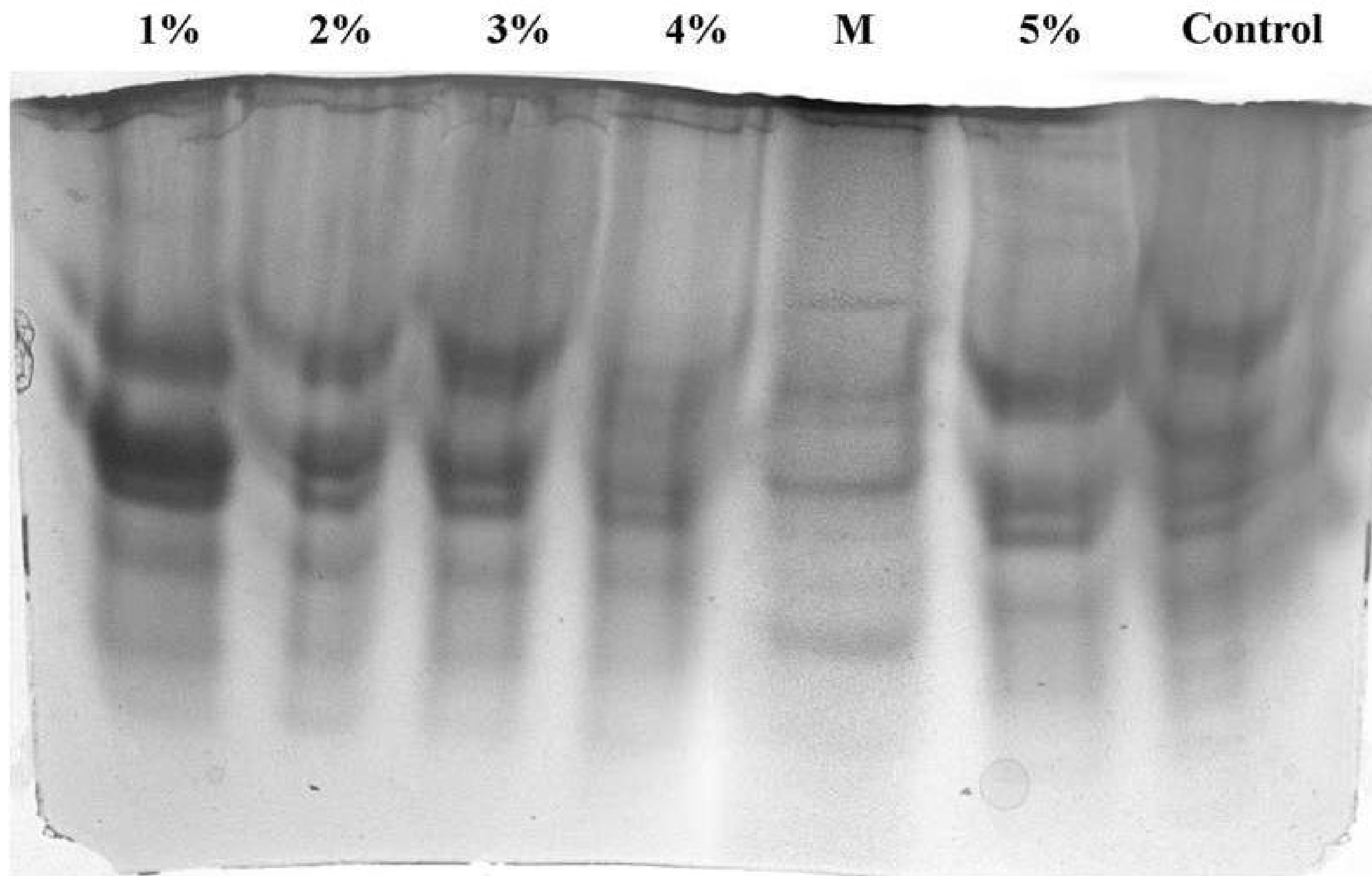
GC-MS analysis of toxic fractions of *Hyptis suaveolens*

PLATE V. 5

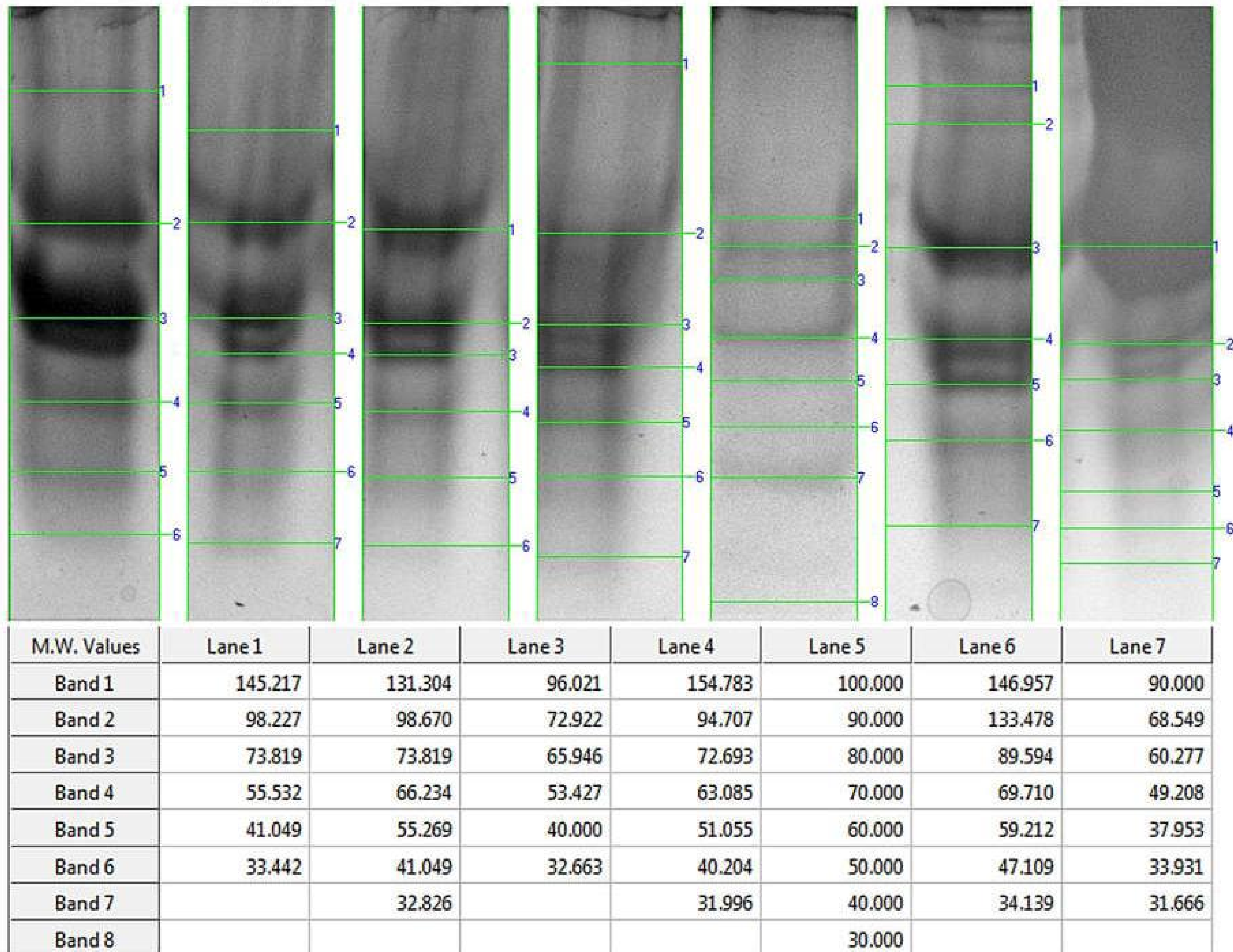


GC- MS analysis of toxic fractions of *Vitex negundo*

PLATE VII. 6



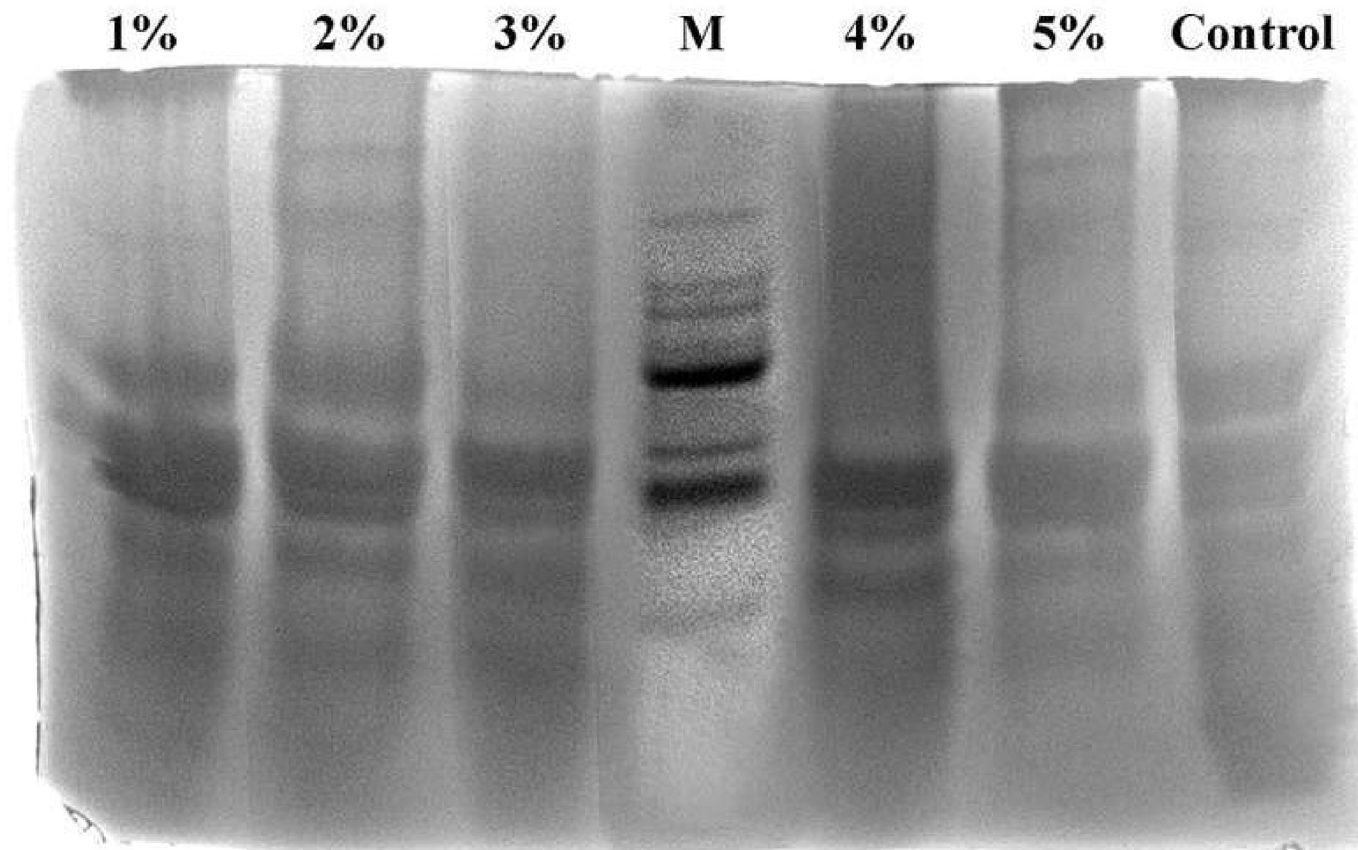
SDS-PAGE gel image showing protein bands of the haemolymph of *O. exvinacea* treated with *V. negundo*.



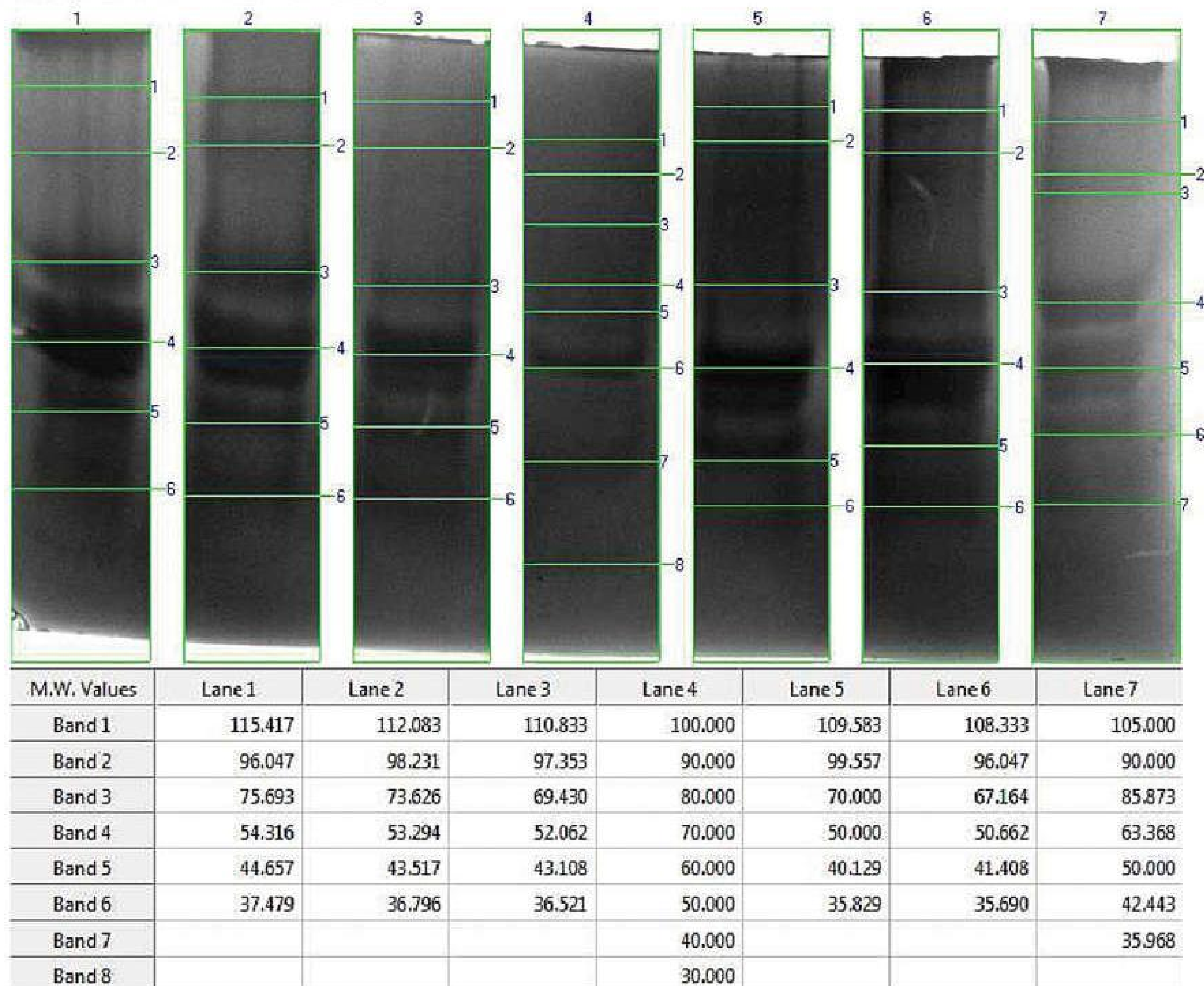
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	145.217	131.304	96.021	154.783	100.000	146.957	90.000
Band 2	98.227	98.670	72.922	94.707	90.000	133.478	68.549
Band 3	73.819	73.819	65.946	72.693	80.000	89.594	60.277
Band 4	55.532	66.234	53.427	63.085	70.000	69.710	49.208
Band 5	41.049	55.269	40.000	51.055	60.000	59.212	37.953
Band 6	33.442	41.049	32.663	40.204	50.000	47.109	33.931
Band 7		32.826		31.996	40.000	34.139	31.666
Band 8					30.000		

Gel documentation showing the molecular weights of protein bands in the haemolymph of *O. exvinacea* treated with *V. negundo*

PLATE VII. 5



SDS- PAGE gel image showing protein bands of the haemolymph of *O. exvinacea* treated with *H. suaveolens*.

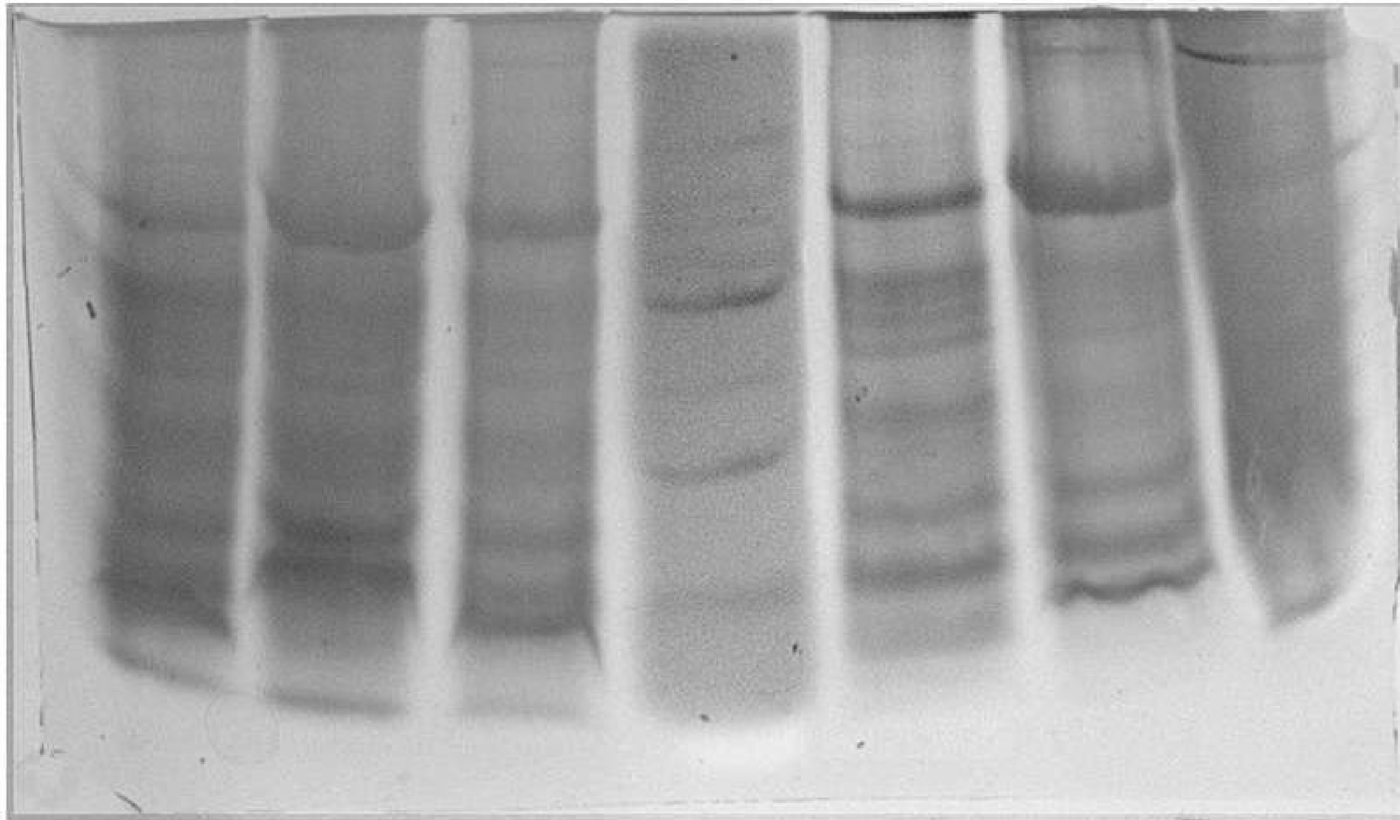


M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	115.417	112.083	110.833	100.000	109.583	108.333	105.000
Band 2	96.047	98.231	97.353	90.000	99.557	96.047	90.000
Band 3	75.693	73.626	69.430	80.000	70.000	67.164	85.873
Band 4	54.316	53.294	52.062	70.000	50.000	50.662	63.368
Band 5	44.657	43.517	43.108	60.000	40.129	41.408	50.000
Band 6	37.479	36.796	36.521	50.000	35.829	35.690	42.443
Band 7				40.000			35.968
Band 8				30.000			

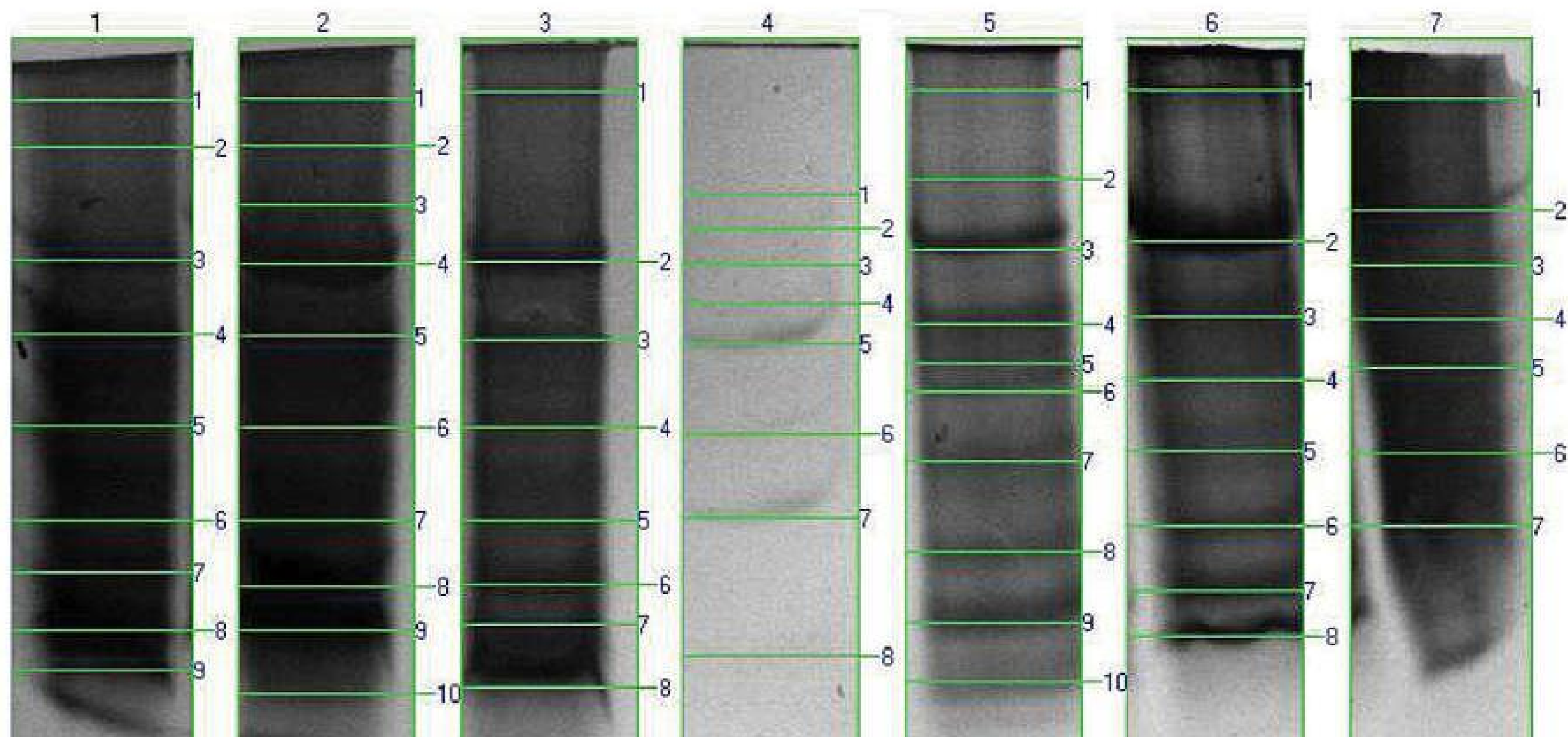
Gel documentation showing the molecular weights of protein bands in the haemolymph of *O. exvinacea* treated with *H. suaveolens*

PLATE VII. 4

1% 2% 3% M 4% 5% Control



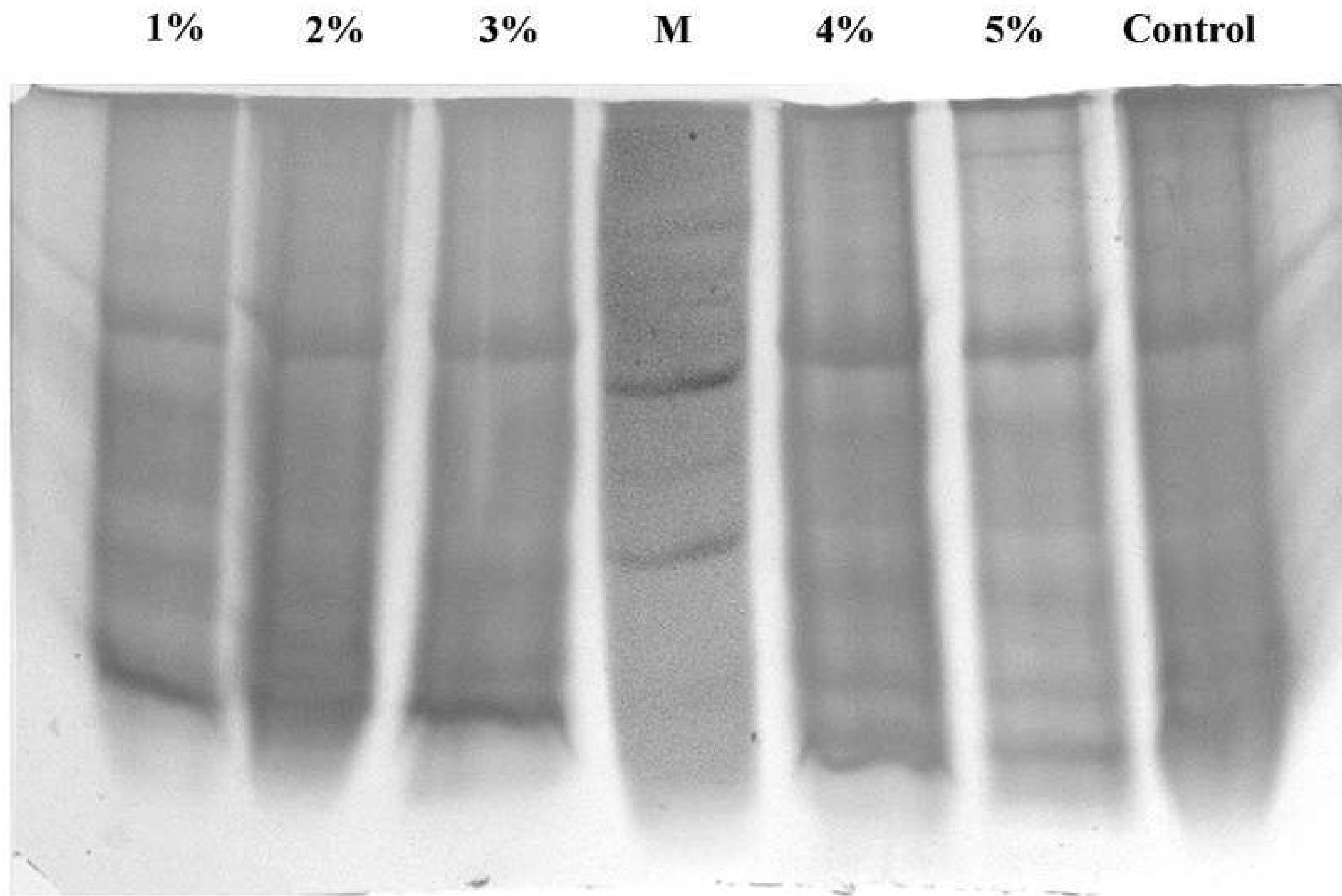
SDS- PAGE gel image showing protein bands of the fat body of *O. exvinacea* treated with *V. negundo*.



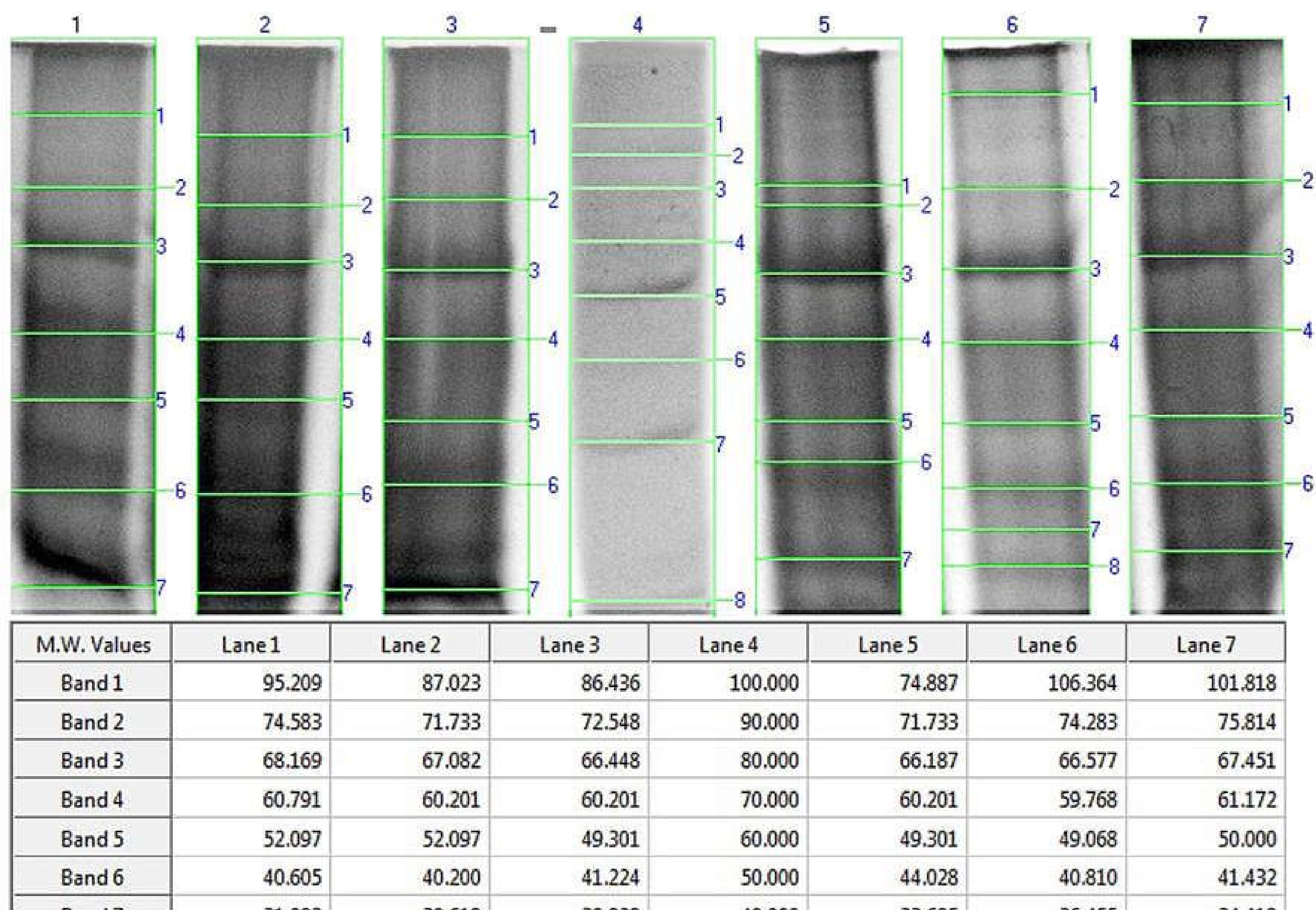
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	128.235	128.824	130.588	100.000	131.176	131.176	128.824
Band 2	114.118	114.706	81.063	90.000	104.706	86.335	95.197
Band 3	81.594	96.987	60.798	80.000	84.234	66.388	80.000
Band 4	62.081	80.532	50.706	70.000	64.405	56.111	65.885
Band 5	50.940	61.643	39.799	60.000	57.848	47.838	57.415
Band 6	39.799	50.706	34.350	50.000	54.793	39.209	47.596
Band 7	35.195	39.799	31.831	40.000	46.625	33.945	39.209
Band 8	31.481	34.214	27.714	30.000	36.894	31.135	
Band 9	29.000	31.481			31.949		
Band 10		27.286			28.143		

Gel documentation showing the molecular weights of protein bands in the fat body of *O. exvinacea* treated with *V. negundo*

PLATE VII. 3



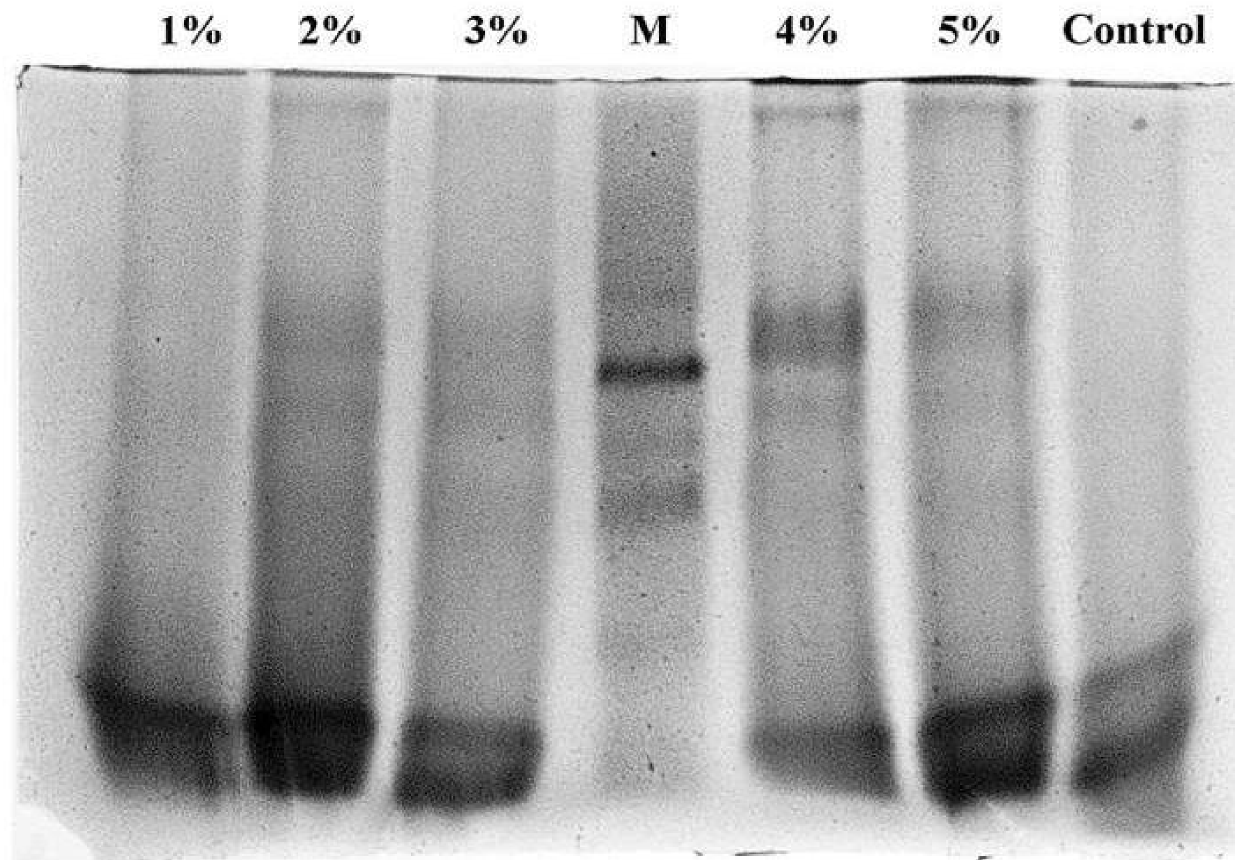
SDS-PAGE gel image showing protein bands of the fat body of *O. exvinacea* treated with *H. suaveolens*.



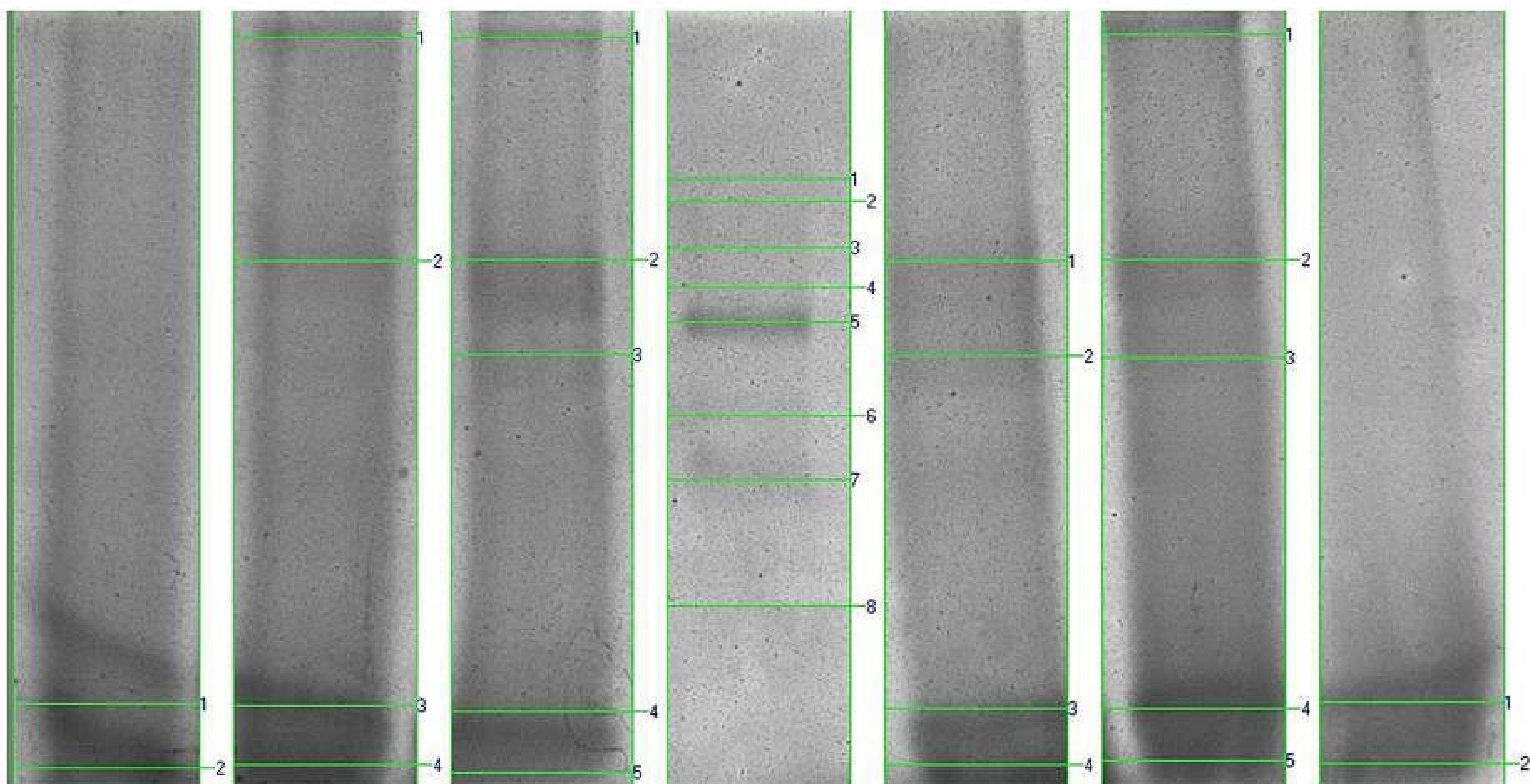
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	95.209	87.023	86.436	100.000	74.887	106.364	101.818
Band 2	74.583	71.733	72.548	90.000	71.733	74.283	75.814
Band 3	68.169	67.082	66.448	80.000	66.187	66.577	67.451
Band 4	60.791	60.201	60.201	70.000	60.201	59.768	61.172
Band 5	52.097	52.097	49.301	60.000	49.301	49.068	50.000
Band 6	40.605	40.200	41.224	50.000	44.028	40.810	41.432

Gel documentation showing the molecular weights of protein bands in the fat body of *O. exvinacea* treated with *H. suaveolens*

PLATE VII. 2



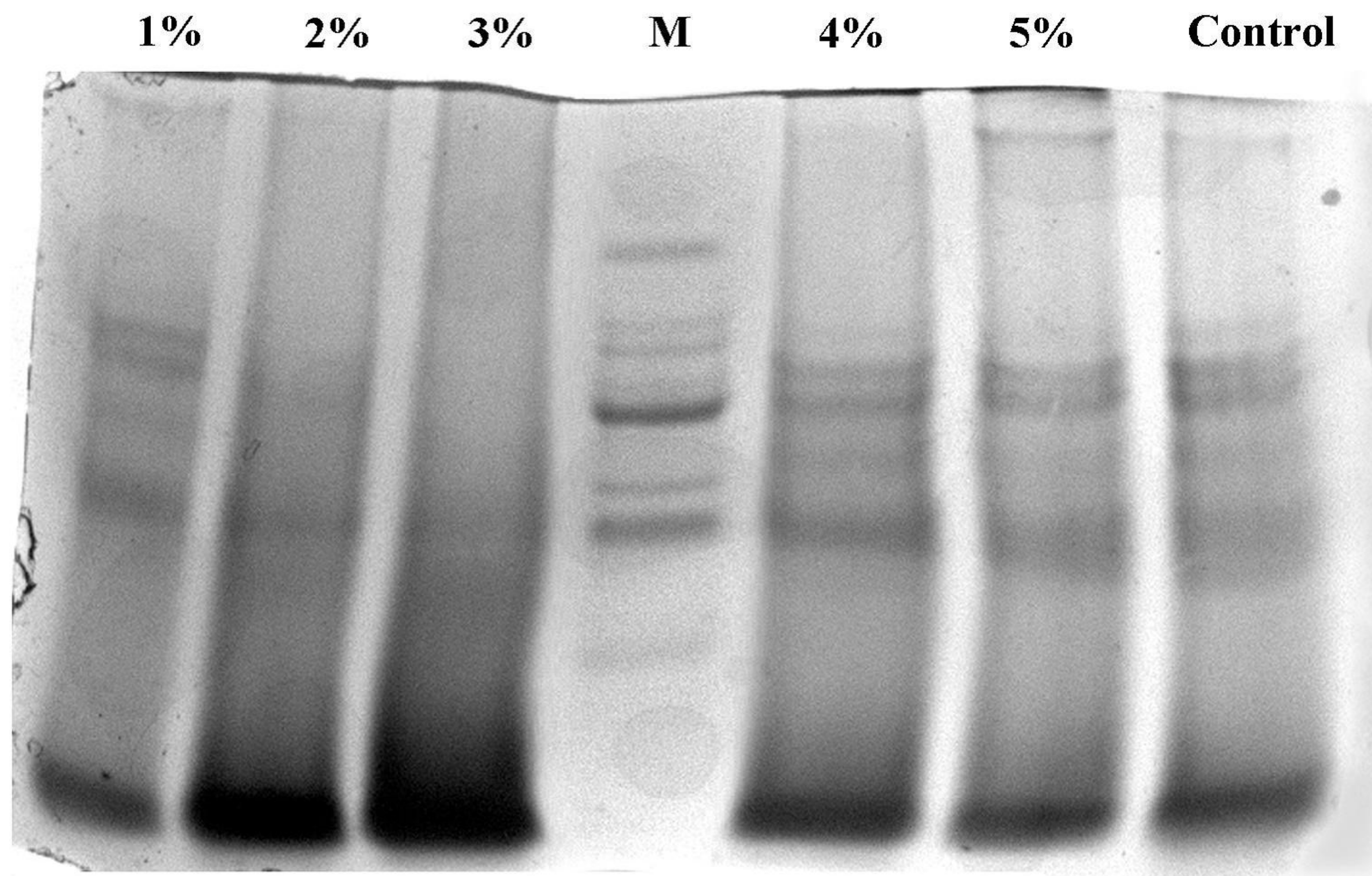
SDS-PAGE gel image showing protein bands of the midgut of *O. exvinacea* treated with *V. negundo*.



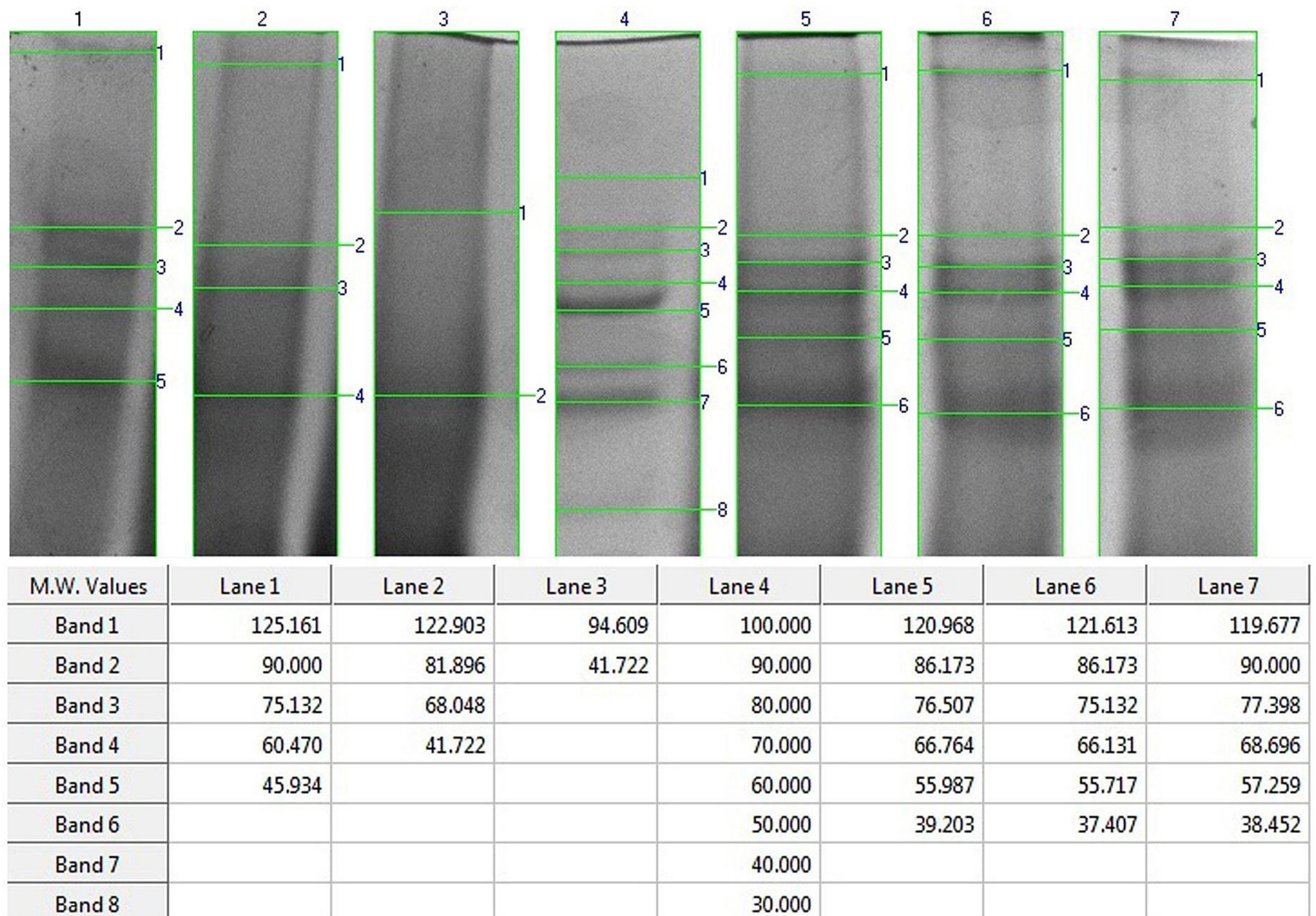
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	22.353	163.333	163.333	100.000	76.892	164.667	22.471
Band 2	17.294	76.892	77.243	90.000	56.920	77.243	17.647
Band 3		22.235	57.061	80.000	22.000	56.778	
Band 4		17.529	21.765	70.000	17.529	22.000	
Band 5			16.941	60.000		17.882	
Band 6				50.000			
Band 7				40.000			
Band 8				30.000			

Gel documentation showing the molecular weights of protein bands in the midgut of *O. exvinacea* treated with *V. negundo*

PLATE-VII. 1



SDS- PAGE gel image showing protein bands of the midgut of *O. exvinacea* treated with *H. suaveolens*.



M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	125.161	122.903	94.609	100.000	120.968	121.613	119.677
Band 2	90.000	81.896	41.722	90.000	86.173	86.173	90.000
Band 3	75.132	68.048		80.000	76.507	75.132	77.398
Band 4	60.470	41.722		70.000	66.764	66.131	68.696
Band 5	45.934			60.000	55.987	55.717	57.259
Band 6				50.000	39.203	37.407	38.452
Band 7				40.000			
Band 8				30.000			

Gel documentation showing the molecular weights of protein bands in the midgut of *O. exvinacea* treated with *H. suaveolens*

Figure IV. 1. Probit analysis graph for the determination of LD_{50} value for *Hyptis suaveolens*

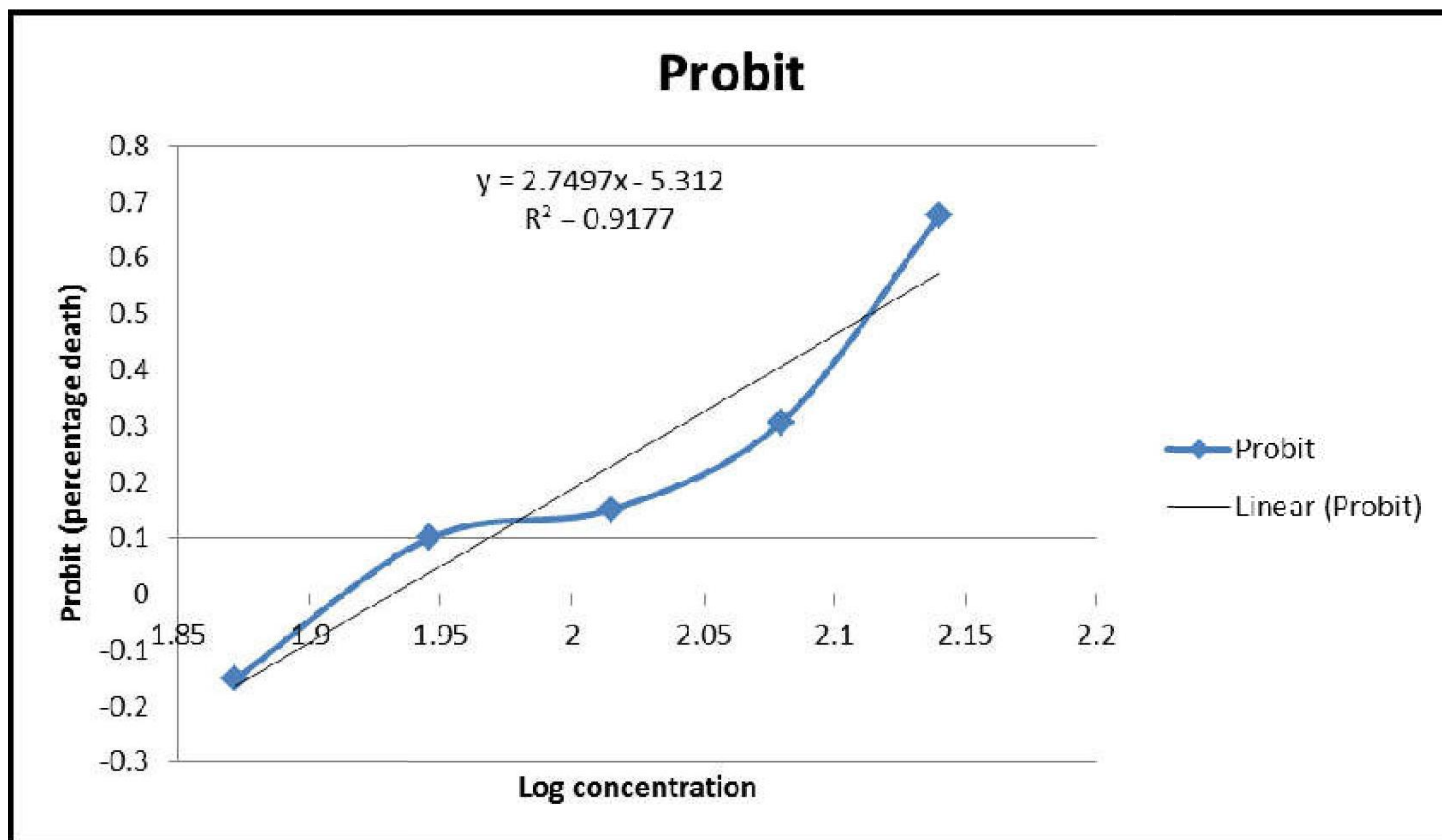


Figure IV. 2. Probit analysis graph for the determination of LD_{50} value for *Vitex negundo*

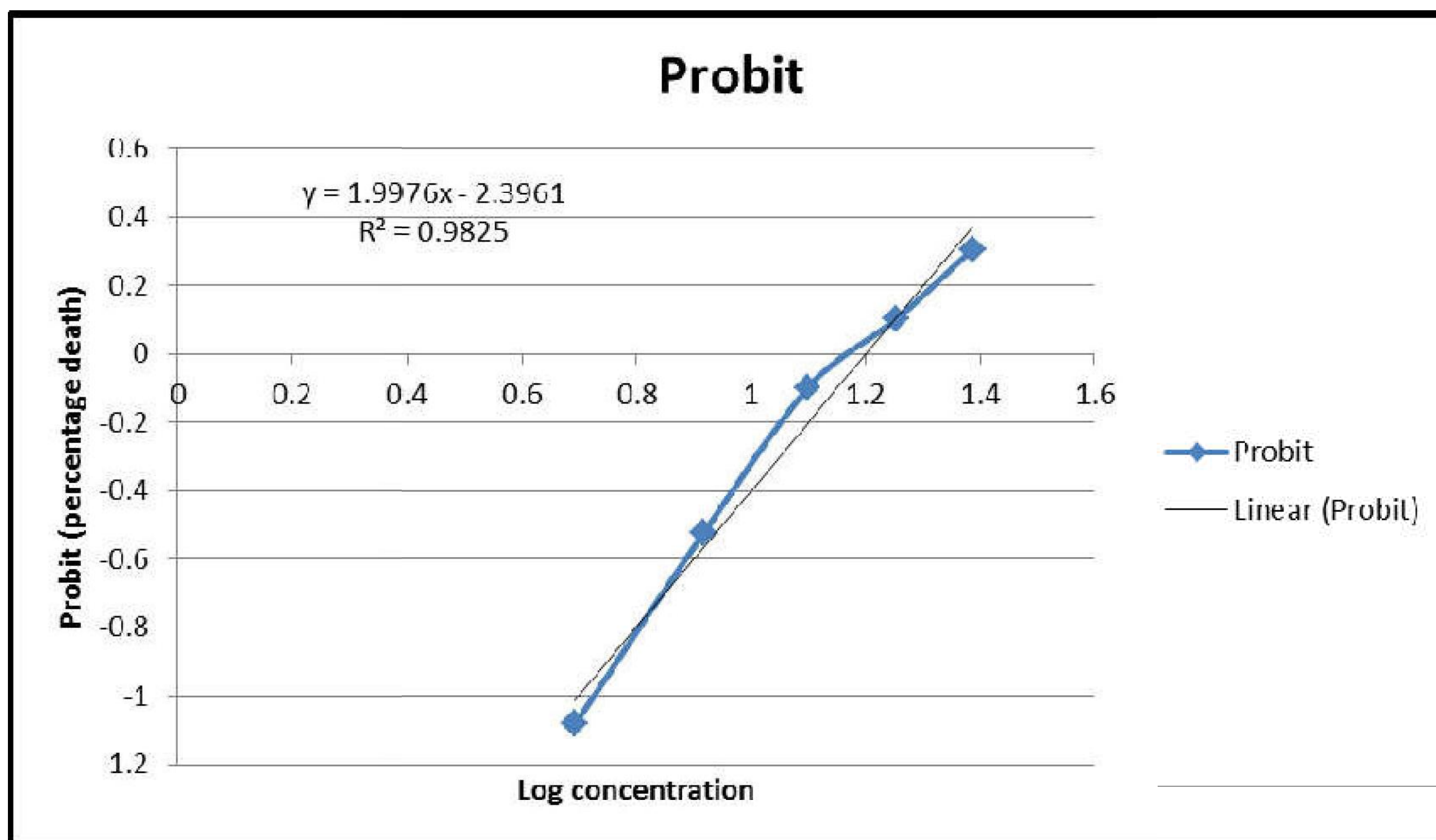
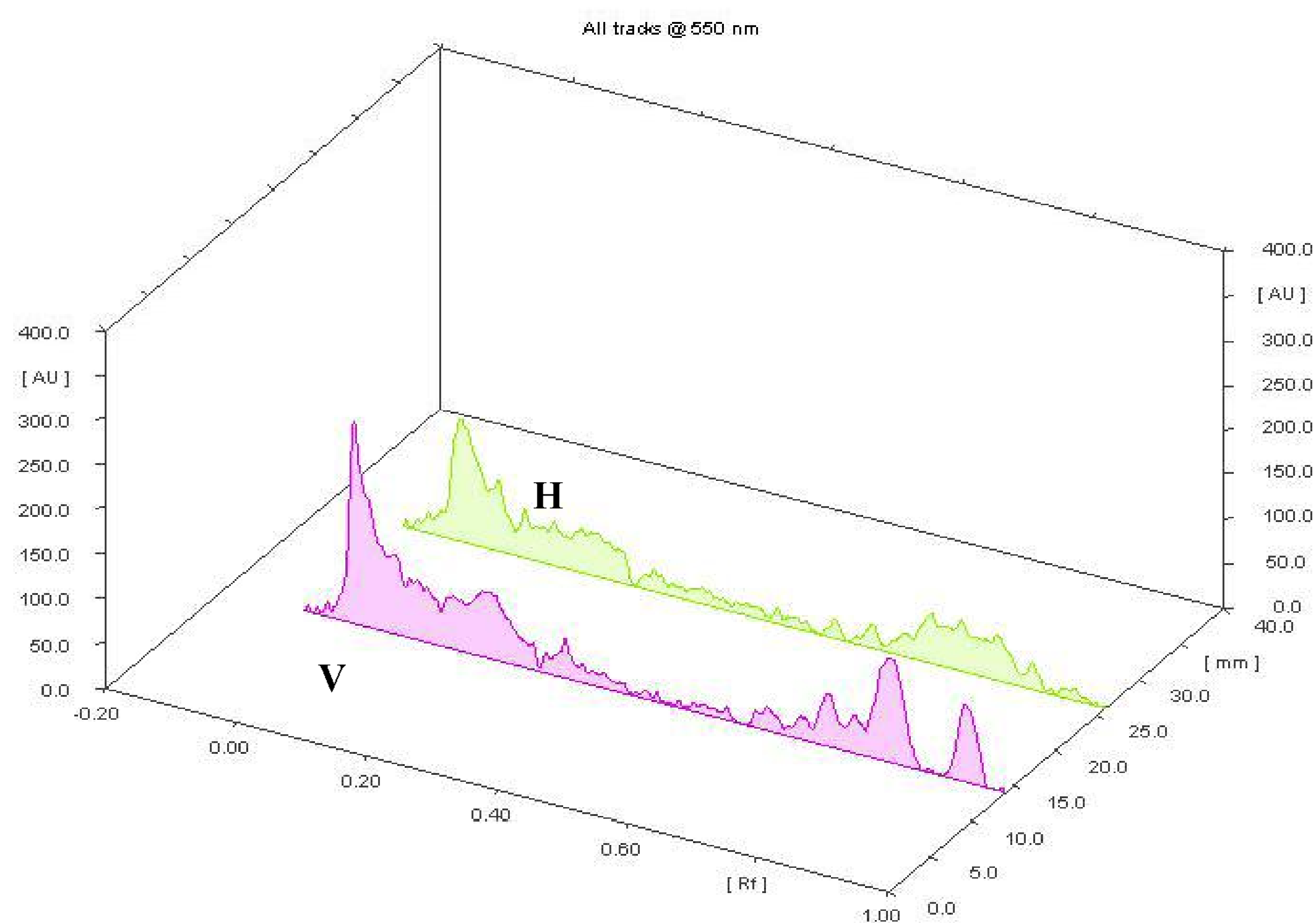


Figure V. 1. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for alkaloids scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 2. HPTLC fingerprint of toxic fraction of *V. negundo* for alkaloids after derivatization showing different peaks of phytoconstituents (550 nm).

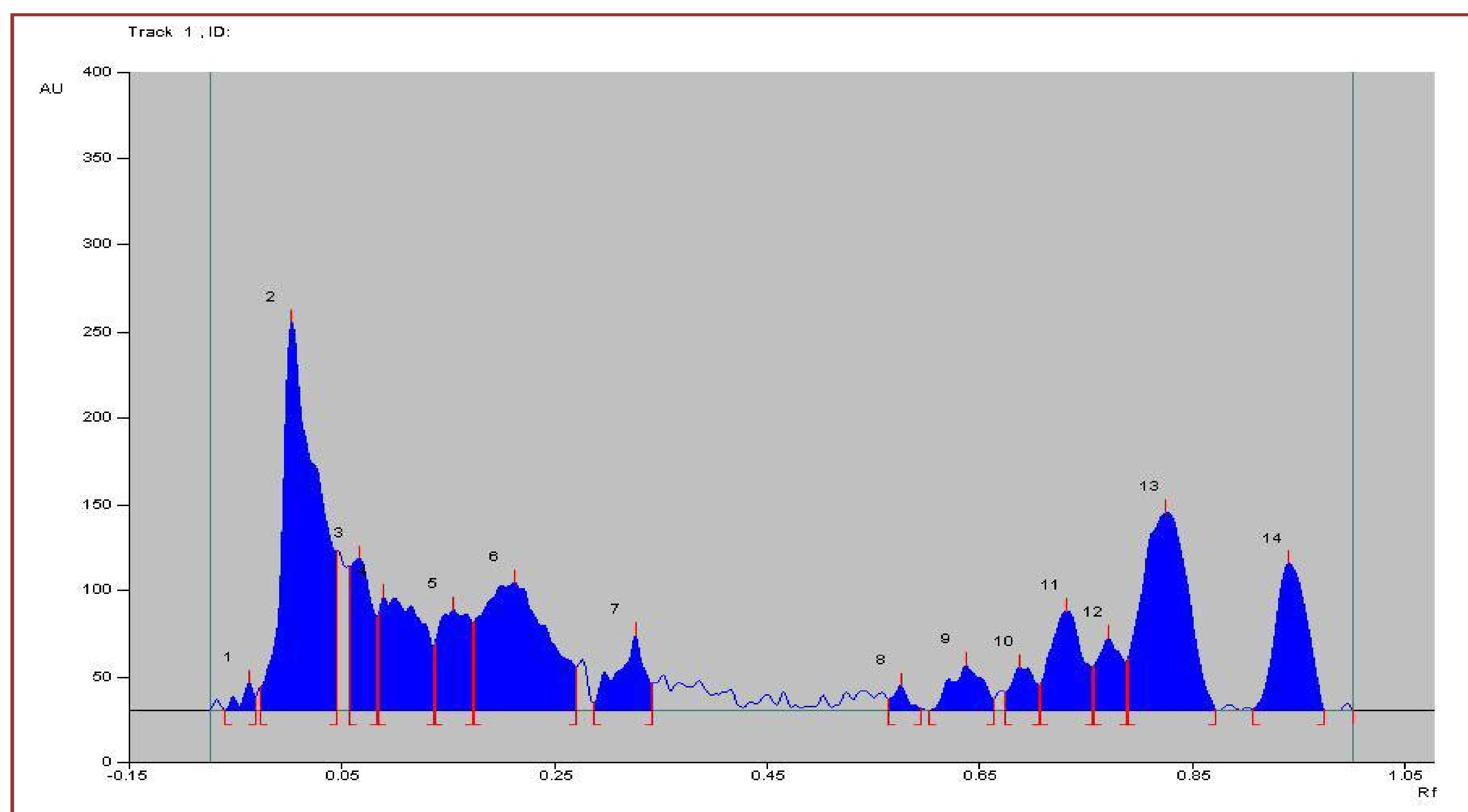


Figure V. 3. HPTLC fingerprint of toxic fraction of *H. suaveolens* for alkaloids showing different peaks of phytoconstituents after derivatization (550 nm).

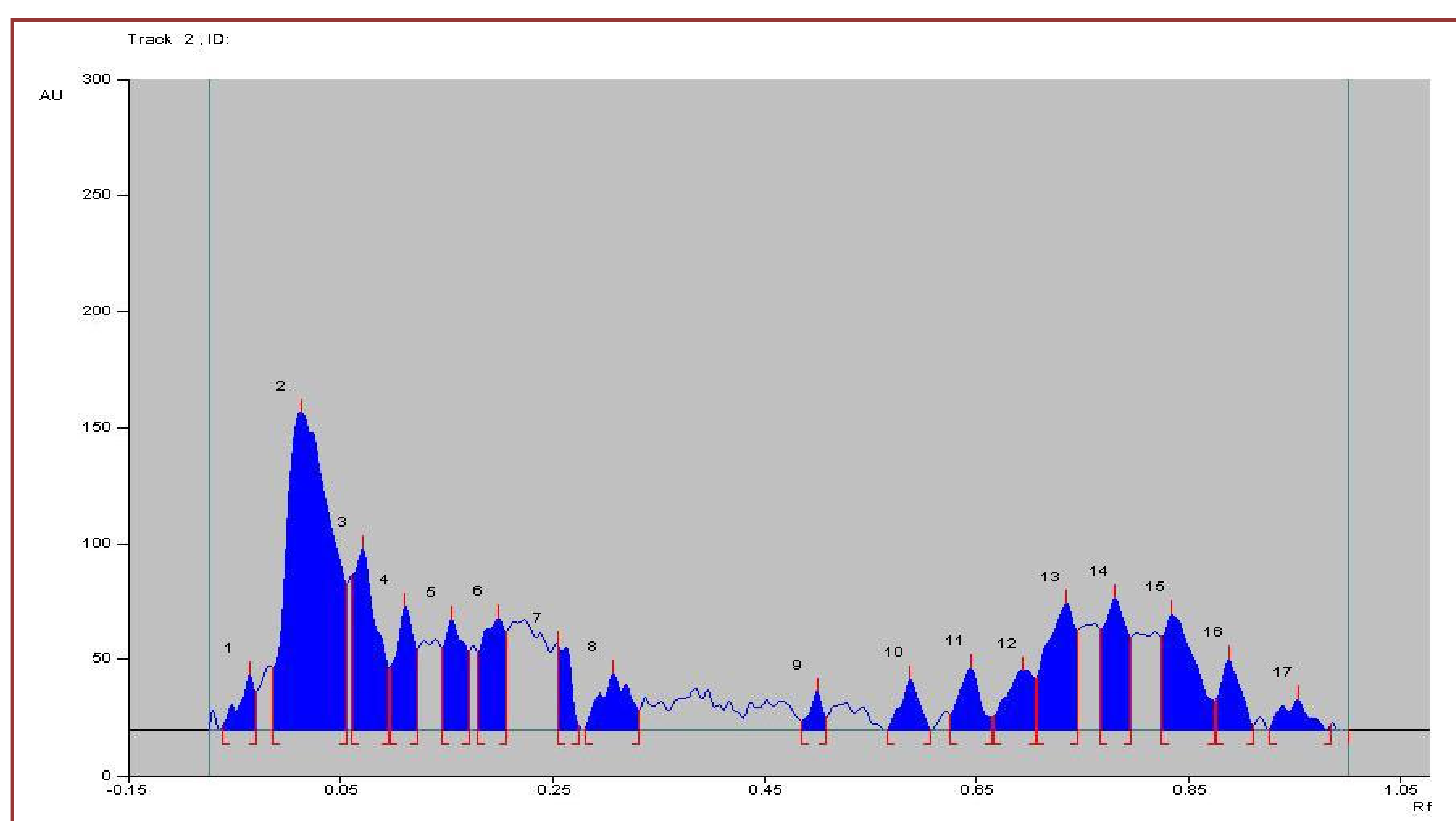
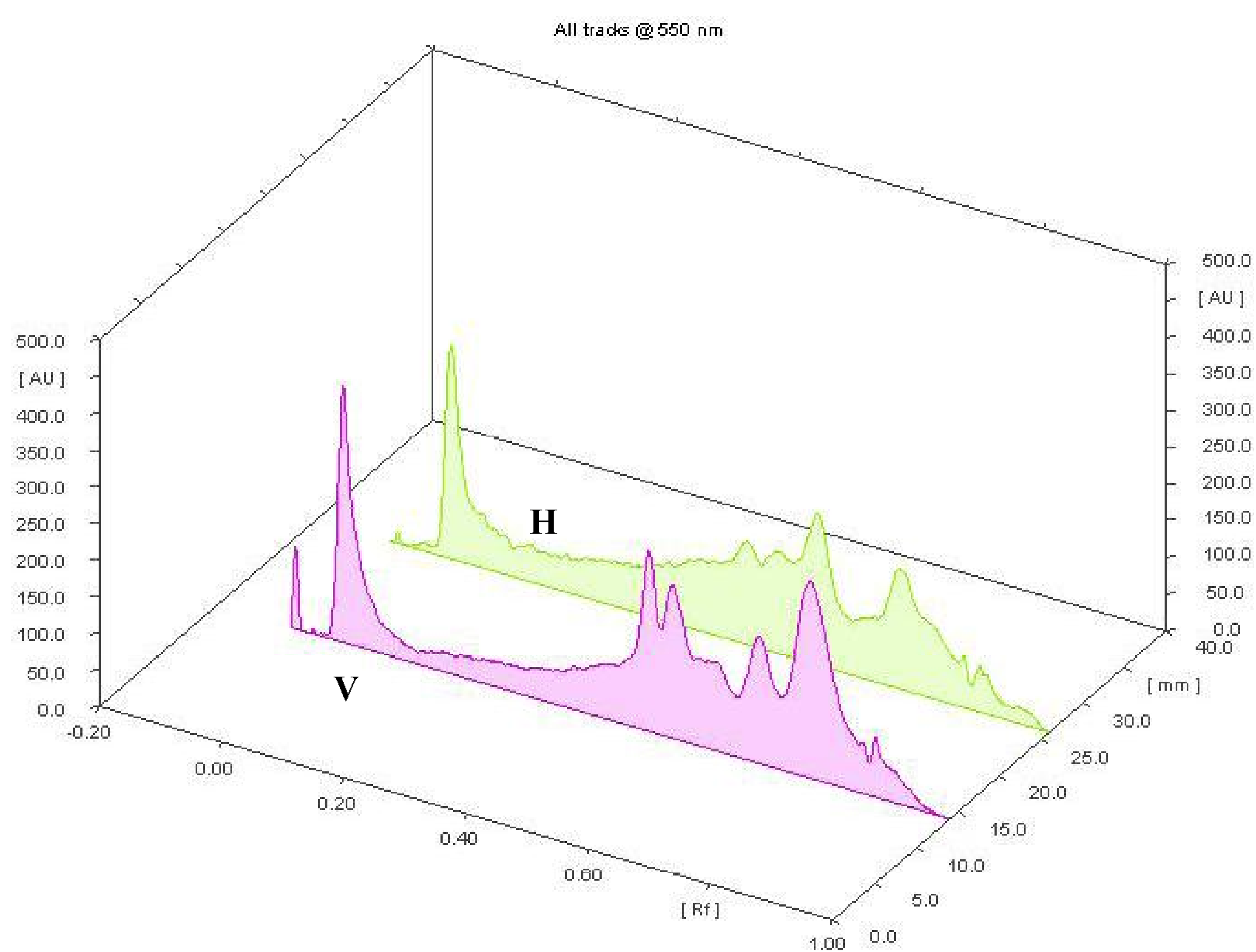


Figure V. 4. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for phenolics scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 5. HPTLC fingerprint of toxic fraction *V. negundo* for phenolics showing different peaks of phytoconstituents after derivatization (550 nm).

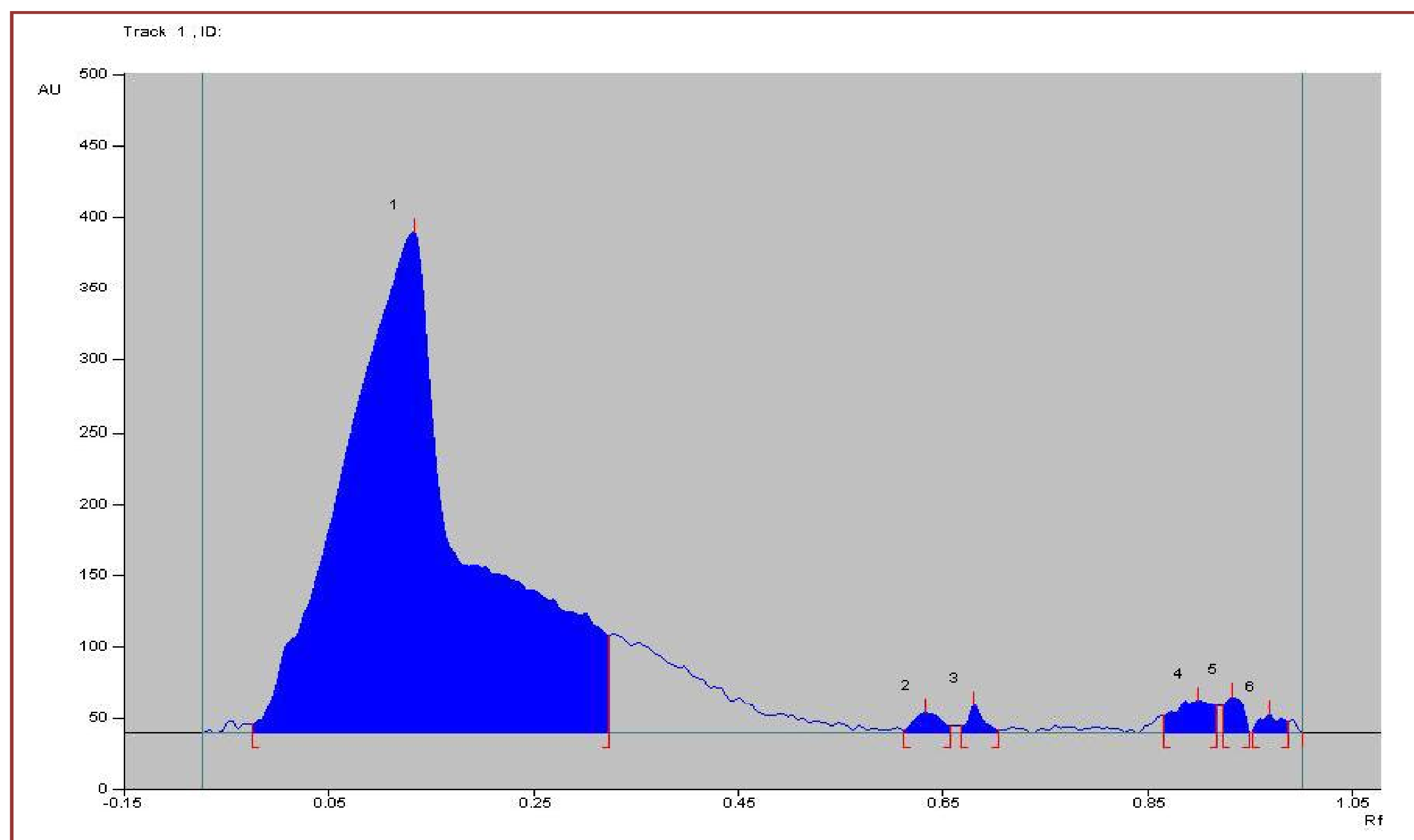


Figure V. 6. HPTLC fingerprint of *H. suaveolens* toxic fraction for phenolics showing different peaks of phytoconstituents after derivatization (550 nm).

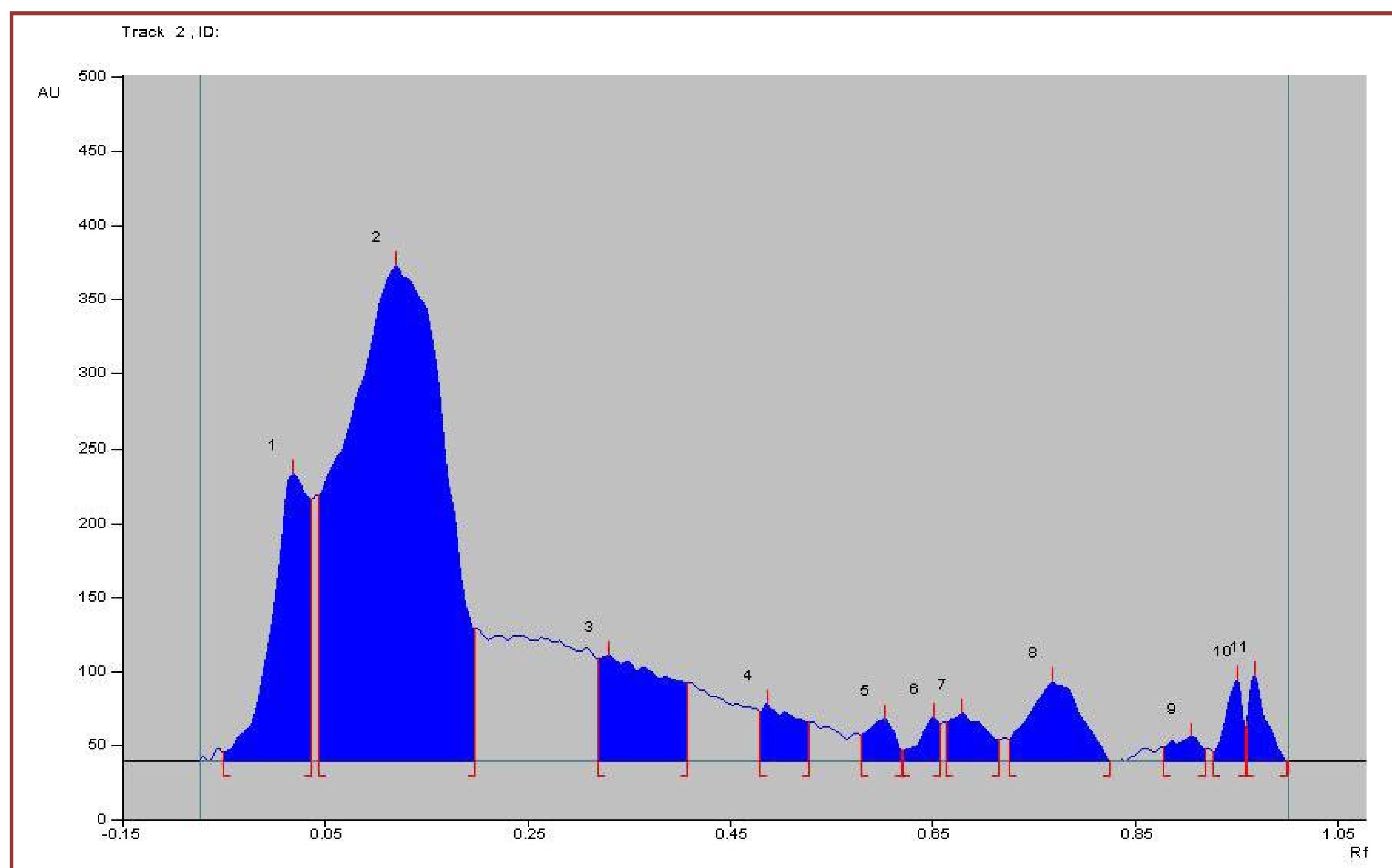
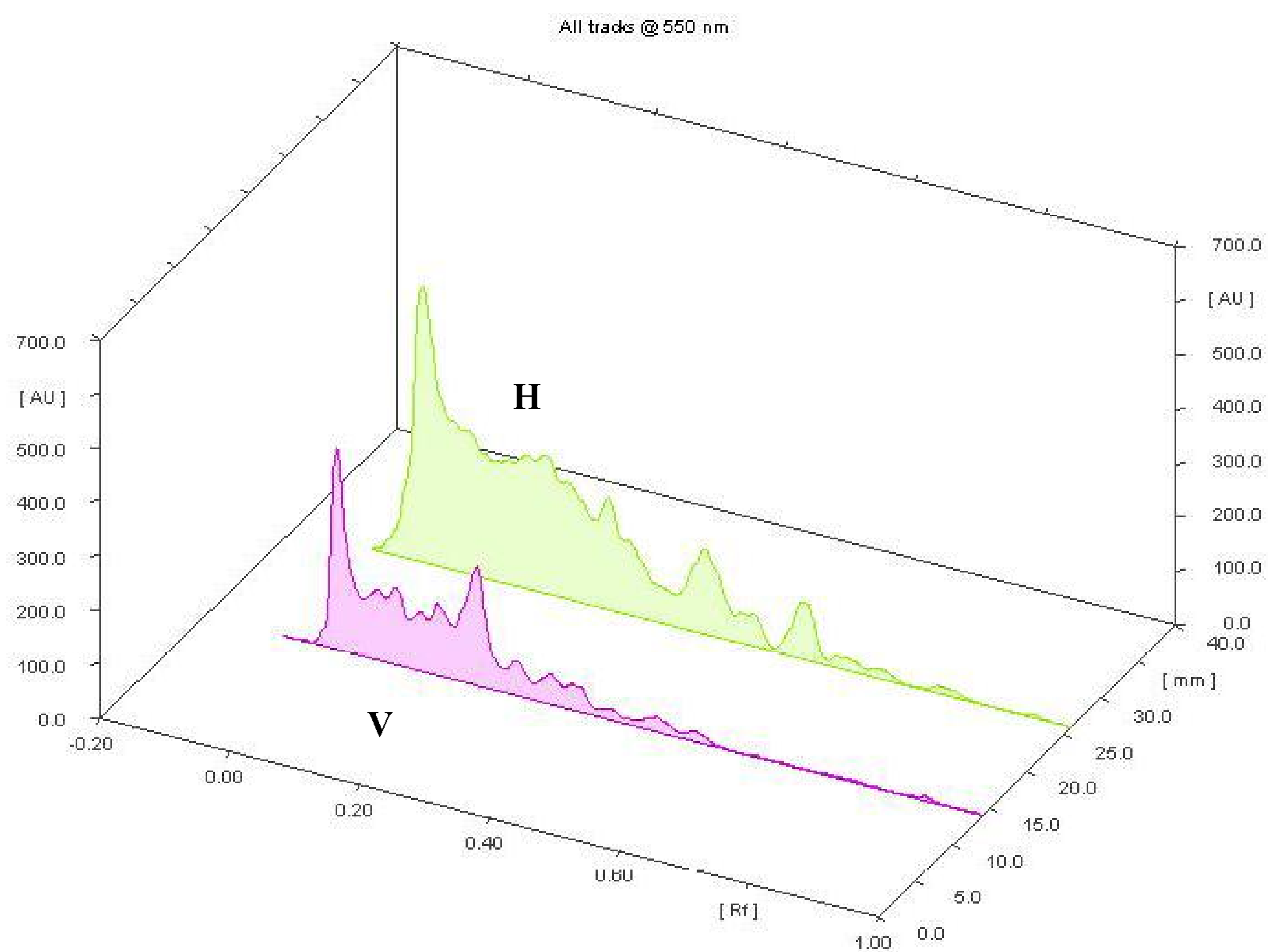


Figure V. 7. Densitometric chromatogram of toxic fractions of both *H. suaveolens* and *V. negundo* for terpenoids scanned at 550 nm (3D view).



V stands for *V. negundo*; H stands for *H. suaveolens*

Figure V. 8. HPTLC fingerprint of toxic fraction of *V. negundo* for terpenoids showing different peaks of phytoconstituents after derivatization (550 nm)

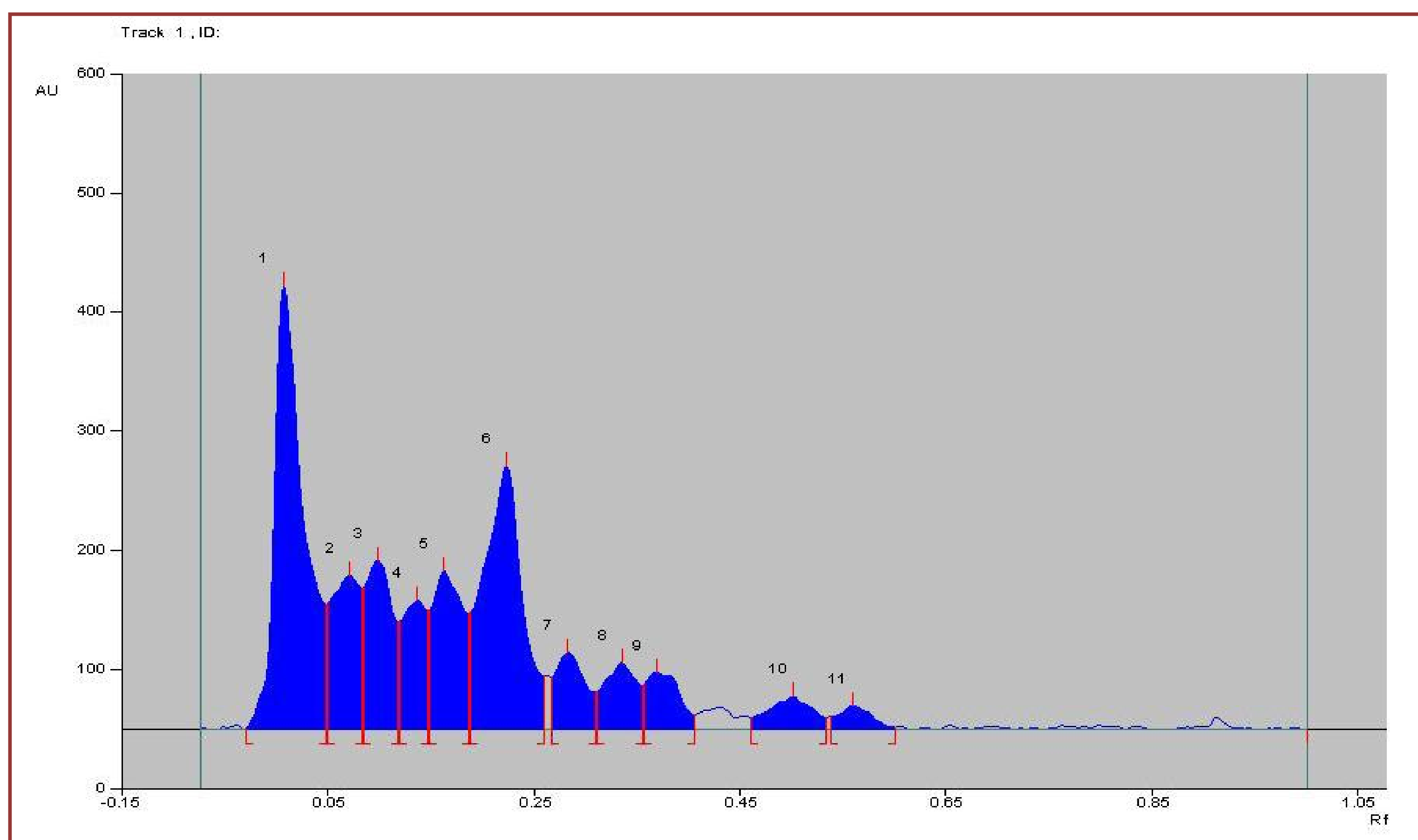


Figure V. 9. HPTLC fingerprint of toxic fraction of *H. suaveolens* for terpenoids showing different peaks of phytoconstituents after derivatization (550 nm)

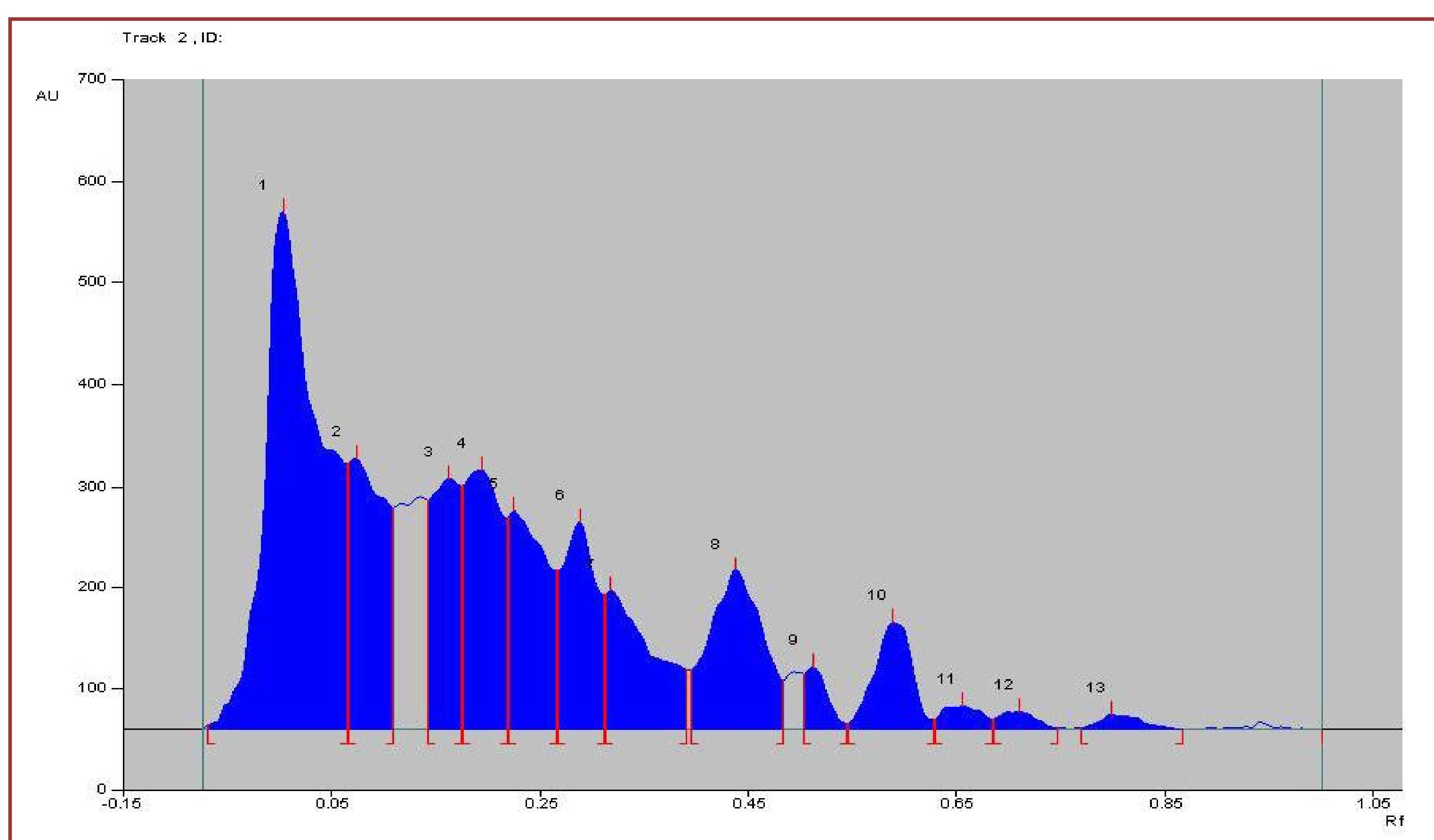


Figure VII. 1. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in the midgut tissue of sixth instar larvae of *O. exvinacea.*

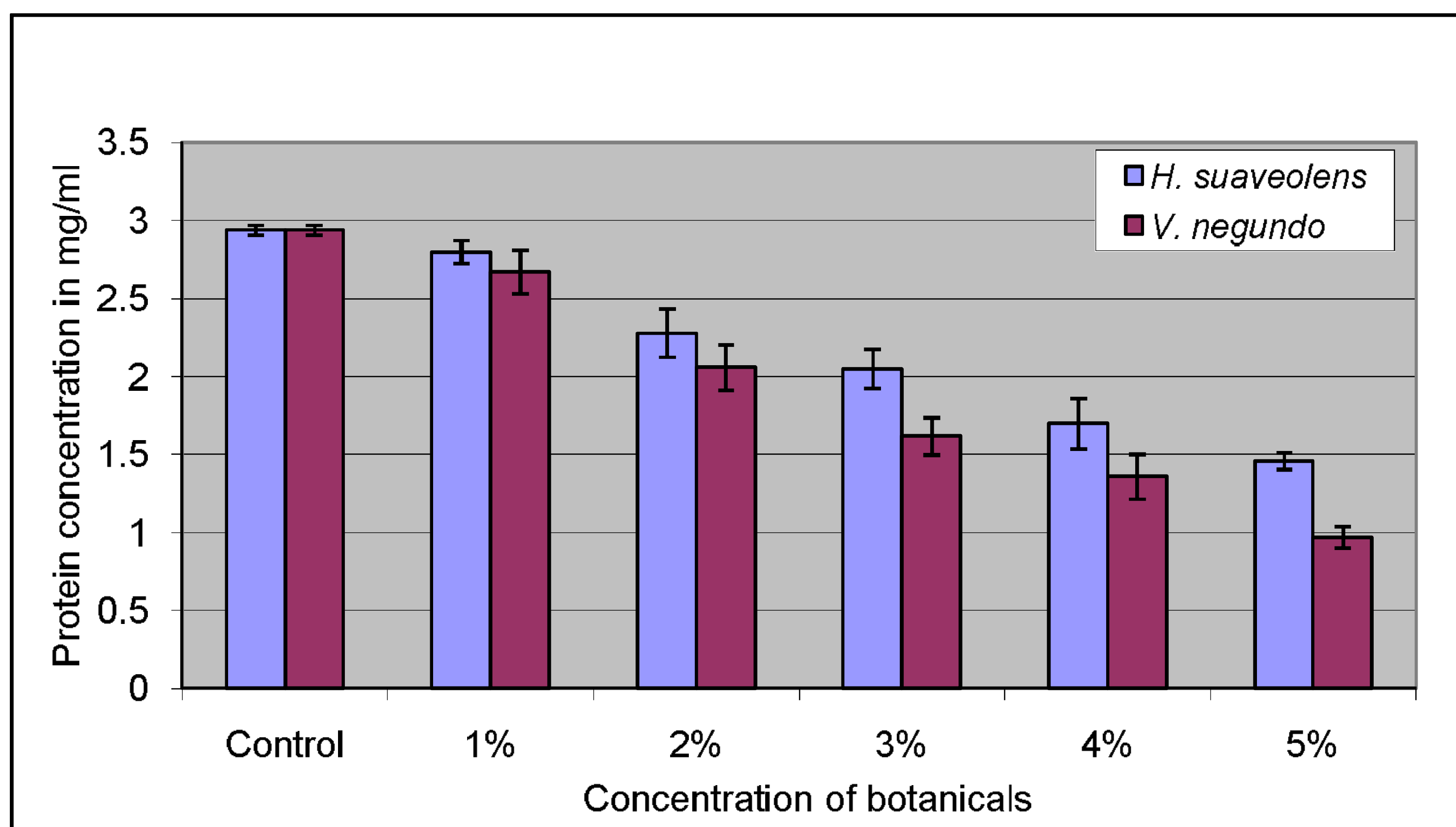


Figure VII. 2. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in fat body of sixth instar larvae of *O. exvinacea.*

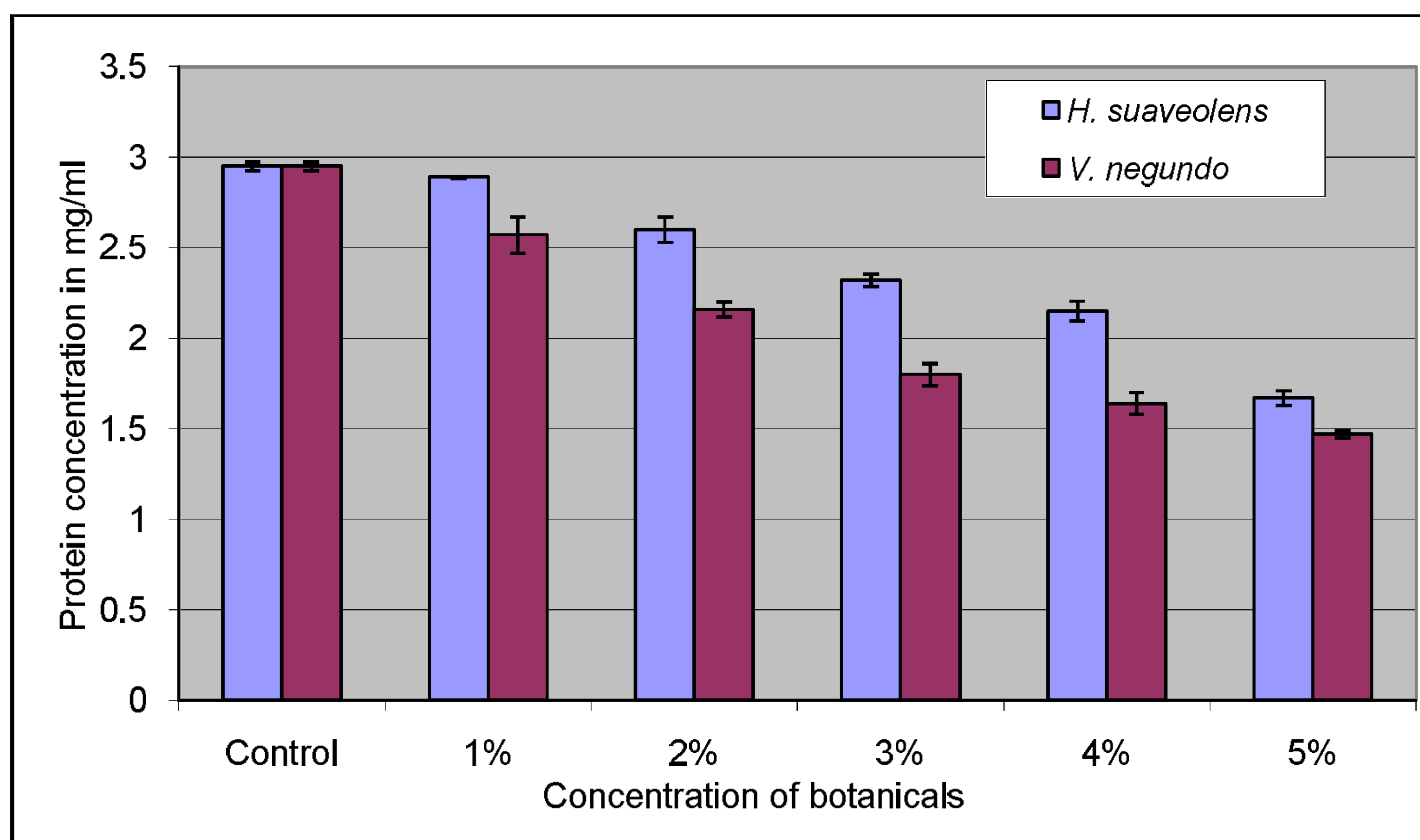


Figure VII. 3. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on protein content in haemolymph of sixth instar larvae of *O. exvinacea.*

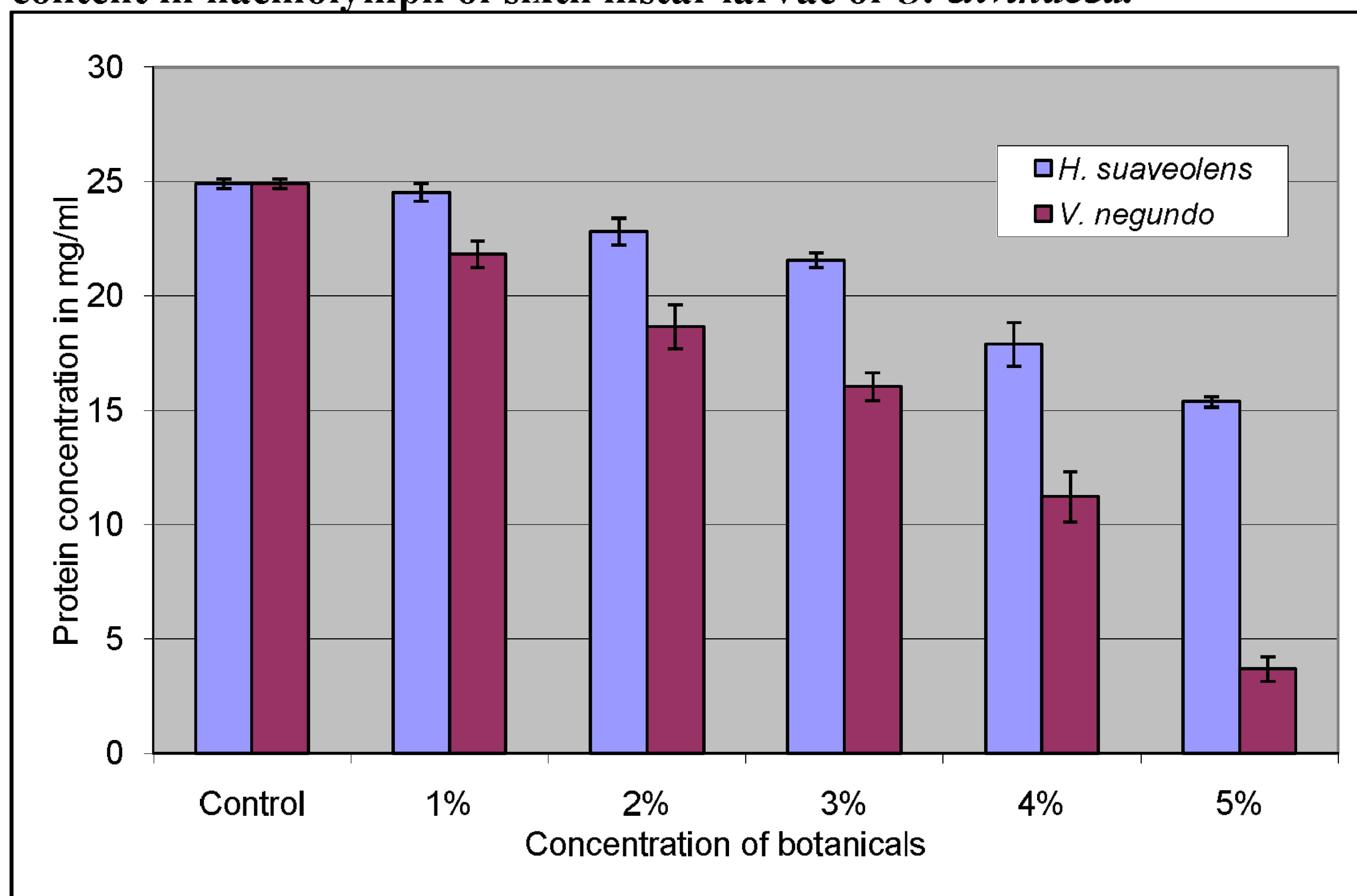


Table VII. 3. Consolidated table showing the percentage of reduction in total protein content over control with respect to mg/ ml mean values of protein concentration for both botanicals.

Conc. of botanicals	Percentage reduction of protein content (%)					
	Hyptis suaveolens treated			*Vitex negundo* treated		
	Midgut	Fat body	haemolymph	Midgut	Fat body	haemolymph
1 %	4.7	2.03	1.53	9.2	12.9	12.4
2 %	22.4	11.9	8.4	29.9	26.8	25.1
3 %	30.3	21.3	13.4	44.9	39	35.6
4 %	42.2	27.1	28.2	53.7	44.4	54.9
5 %	50.3	43.4	38.8	67	50.2	85.1

For 1 % treatment of *H. suaveolens*, the midgut tissue showed only 4.7 % reduction of protein concentration when compared to control midgut tissue while that of *V. negundo* exhibited 9.2 % of reduction to that of control larval tissue. In the case of samples treated with 5 % of *H. suaveolens*, the

Figure VII. 4. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in the midgut tissue of sixth instar larvae of *O. exvinacea.*

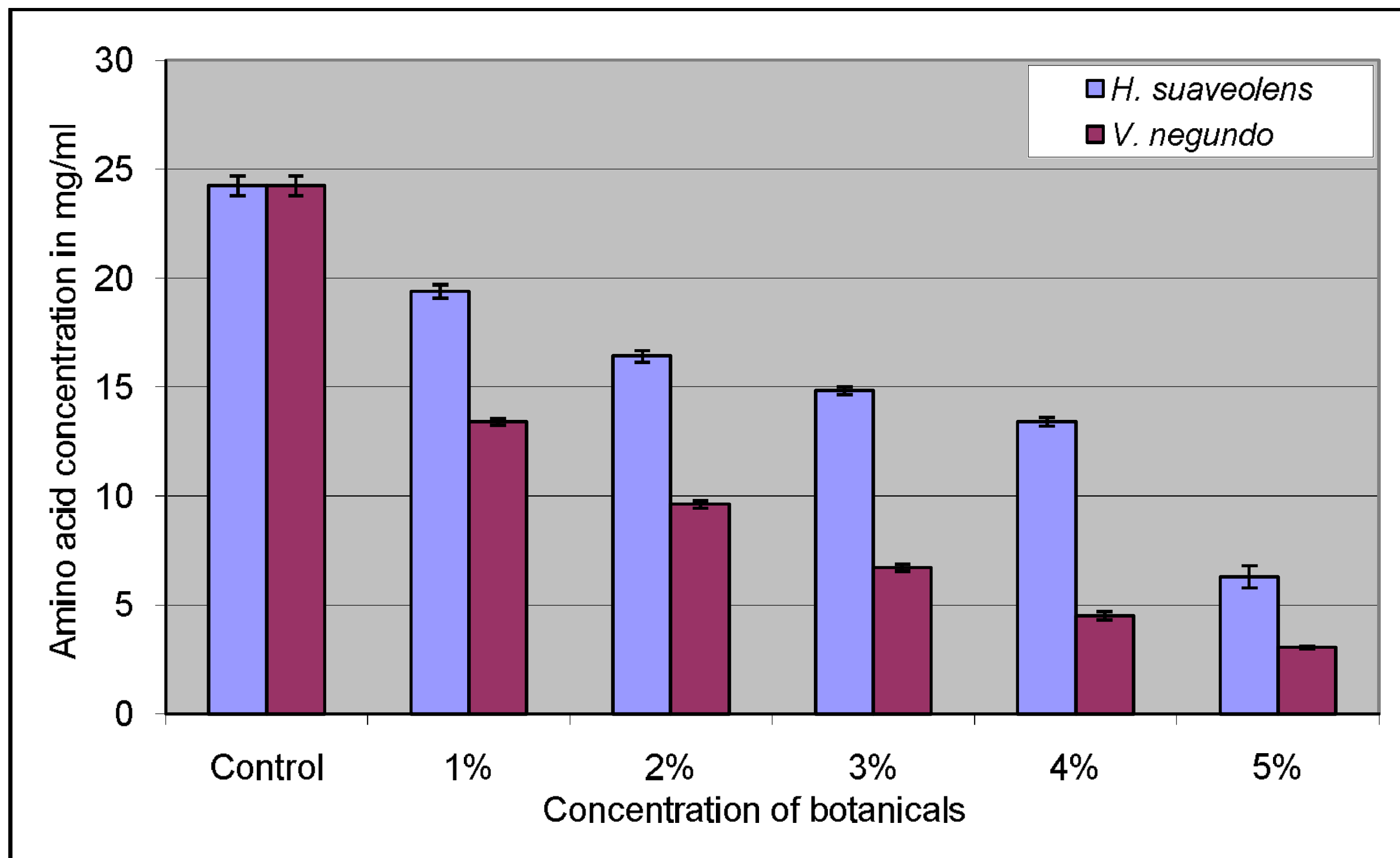


Figure VII. 5. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in fat body tissue of sixth instar larvae of *O. exvinacea.*

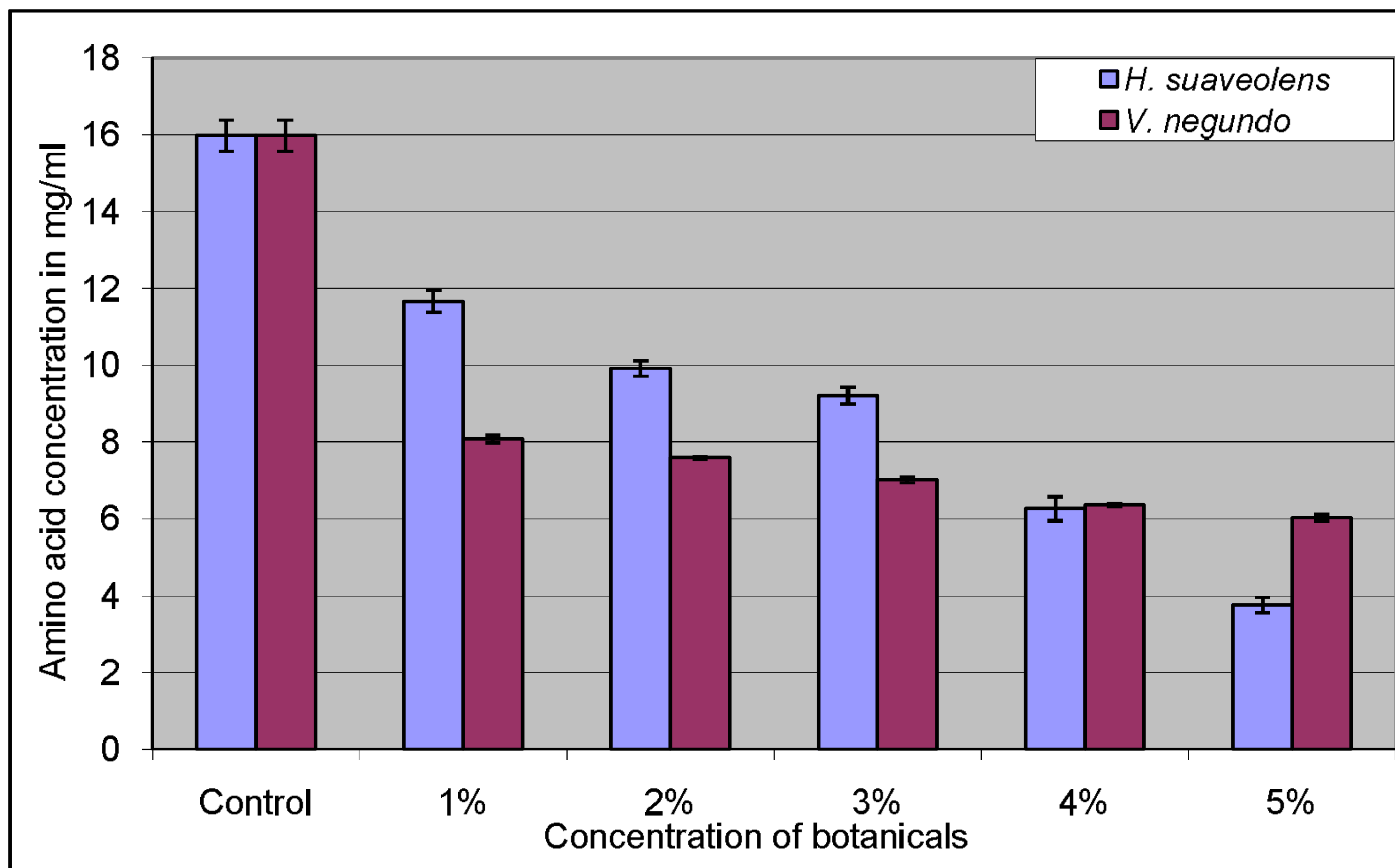


Figure VII. 6. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* at different concentrations on amino acid content in haemolymph of sixth instar larvae of *O. exvinacea.*

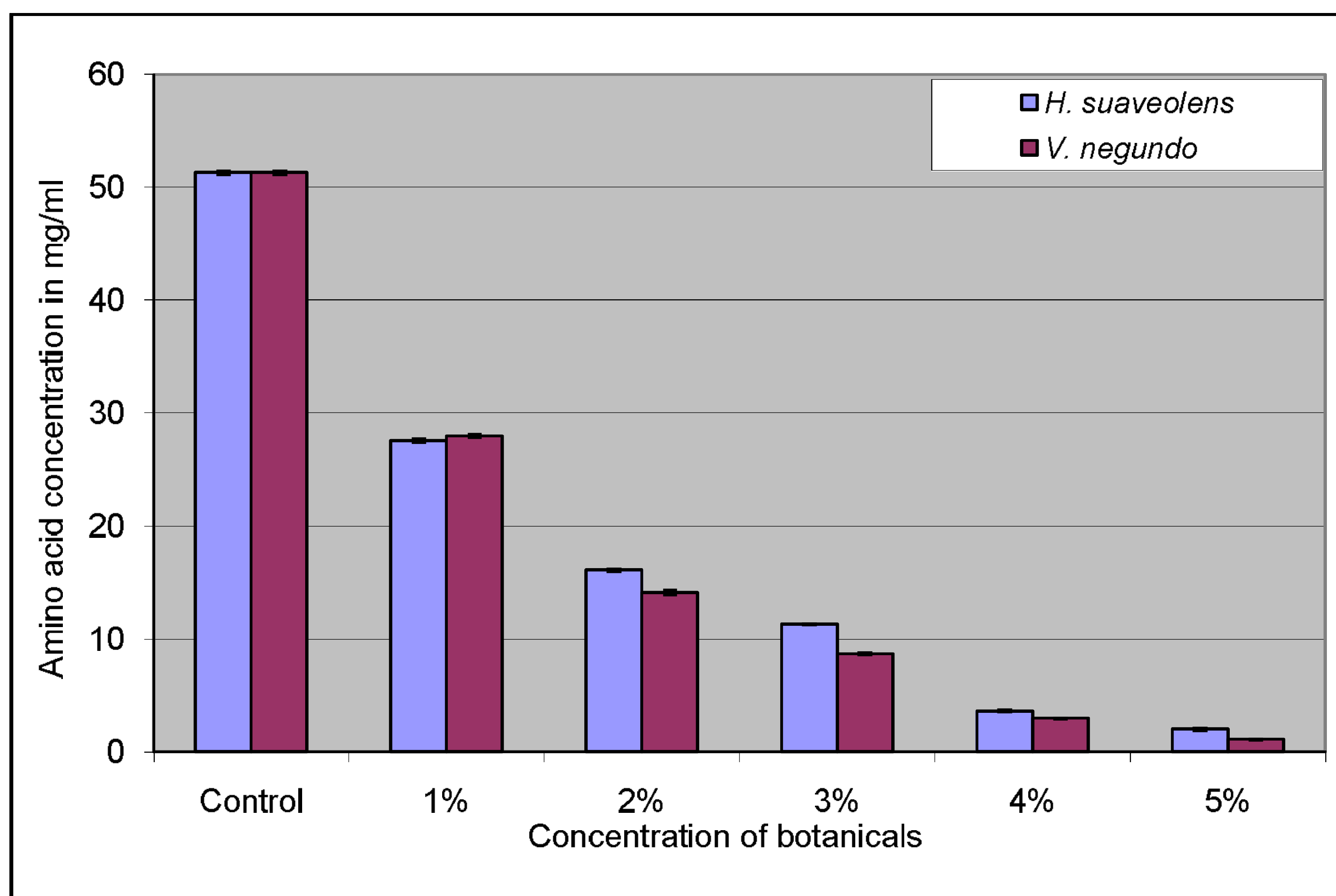


Table VII. 6. Consolidated table showing the percentage of reduction in total amino acid content over control with respect to mg/ ml mean values of amino acid concentration for both botanicals.

Conc. of botanicals	Percentage reduction of amino acid content (%)					
	Hyptis suaveolens treated			*Vitex negundo* treated		
	Midgut	Fat body	haemolymph	Midgut	Fat body	Haemolymph
1 %	20	27	46.2	44.6	49.4	45.4
2 %	32.3	37.9	68.6	60.3	52.5	72.5
3 %	38.8	42.4	78	72.2	56.1	83
4 %	44.6	60.8	93	81.4	60.1	94.1
5 %	74	76.5	96.1	87.3	62.2	97.8

Figure VIII. 1. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on catalase activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

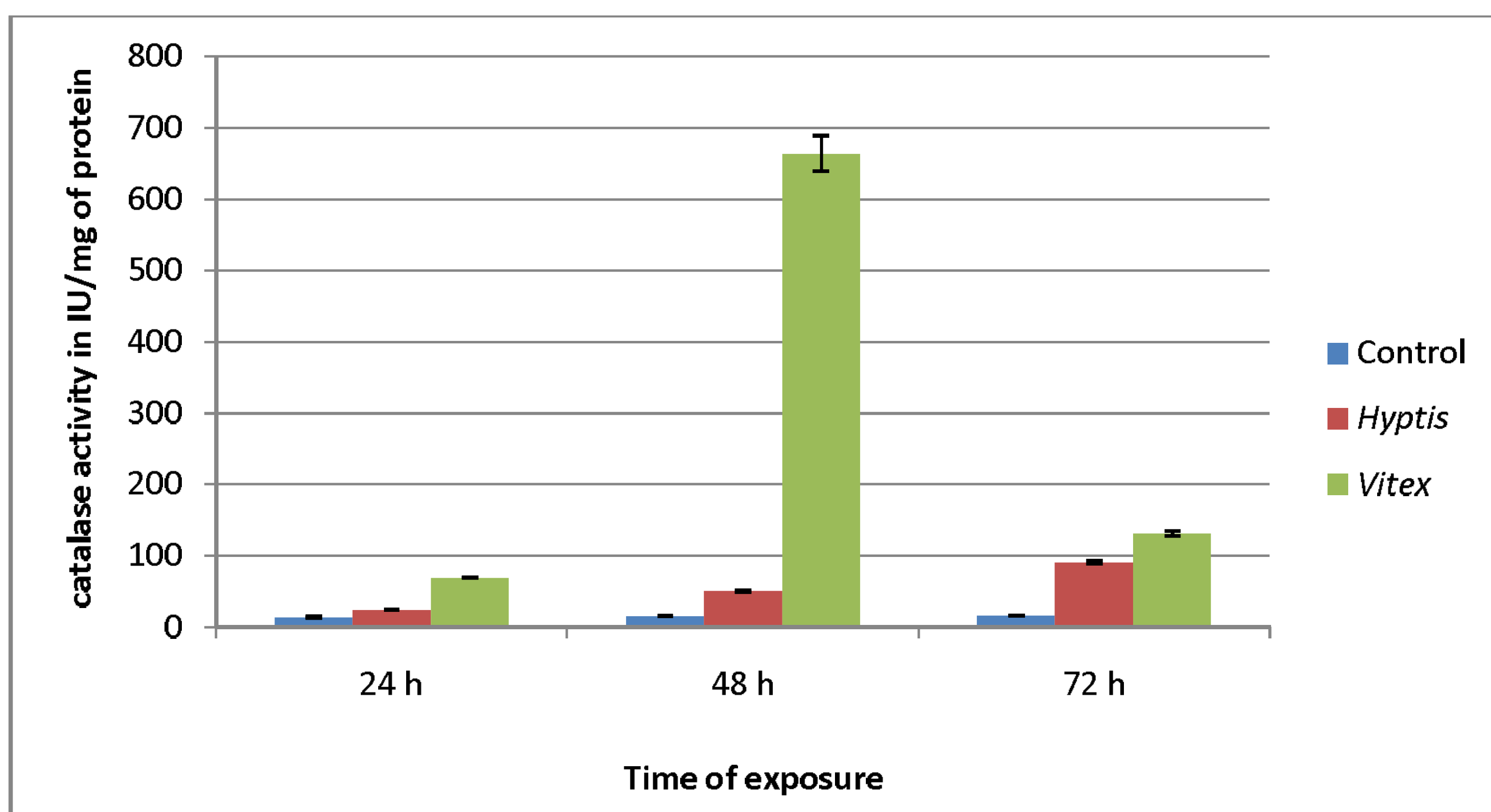


Figure VIII. 2. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

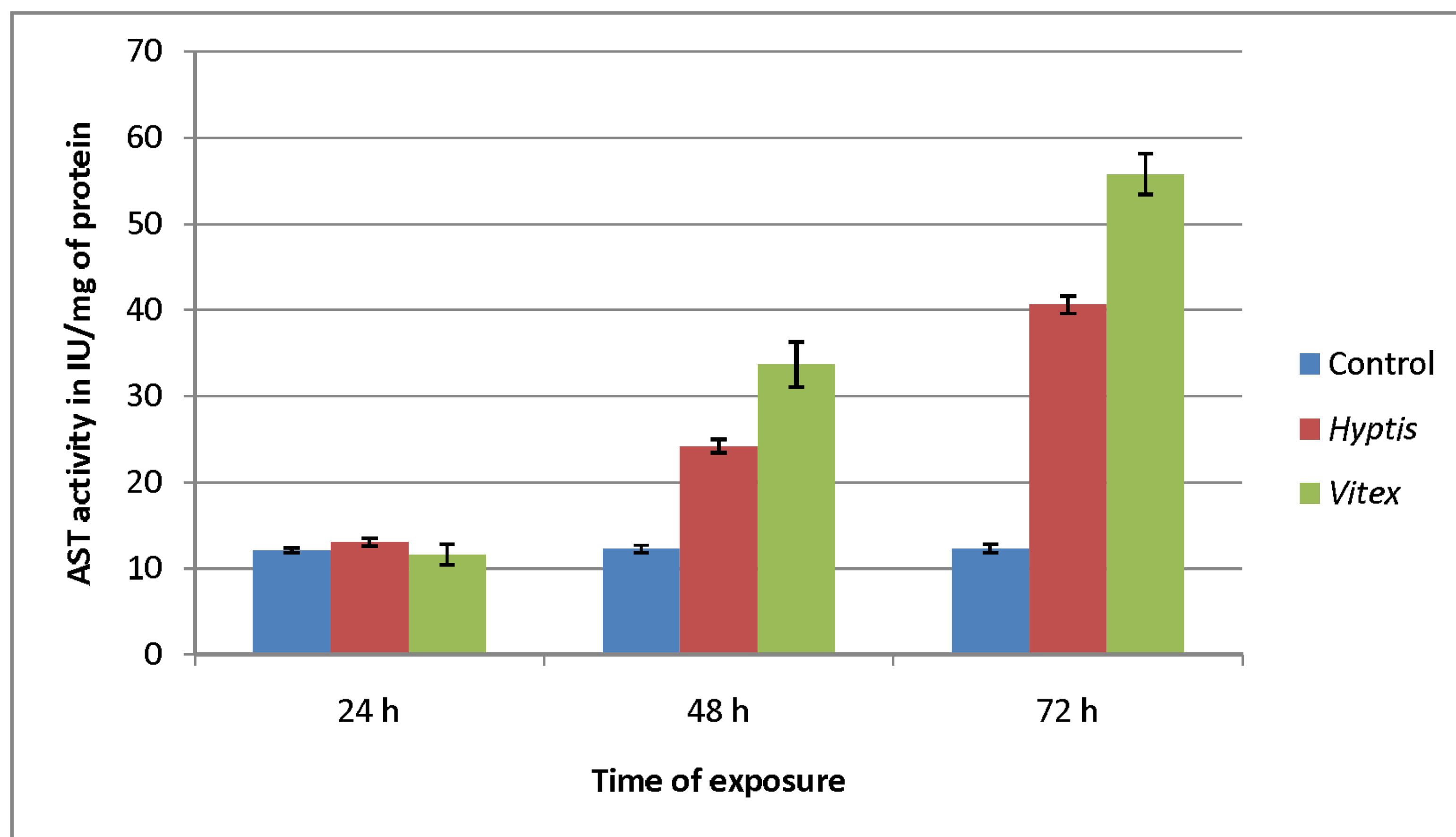


Figure VIII. 3. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

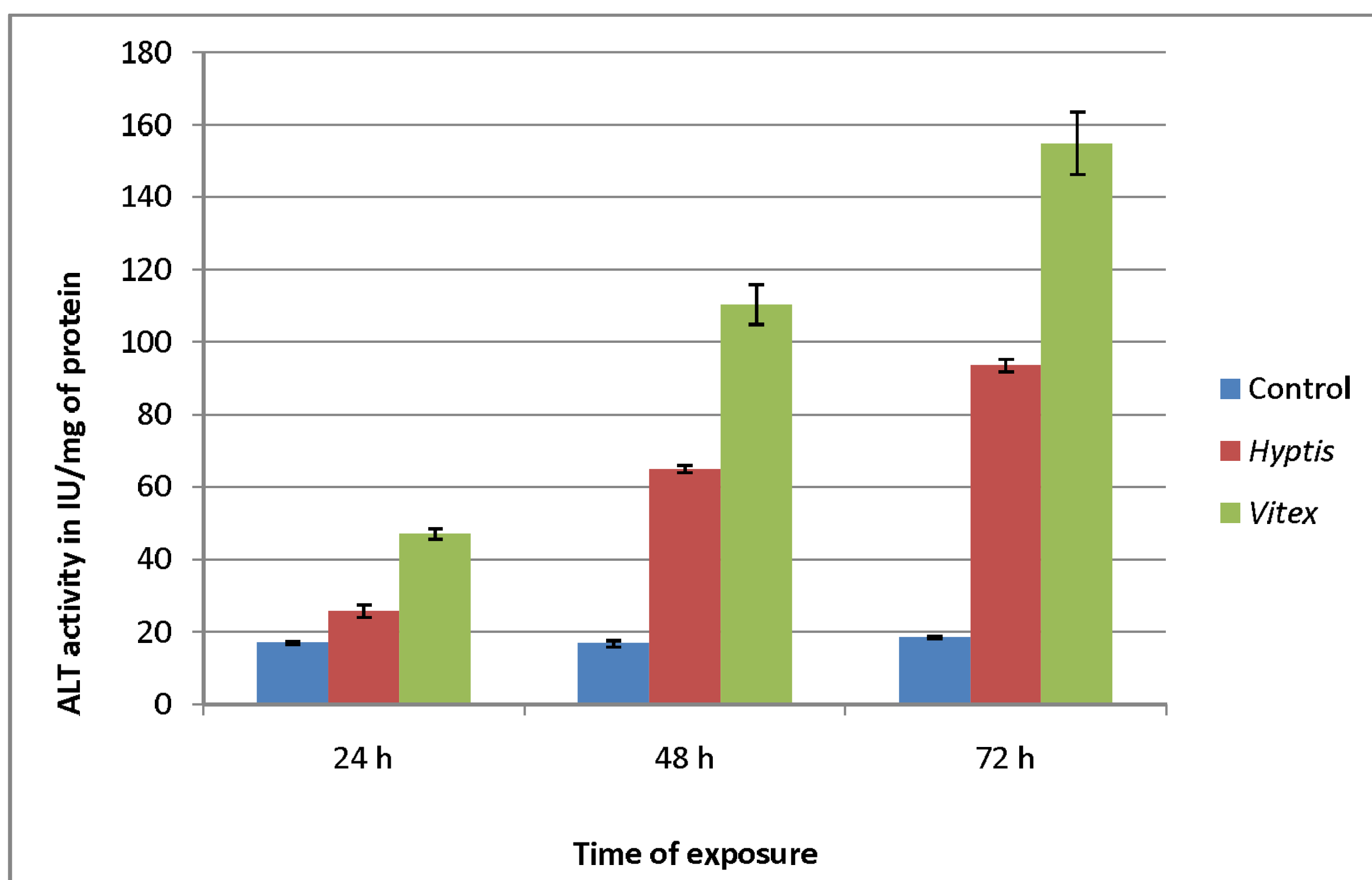


Figure VIII. 4. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

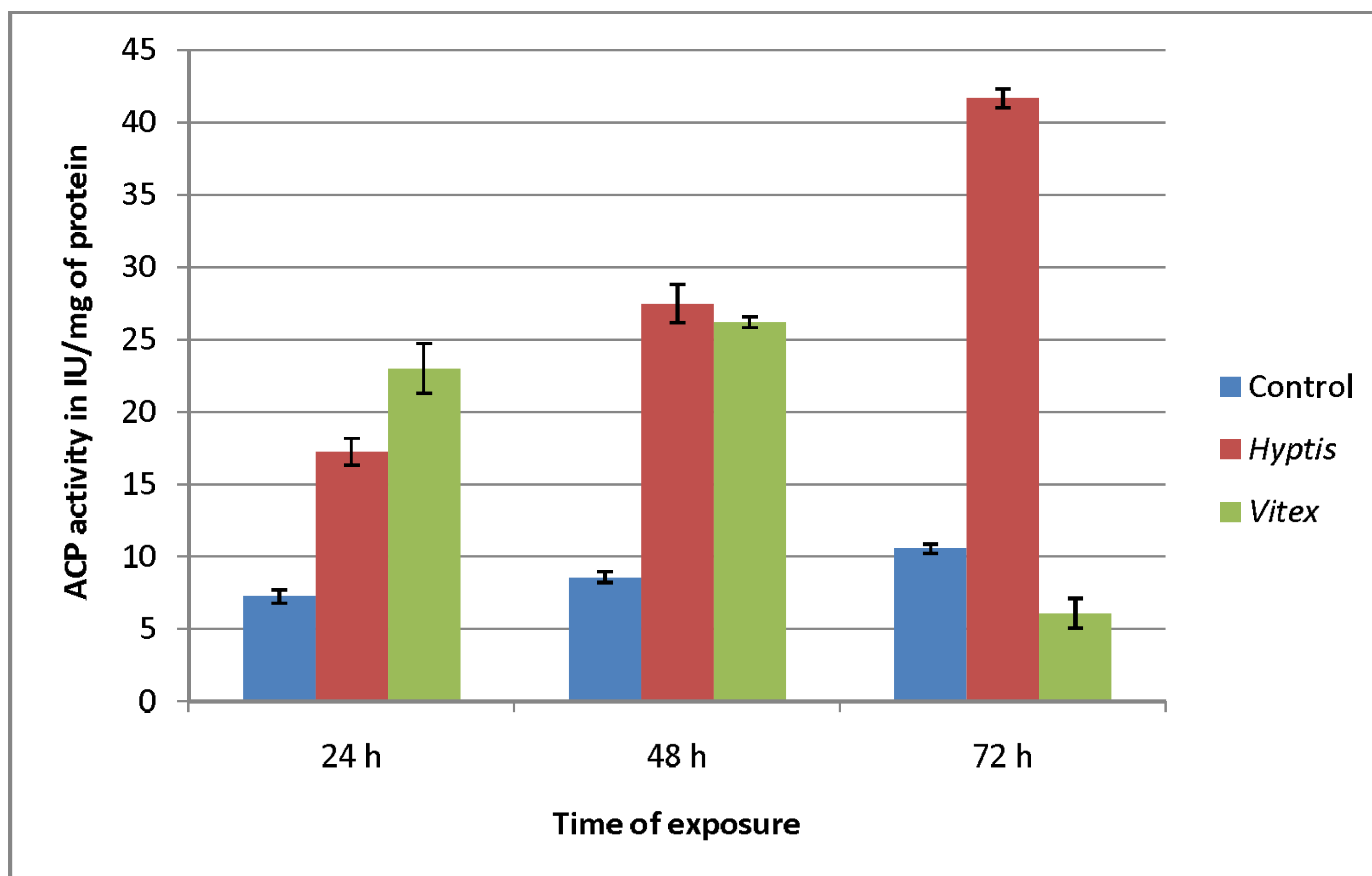


Figure VIII. 5. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in midgut tissue of sixth instar larvae of *O. exvinacea* at different time of exposures.

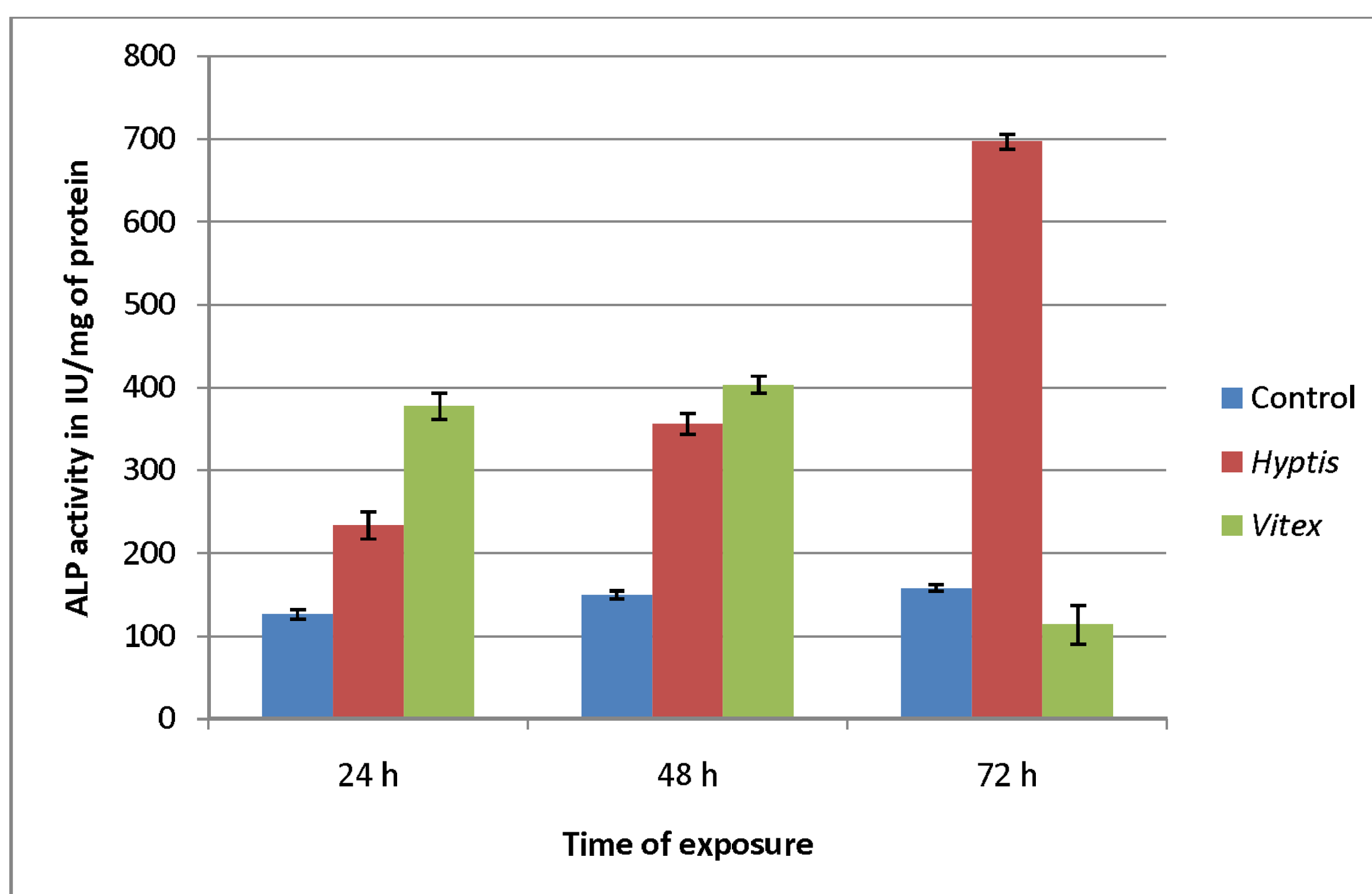
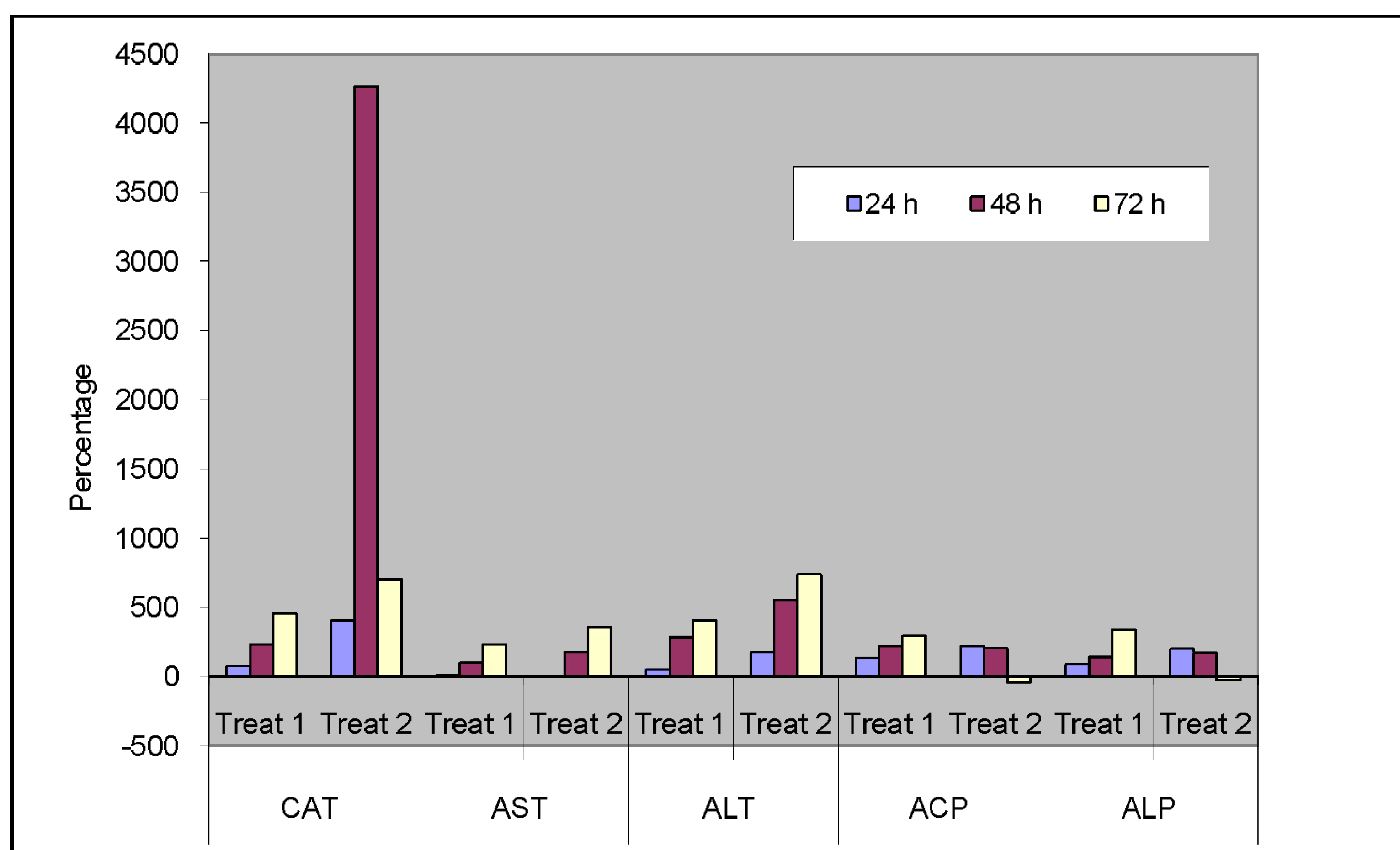


Table VIII. 2. Percentage of enzyme activity in the larval midgut tissue at different time of exposure for both treatments with respect to control.

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	73.98	7.59	51.05	137.4	85.06
	Treatment 2	403.8	-4.12	175.6	216.4	198.9
48 h	Treatment 1	230.3	97.8	285.7	220.1	137.8
	Treatment 2	4266	175.1	555.2	205	169.1
72 h	Treatment 1	457.2	230.4	405	295	340
	Treatment 2	703.9	353.6	735.5	-42.4	-28.14

Treatment 1- treatment with *H. suaveolens*
Treatment 2- treatment with *V. negundo.*

Figure VIII. 6. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval midgut tissue at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.



exposure, a slight declining tendency was noticed in the specific activity of catalase. The percentage of enzyme activity for this treatment, showed above four folds increased activity to that of control on 24 h of treatment. This change in activity was found to be much more increased (above 34 times that of control) on 48 h of exposure and at 72 h, though there was an increase in the activity of catalase when compared to control (above 7 times with that of control), the activity was noticed to be slightly decreased in comparison with the catalase activity for 48 h (Table VIII. 4 and Figure VIII. 12).

Figure VIII. 7. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on CAT activity in fat body of sixth instar larva of *O. exvinacea* at different time of exposures.

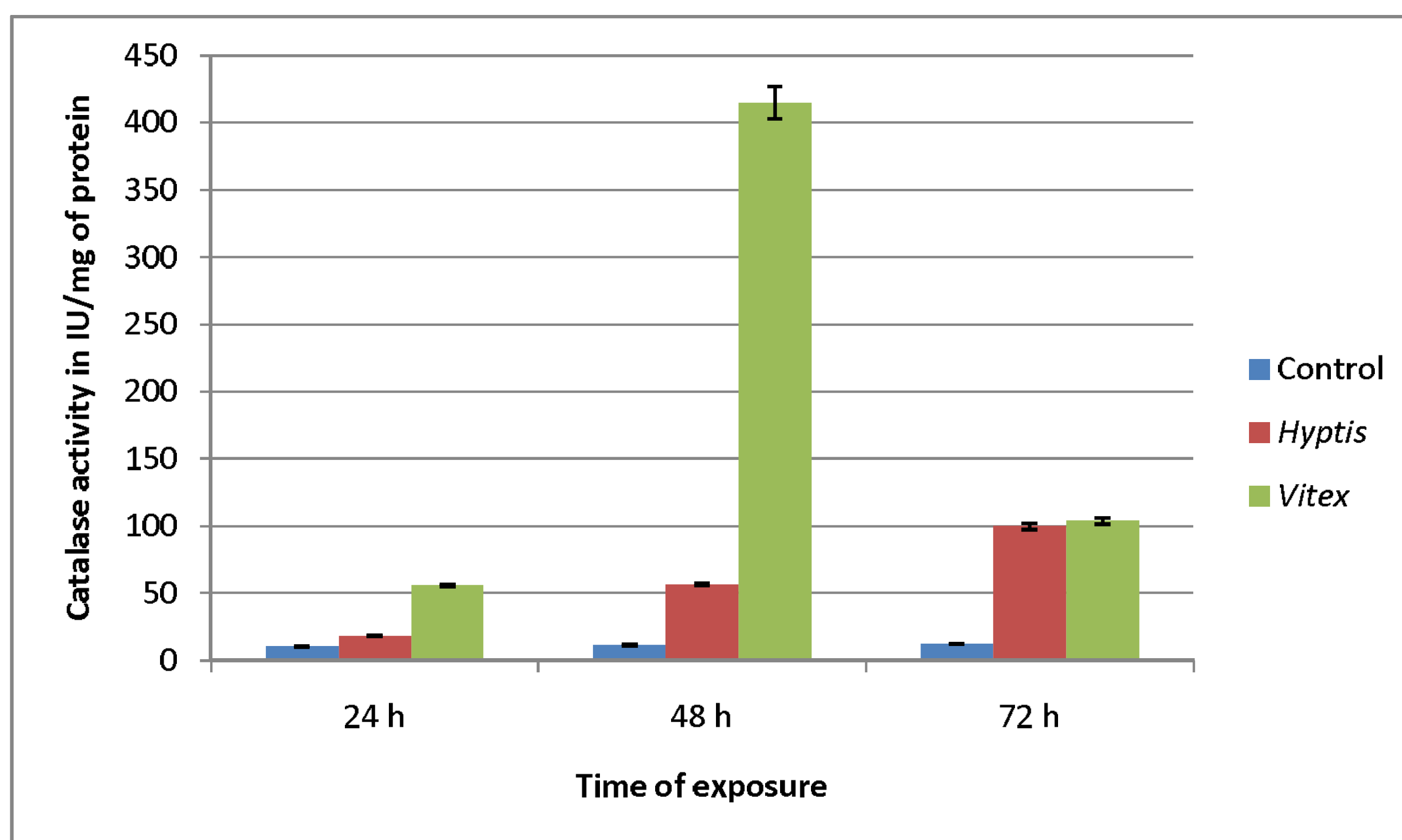


Figure VIII. 8. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

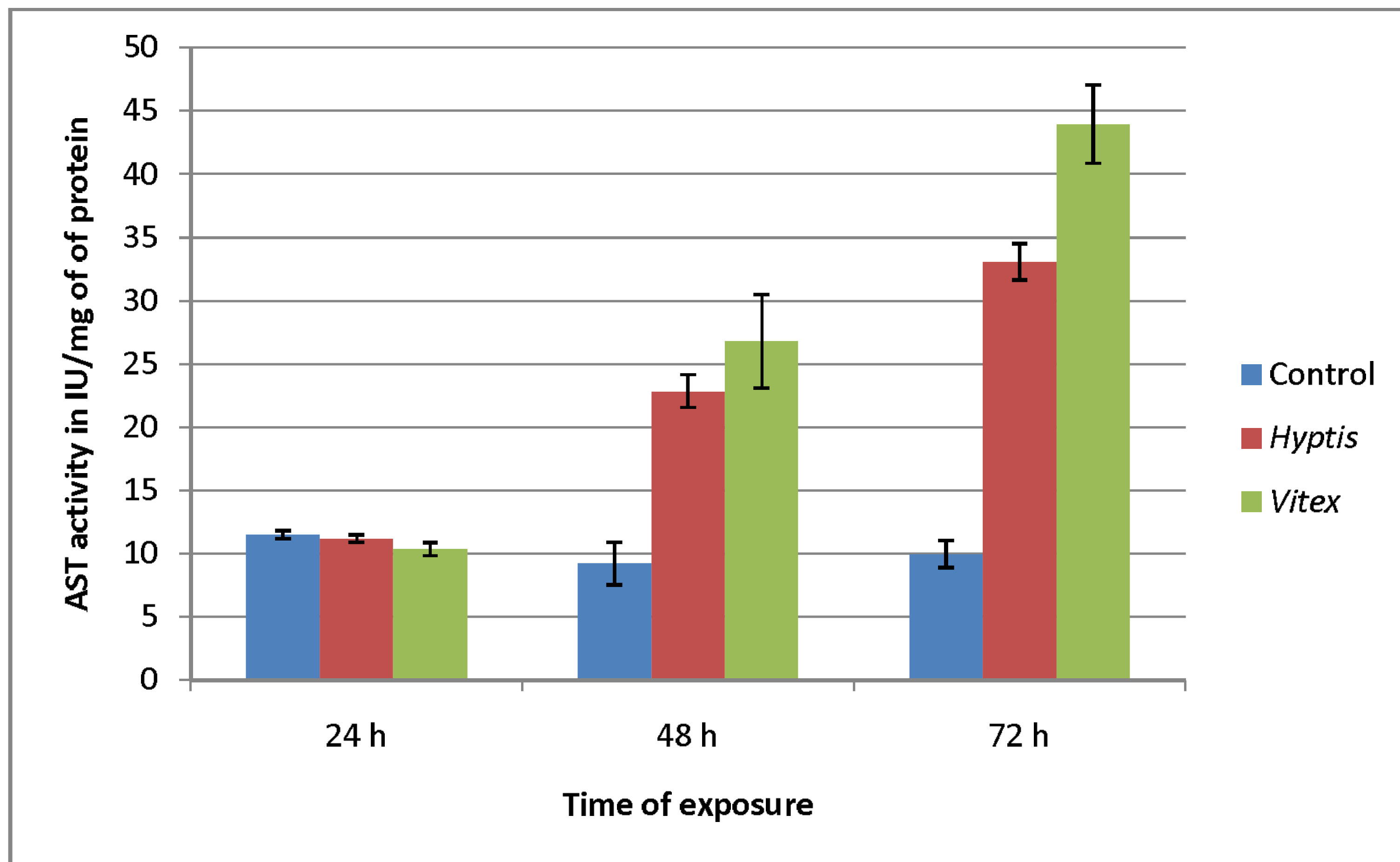


Figure VIII. 9. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

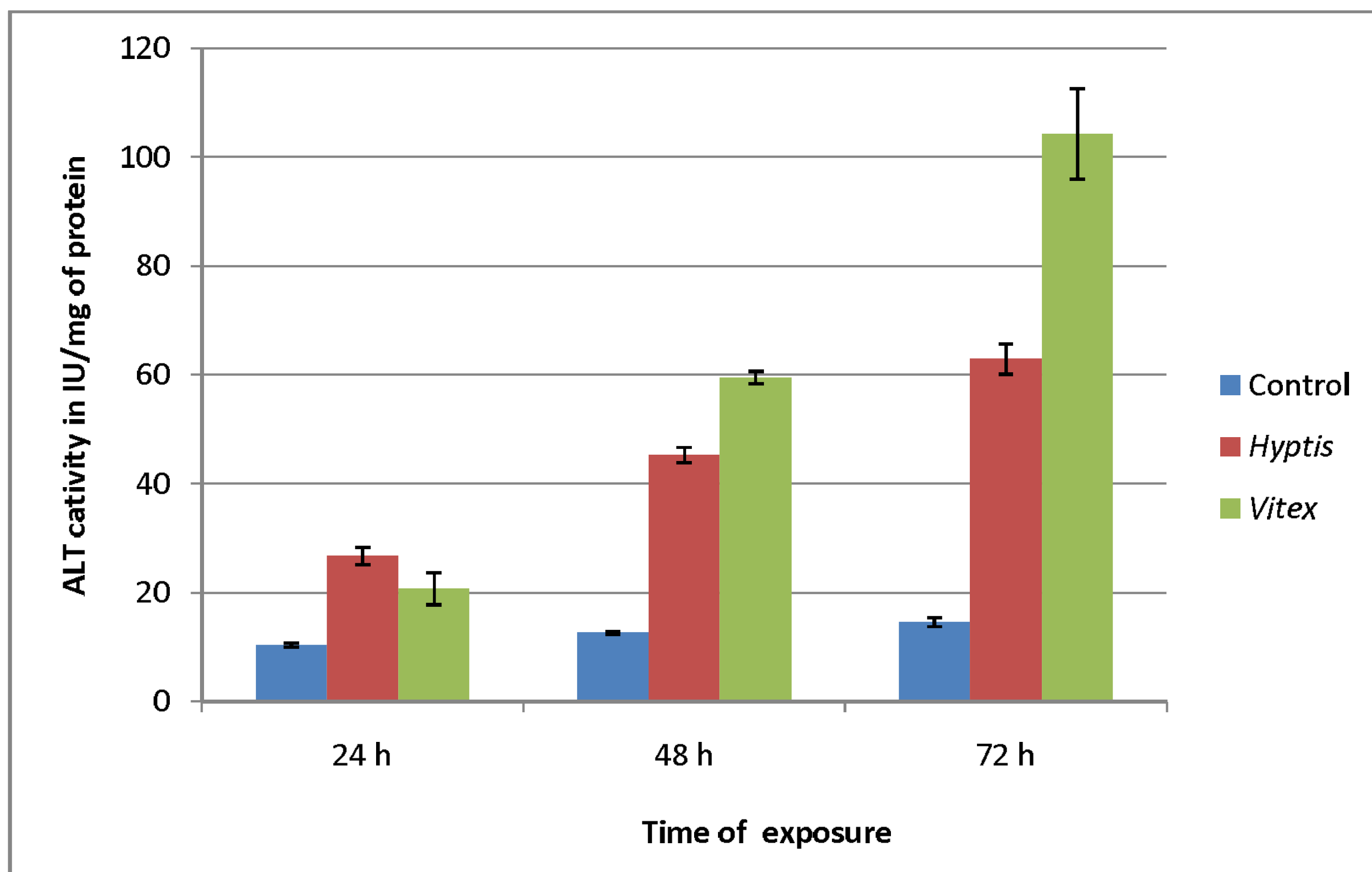


Figure VIII. 10. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

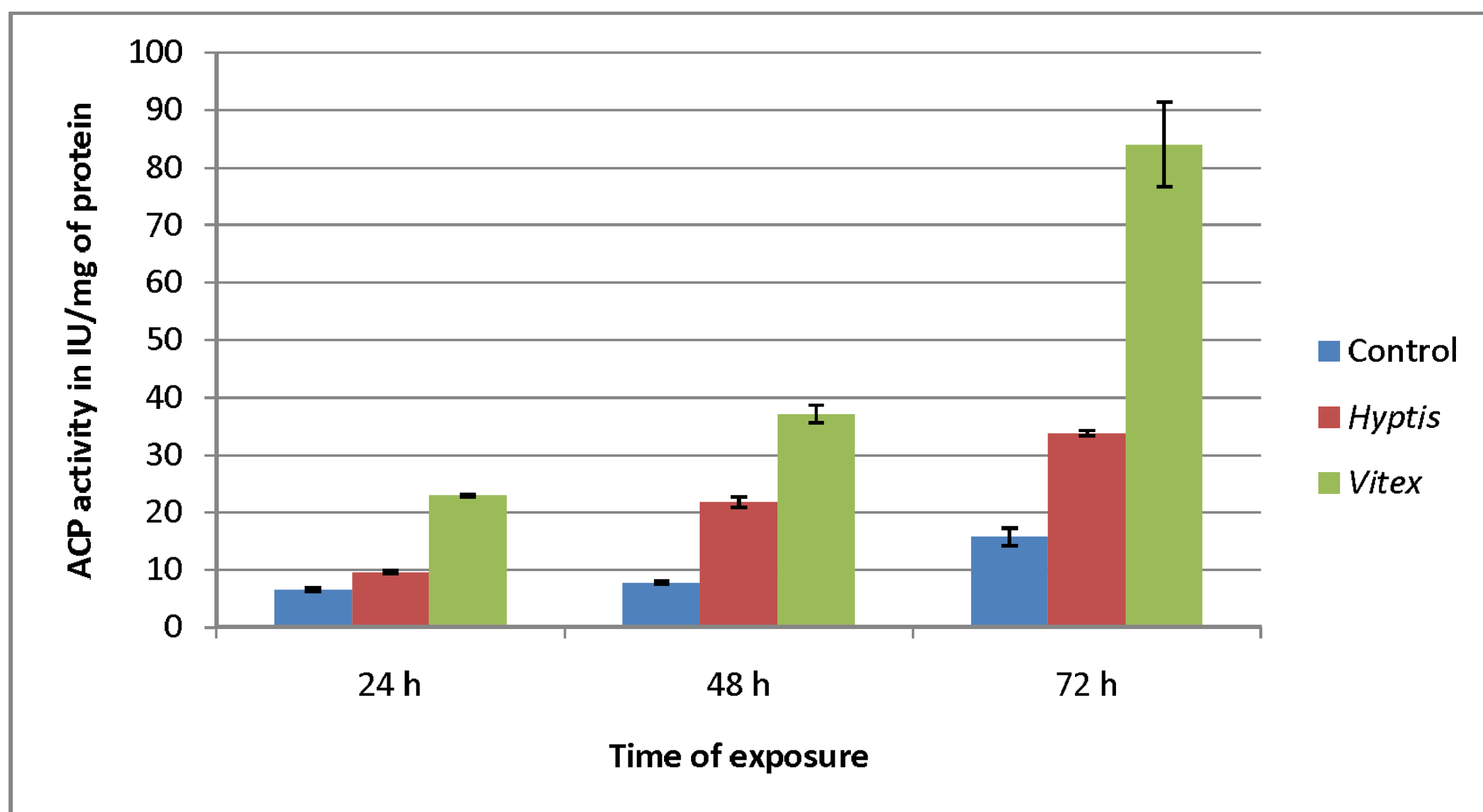


Figure VIII. 11. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in fat body of sixth instar larvae of *O. exvinacea* at different time of exposures.

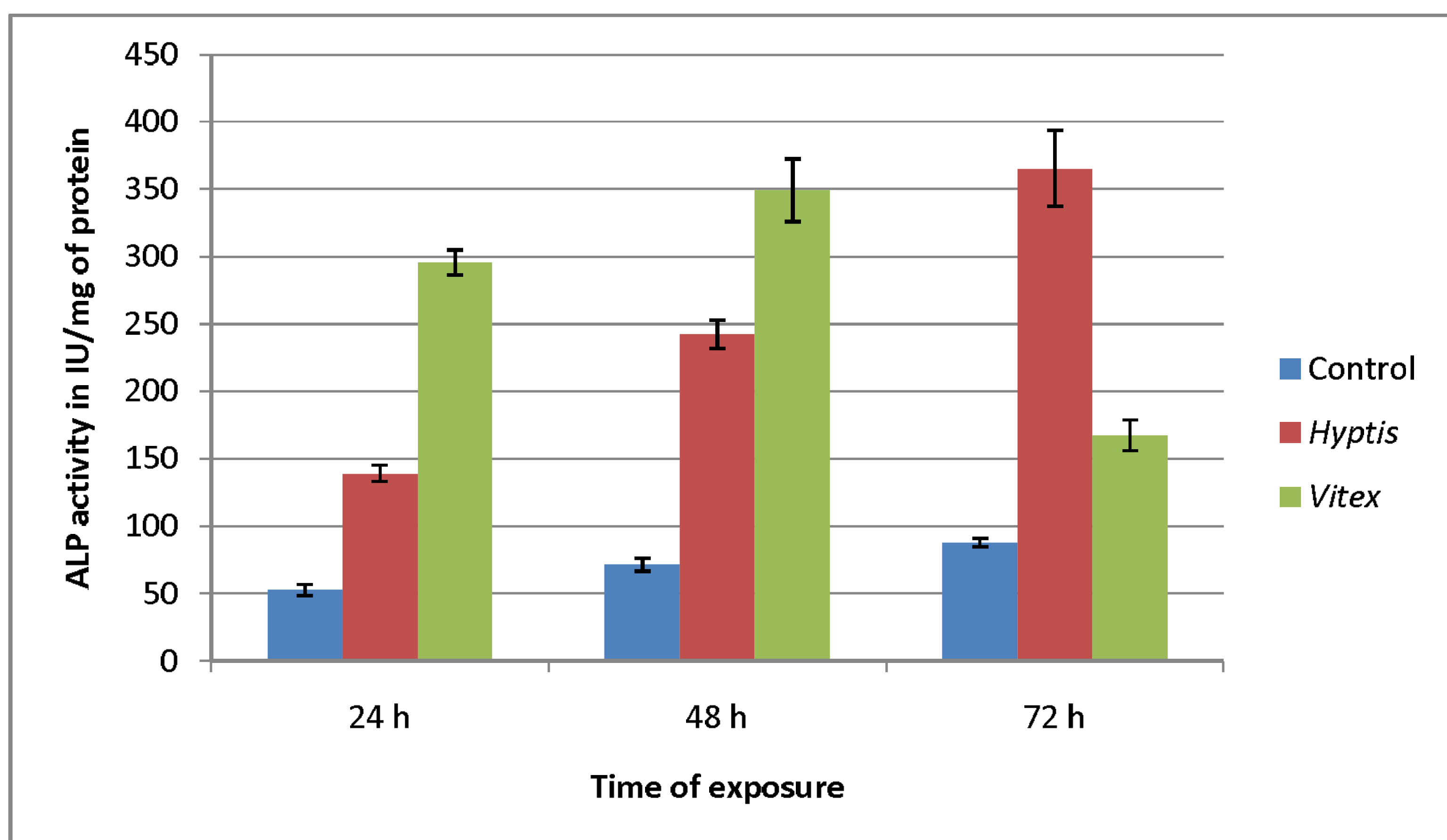


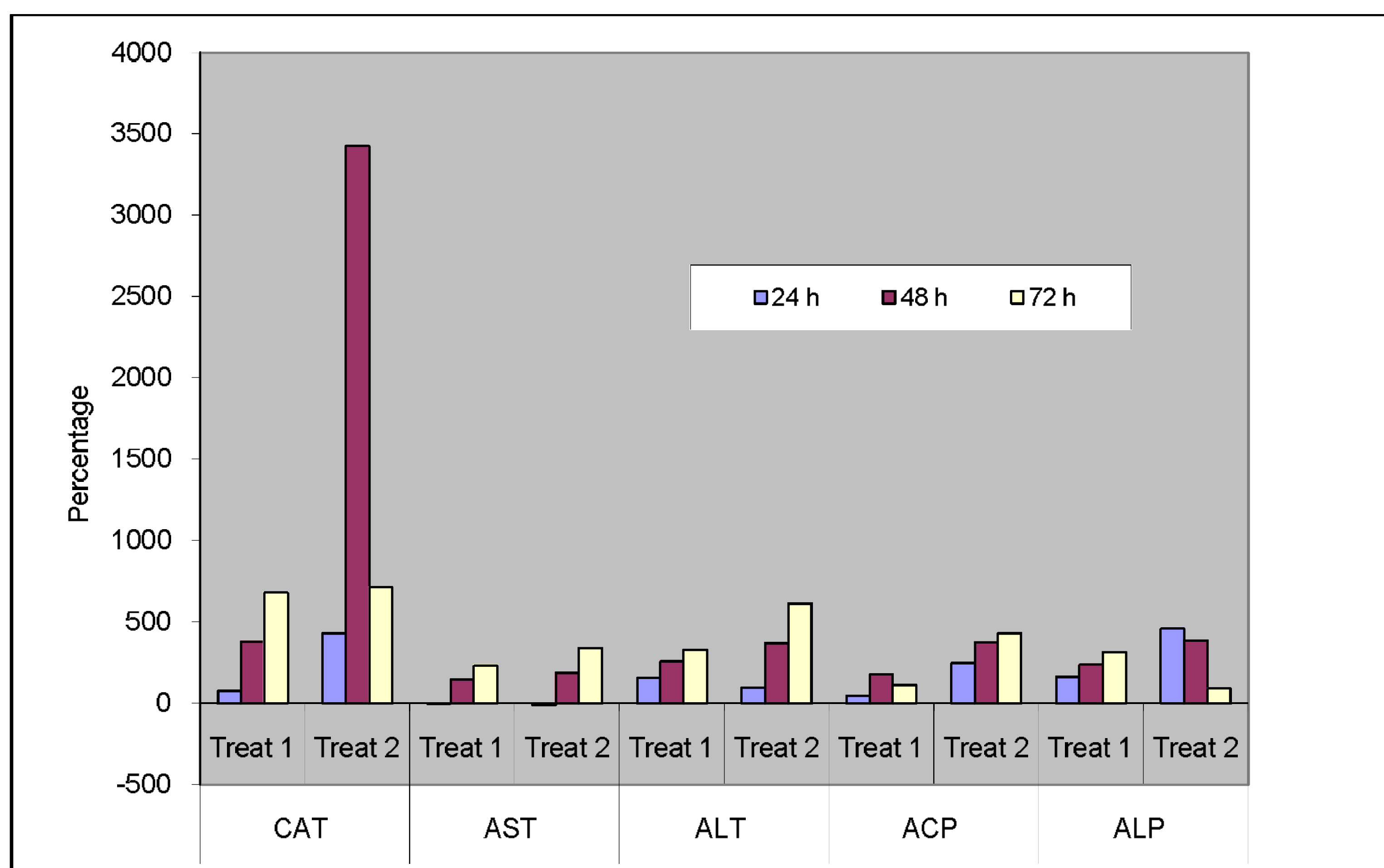
Table VIII. 4. Percentage of enzyme activity in the larval fat body at different time of exposure for both treatments with respect to control.

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	74.67	-2.53	156.8	46.82	163.5
	Treatment 2	432.6	-9.67	99.2	248.2	459.5
48 h	Treatment 1	381.7	147.2	257.8	180	238.4
	Treatment 2	3425	189.9	370.6	376	386.9
72 h	Treatment 1	680.8	231.7	330.6	114	316.4
	Treatment 2	710.9	340.8	613	432.3	90.95

Treatment 1- treatment with *Hyptis suaveolens*

Treatment 2- treatment with *Vitex negundo*.

Figure VIII. 12. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval fat body at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.



of control catalase activity. Thereafter an increased activity of more than 23 folds with that of control was demarcated for 48 h exposure period. Even though, increase in the activity of catalase was recognized for 72 h of exposure (6 times to that of control) the activity was found to be decreased to some extent when compared with the catalase activity for 48 h (Table VIII. 6 and Figure VIII. 18).

Figure VIII. 13. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on CAT activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

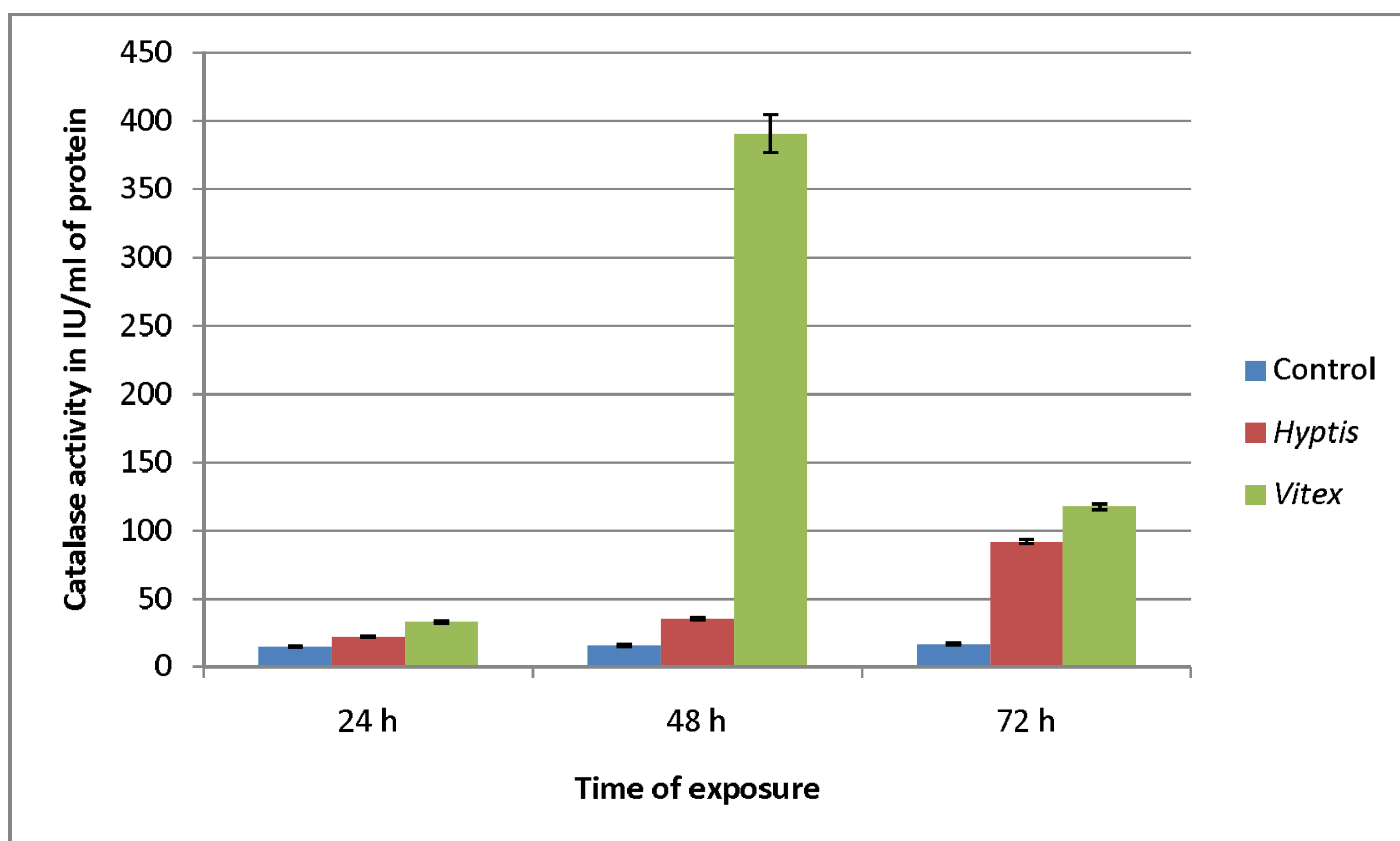


Figure VIII. 14. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on AST activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

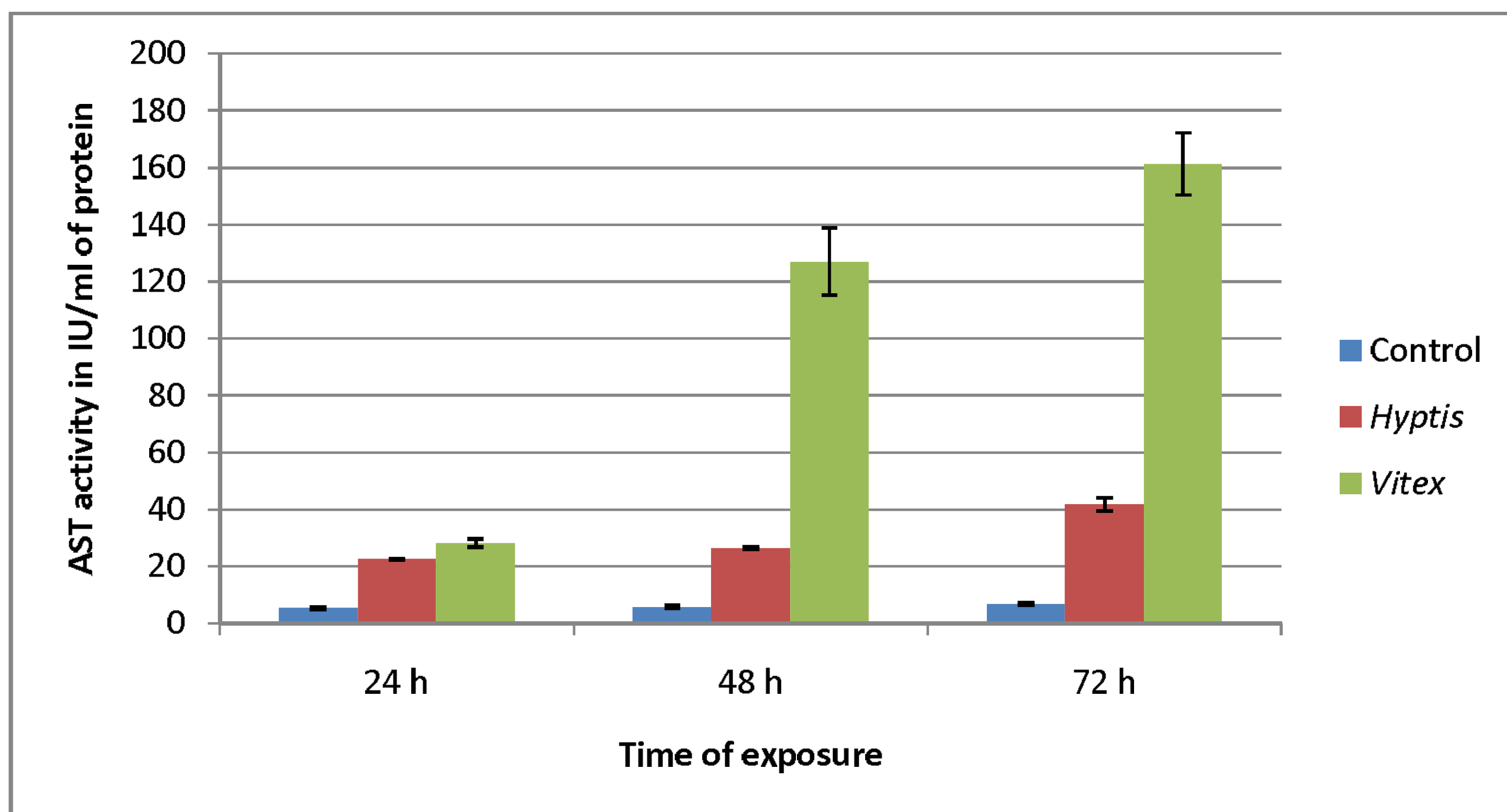


Figure VIII. 15. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALT activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

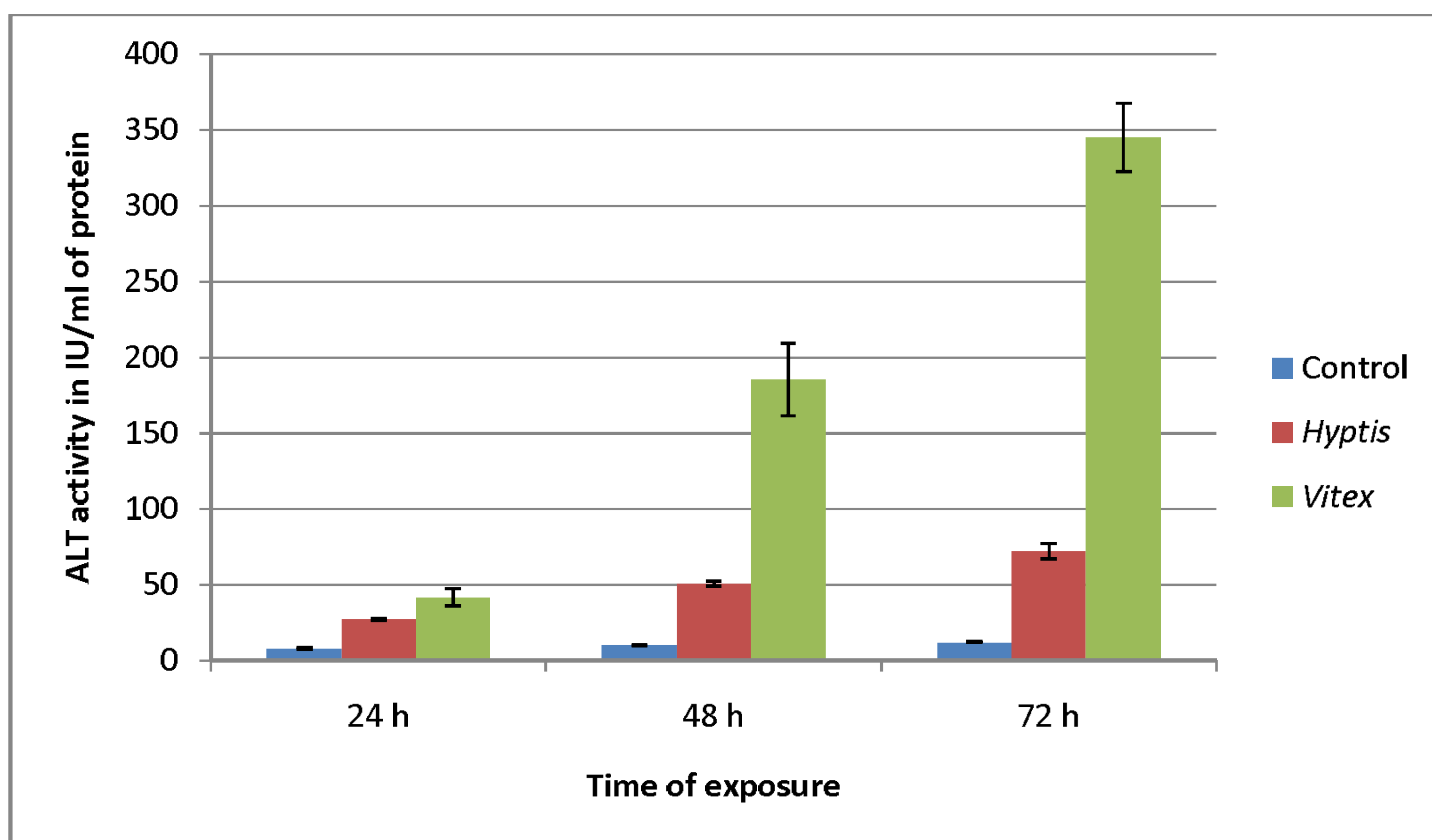


Figure VIII. 16. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ACP activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

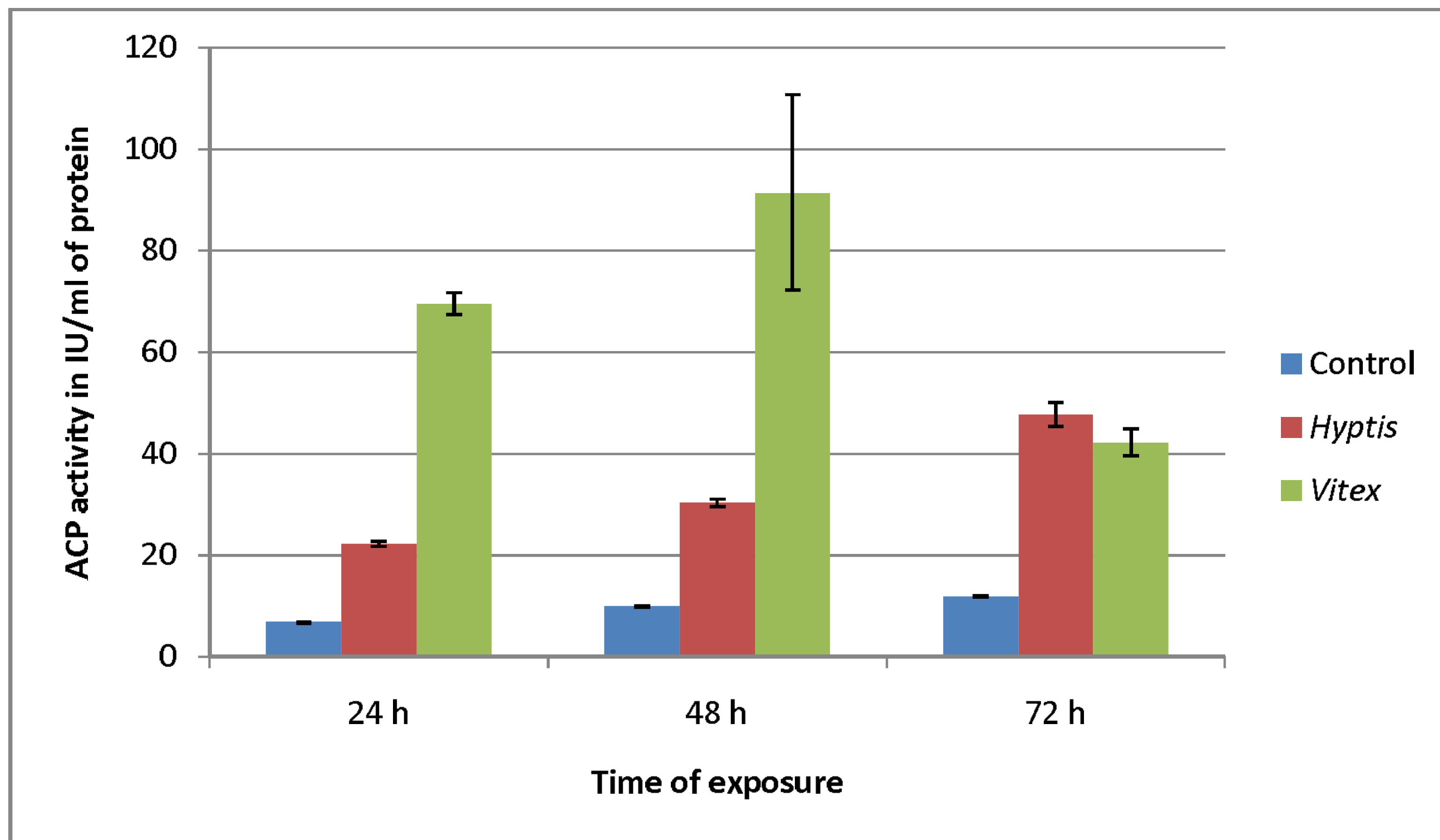


Figure VIII. 17. The effect of methanolic leaf extracts of both *H. suaveolens* and *V. negundo* on ALP activity in haemolymph of sixth instar larvae of *O. exvinacea* at different time of exposures.

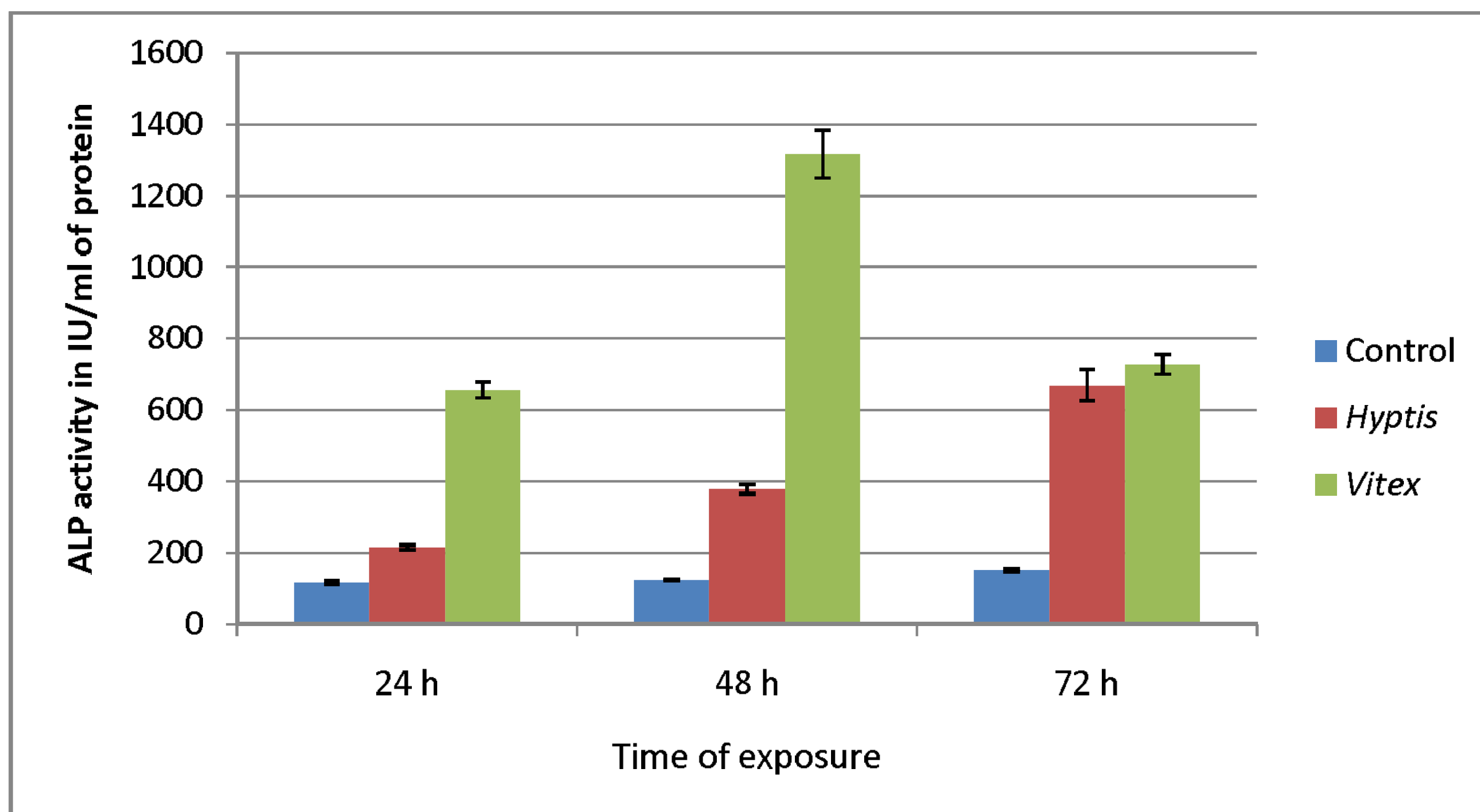


Table VIII. 6. Percentage of enzyme activity in the larval haemolymph at different time of exposure for both treatments with respect to control

Time of exposure	Treatments	CAT (%)	AST (%)	ALT (%)	ACP (%)	ALP (%)
24 h	Treatment 1	47.9	318.9	237	227	85
	Treatment 2	122.2	425.3	421	923.5	462
48 h	Treatment 1	126.4	347.4	404	204.7	204.6
	Treatment 2	2391	2042	1738	817.7	957.2
72 h	Treatment 1	451.3	509	493.1	300	341.6
	Treatment 2	603.4	2248	2747	254.5	380.5

Treatment 1- treatment with *Hyptis suaveolens*
Treatment 2- treatment with *Vitex negundo*.

Figure VIII. 18. The percentage of enzyme activities for CAT, AST, ALT, ACP and ALP in the larval haemolymph at different time of exposure for both *H. suaveolens* and *V. negundo* with respect to control.

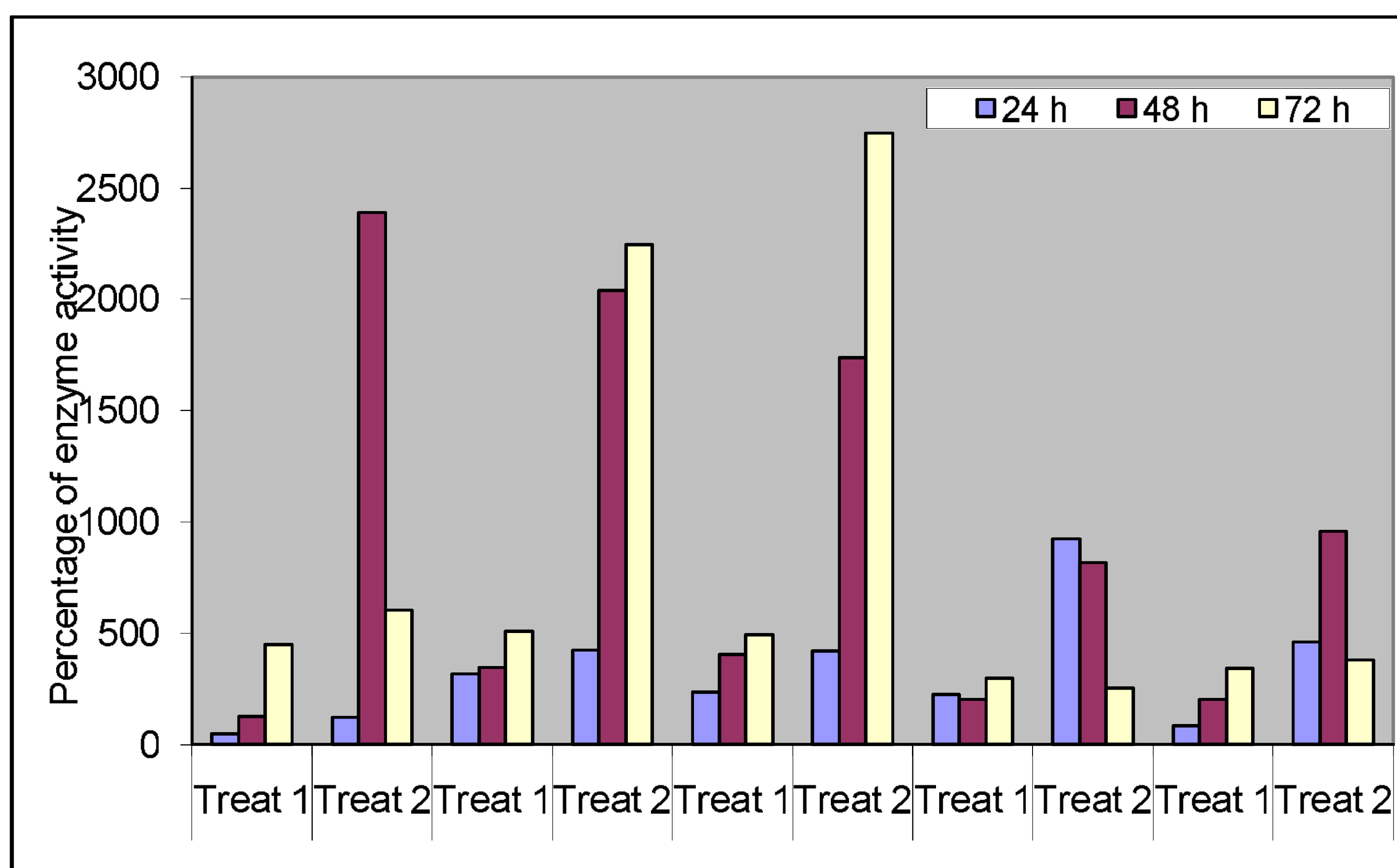


PLATE VI.1

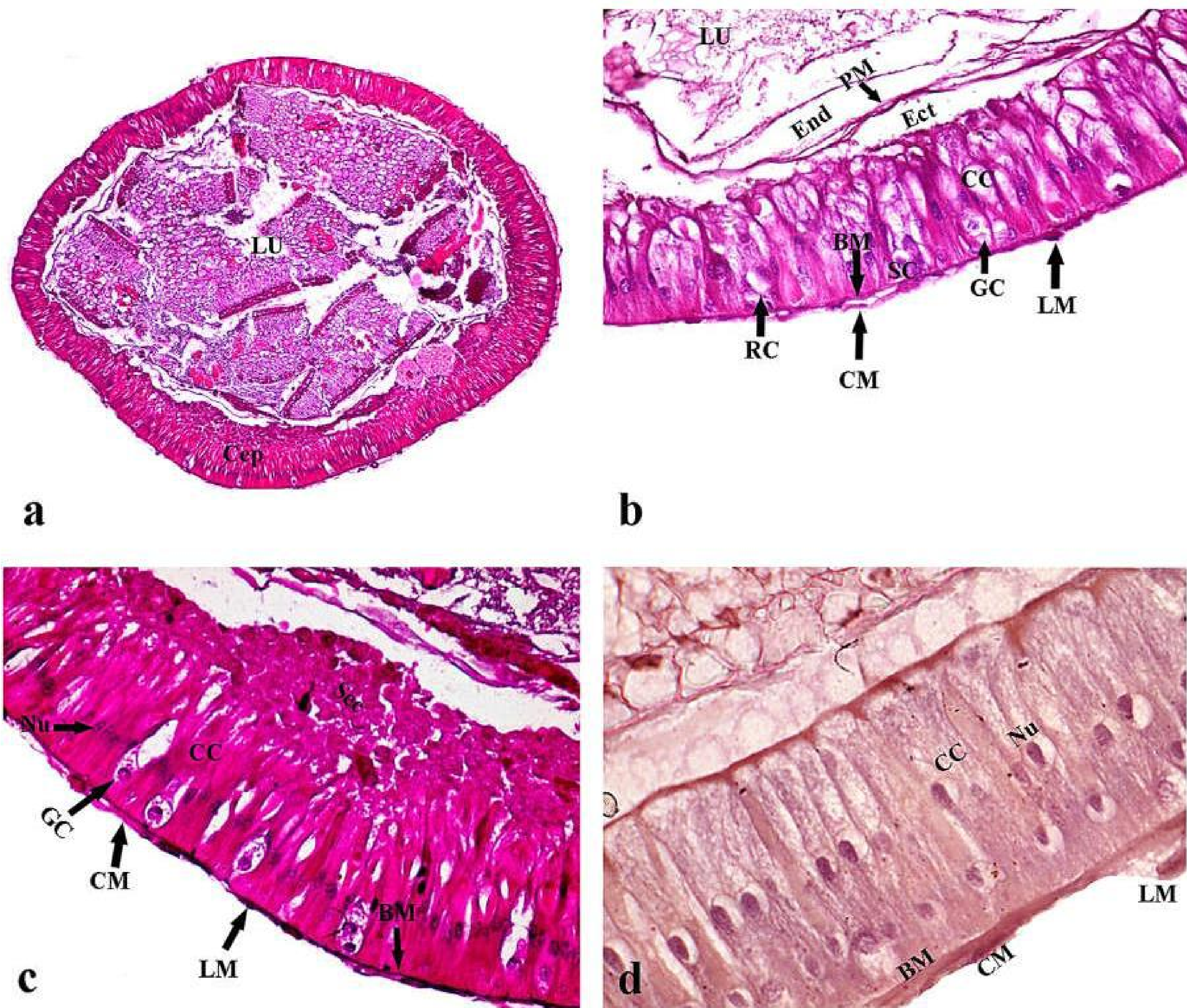


Fig. 1. a. T.S of the midgut tissue of *O. exvinacea* showing the general histomorphology of control larva (100X); b. control larval midgut with intact midgut epithelium (400X); c. A portion of larval midgut epithelium showing secretory nature (400X); d. enlarged view of the midgut epithelium (1000X). Longitudinal muscle layer (LM); Circular muscle layer (CM); Basement membrane (BM); Regenerative cells (RC); Columnar epithelial cells (CC); Goblet cells (GC);brush border (BB);Ectoperitrophic space (Ect); Peritrophic membrane (PM); Endoperitrophic space (End); Lumen(LU); Columnar Epithelium (Cep); Nucleus (Nu); Secretory vesicle (Sec).

PLATE VI. 14

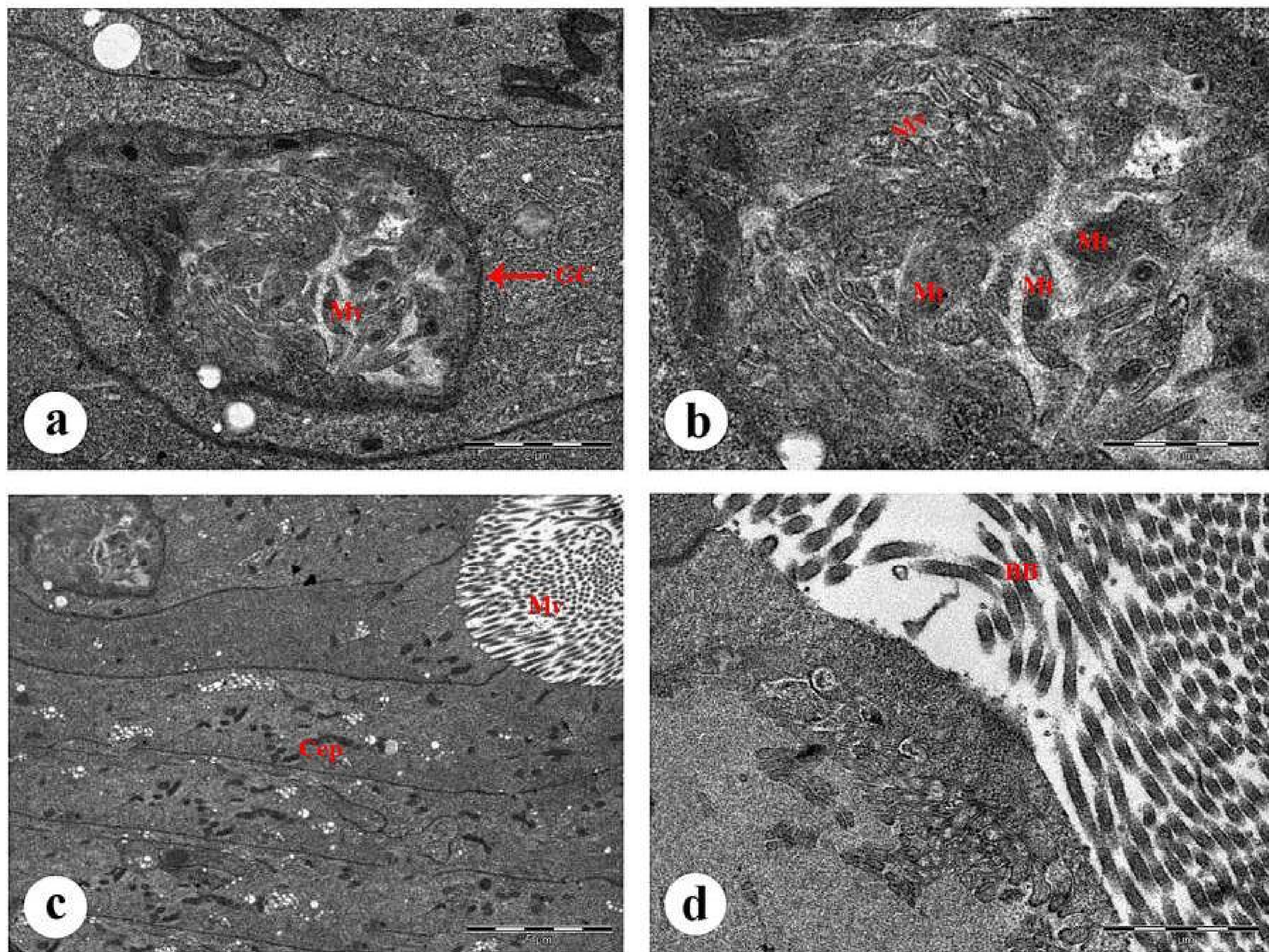


Fig. 9. Showing the details of larval midgut tissue treated with *V. negundo* a. Goblet cell showing extreme degeneration (11000X); b. Enlarged view of goblet with ruptured microvilli (Mv) and shrunken mitochondria (Mt) (23000X); c. Apical part of columnar epithelium (Cep) showing disruption in the brush border (BB) (4800X); d. Magnified view of apical region showing disruption in the BB (11000X).

PLATE VI. 13

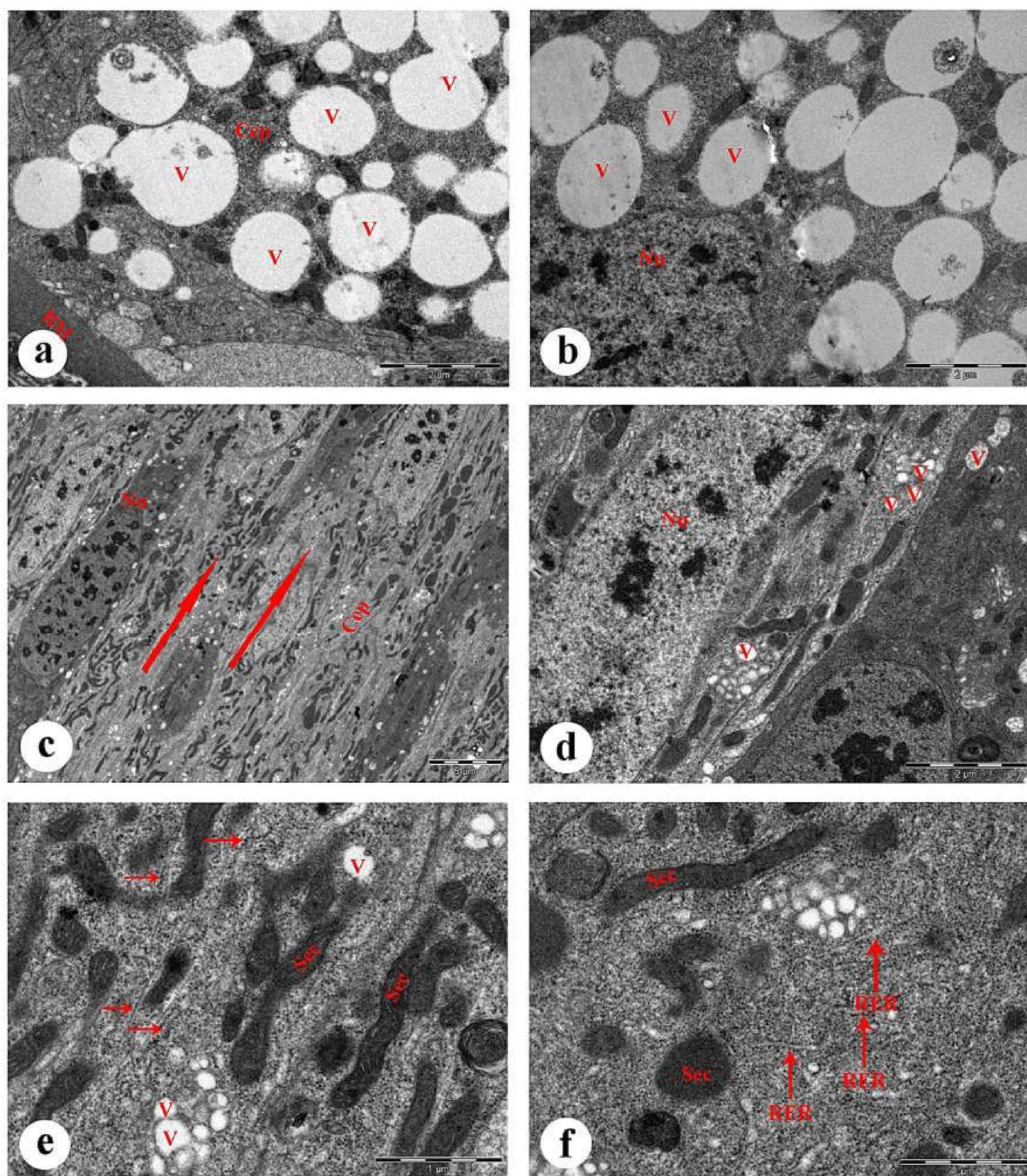


Fig. 8. Showing the details of larval midgut tissue treated with *V. negundo* a. Vacuolization (V) in the cytoplasmic area of epithelial cell layer (Cep) towards the basal region (6800X); b. Enlarged view of vacuolized epithelial cytoplasm with a part of nucleus (Nu) (11000X); c. Elongated columnar epithelial cells with their nuclei (thin arrows) (2900X); d. Magnified view of columnar epithelial cell with nucleus (11000X); e. Columnar cytoplasm showing reduced cell organelles (23000X); f. Apical part of columnar cells with very few cell organelles (23000X).

PLATE VI. 12

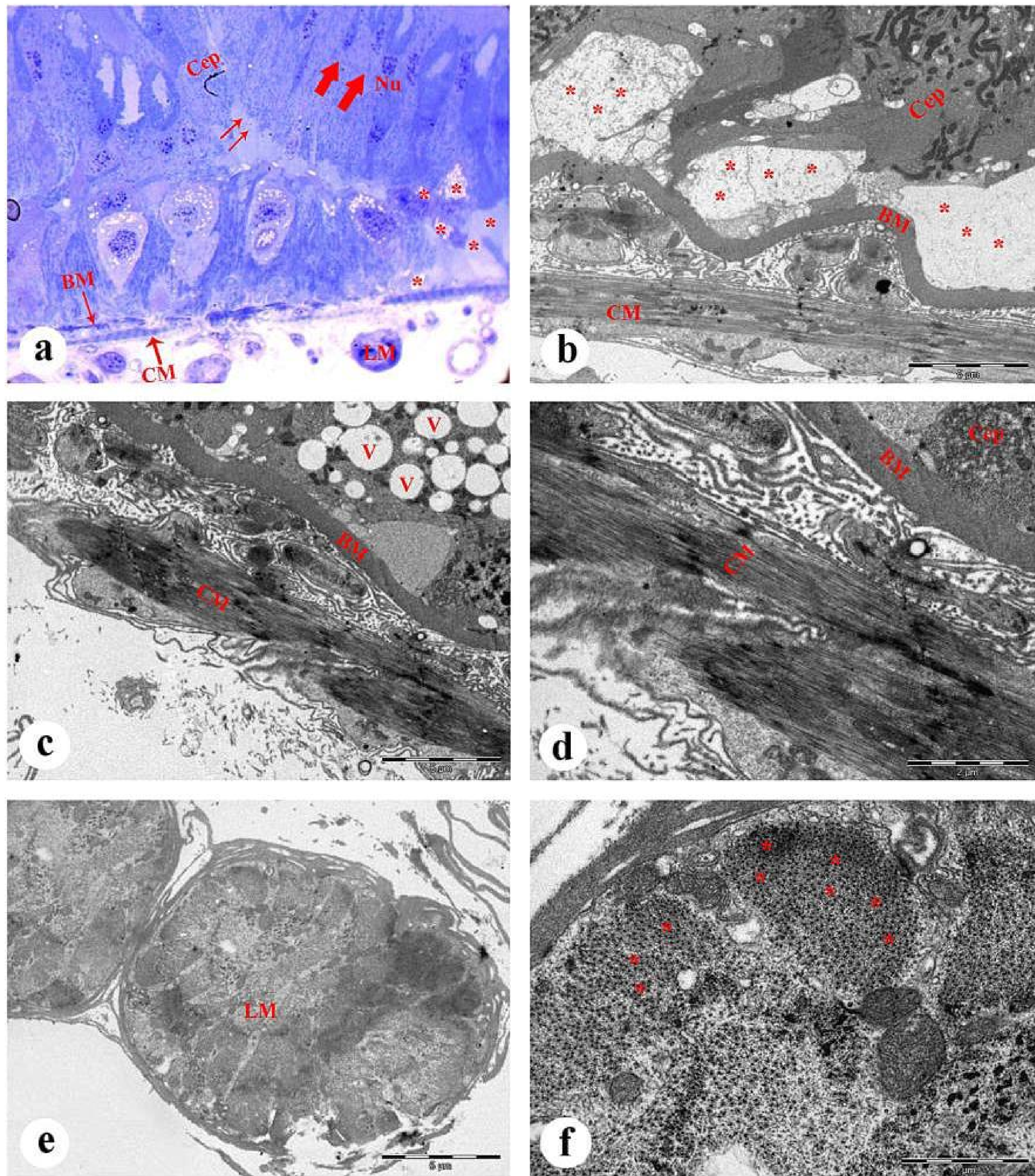


Fig 7. a. Semithin section of larval midgut tissue treated with *V. negundo* extract showing the complete detachment of Columnar epithelial layer (Cep) from the basement membrane (BM) (thin arrows) due to excessive vacuolization between BM and epithelial layer (asterik). Epithelial nuclei shows apical movement towards the lumen due to elongation of epithelial cell and its nucleus (thick arrows) (1000X); b. Ultrathin section showing detached epithelial layer from BM due to vacuolization (asterik) (4800X); c. Showing epithelial vacuoles (V), BM and Circular muscle (CM) (4800X); d. Magnified view of CM (11000X); e. Longitudinal Muscle layer (LM) (4800X); f. Enlarged view of LM with myofibrils (asterik) (23000X).

PLATE VI. 11.

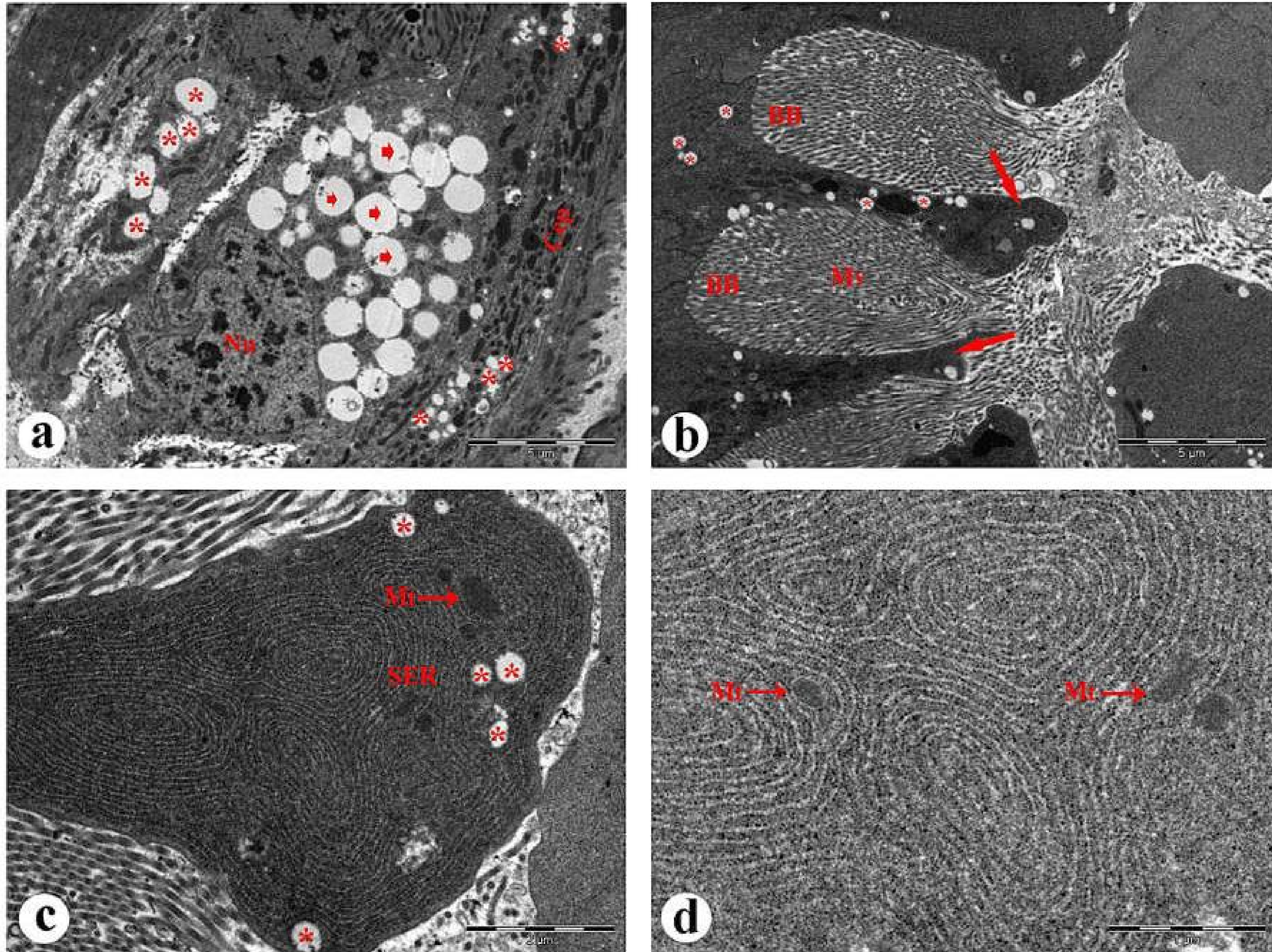


Fig. 6. Showing the details of larval midgut tissue treated with *H. suaveolens*. a. Large vacuoles in the cytoplasm of the secretory cell (arrow head) along with cytoplasmic vacuolization of Columnar epithelium (Cep) (asterik) (4800X); b. Brush border (BB) with apical blebbing of Cep (arrows) with vacuolization (asterik) (4800X); c. Apical part of blebbed columnar with rich SER and microvilli along with very few mitochondria (Mt) (11000X); d. Enlarged view of SER inside Cep apical bleb (23000X)

PLATE VI. 10

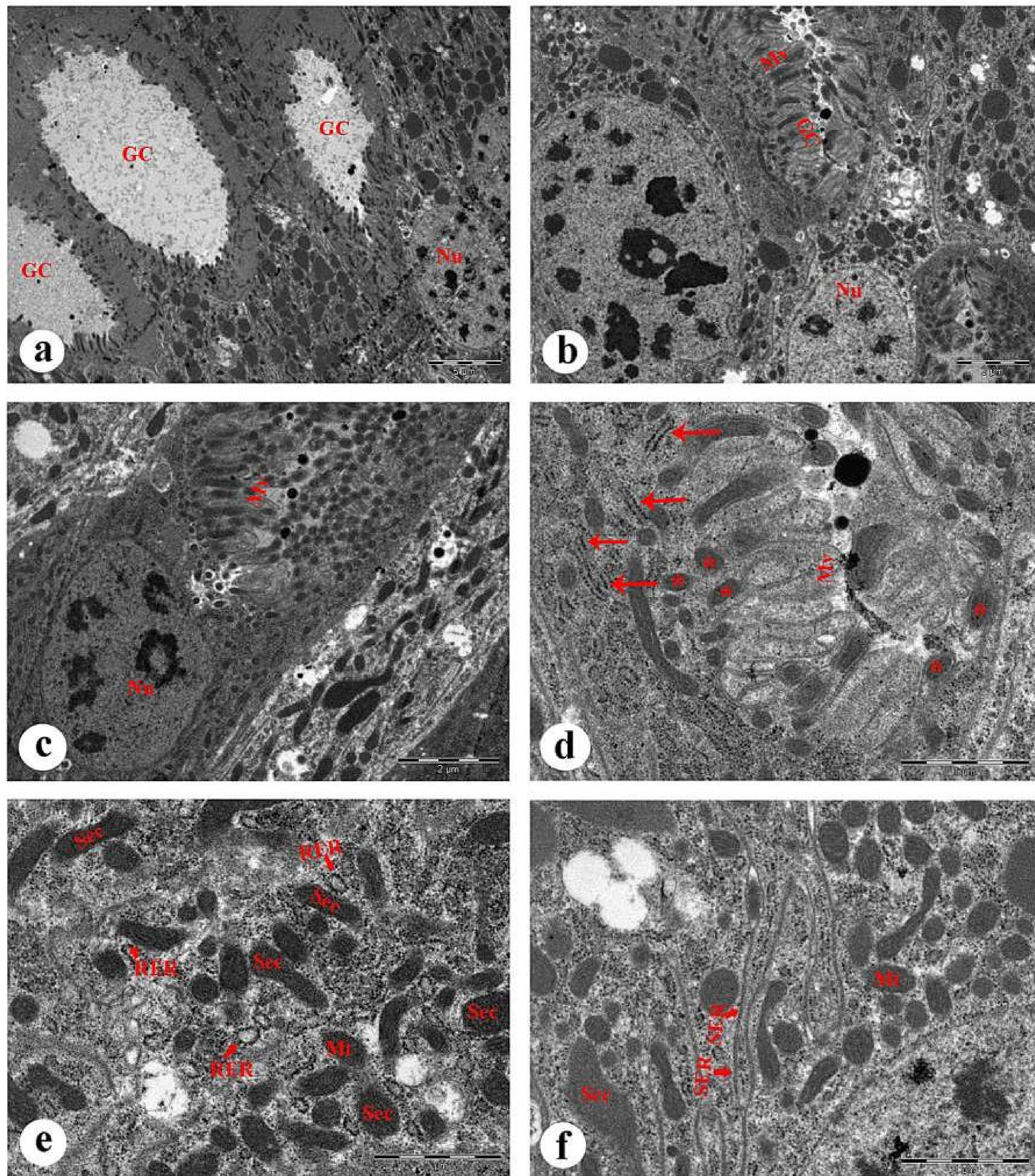


Fig. 5. Showing the details of larval midgut tissue treated with *H. suaveolens*. a. Epithelial layer with adjacent goblet cells (GC) (2900X); b. A part of nuclei (Nu) of adjacent epithelial and goblet respectively (6800X); c. Goblet cell nucleus with dense microvilli (Mv) (9300X); d. Goblet cell rich in microvilli, mitochondria (Mt) and RER (23000X); e. Columnar epithelium rich in mitochondria, RER and secretory vesicles (Sec) (23000X); f. Columnar cytoplasm with SER and mitochondria (23000X).

PLATE VI. 8

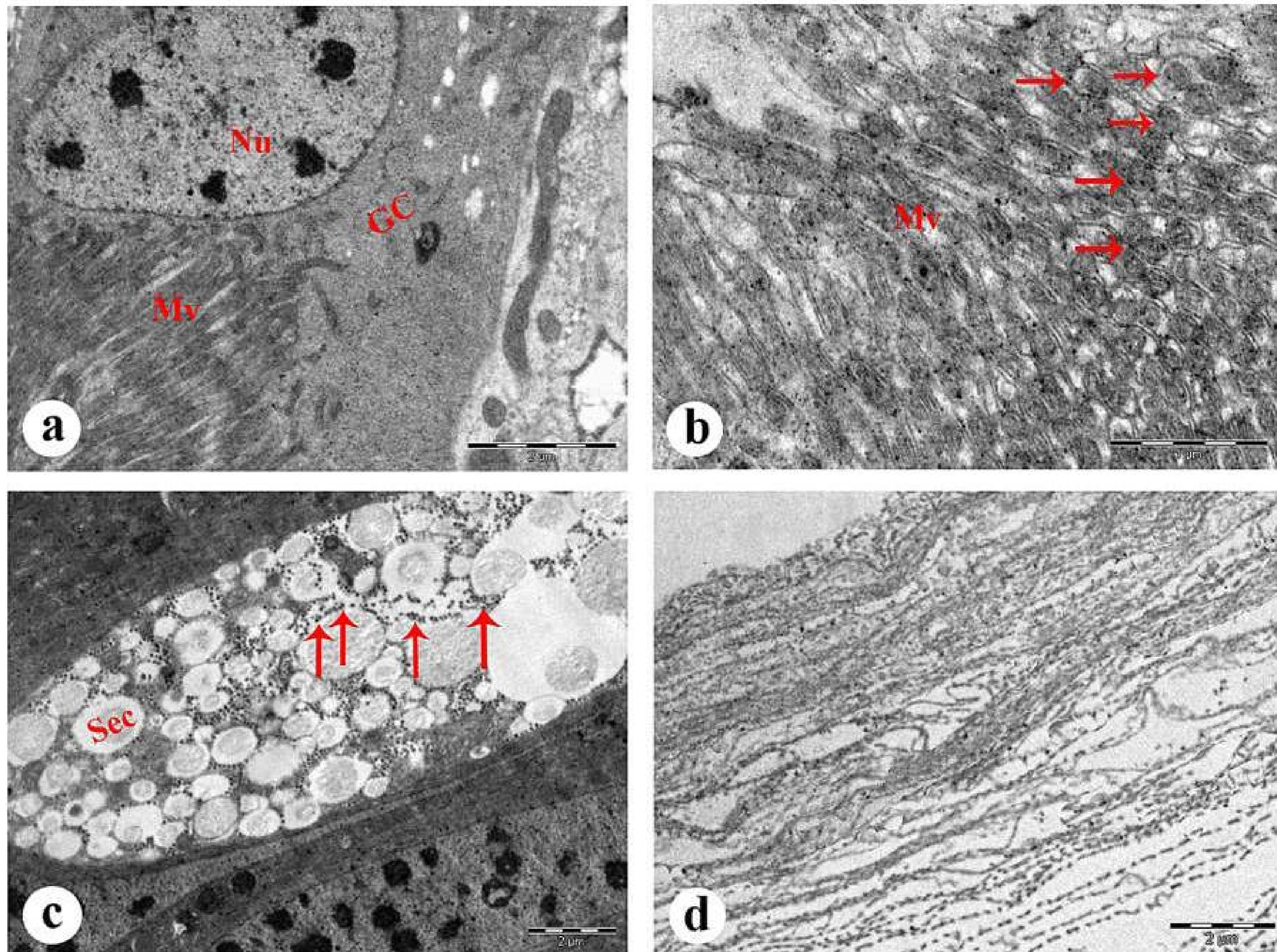


Fig. 3. Showing details of control midgut epithelium. a. Part of goblet cell (GC) with its nuclei (Nu) (11000X); b. Enlarged view of microvilli (Mv) in goblet cell with mitochondria (arrows) (23000X) c. Secretory cell in the epithelial layer with its secretions as secretory granules (arrows) (6800X); d. Peritrophic membrane (6800 X).

PLATE VI. 7

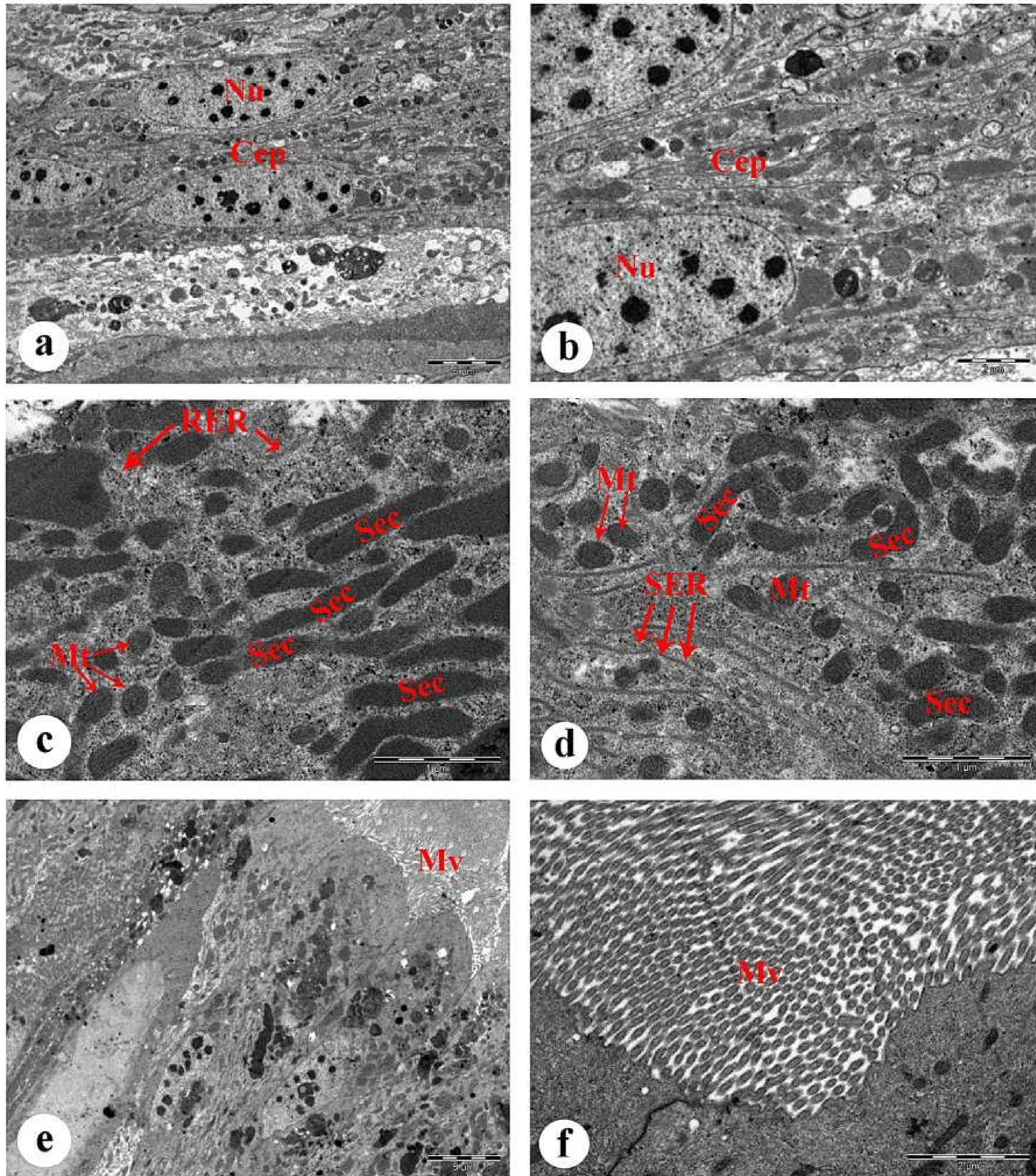


Fig. 2. Showing the details of epithelium of control midgut tissue. a. Columnar epithelial layer (Cep) with nucleus (Nu) (2900X); b. Magnified view of columnar epithelial cell with nucleus (6800X); c. Apical part of Cep rich in mitochondria (Mt) and secretory vesicles (Sec) (23000X); d. Cep with mitochondria and smooth endoplasmic reticulum (SER) (23000X); e. Apical part of columnar epithelial cells (2900X); f. An enlarged view of apical part of columnar epithelial cells rich in microvilli (Mv) (11000 X).

PLATE VI. 6

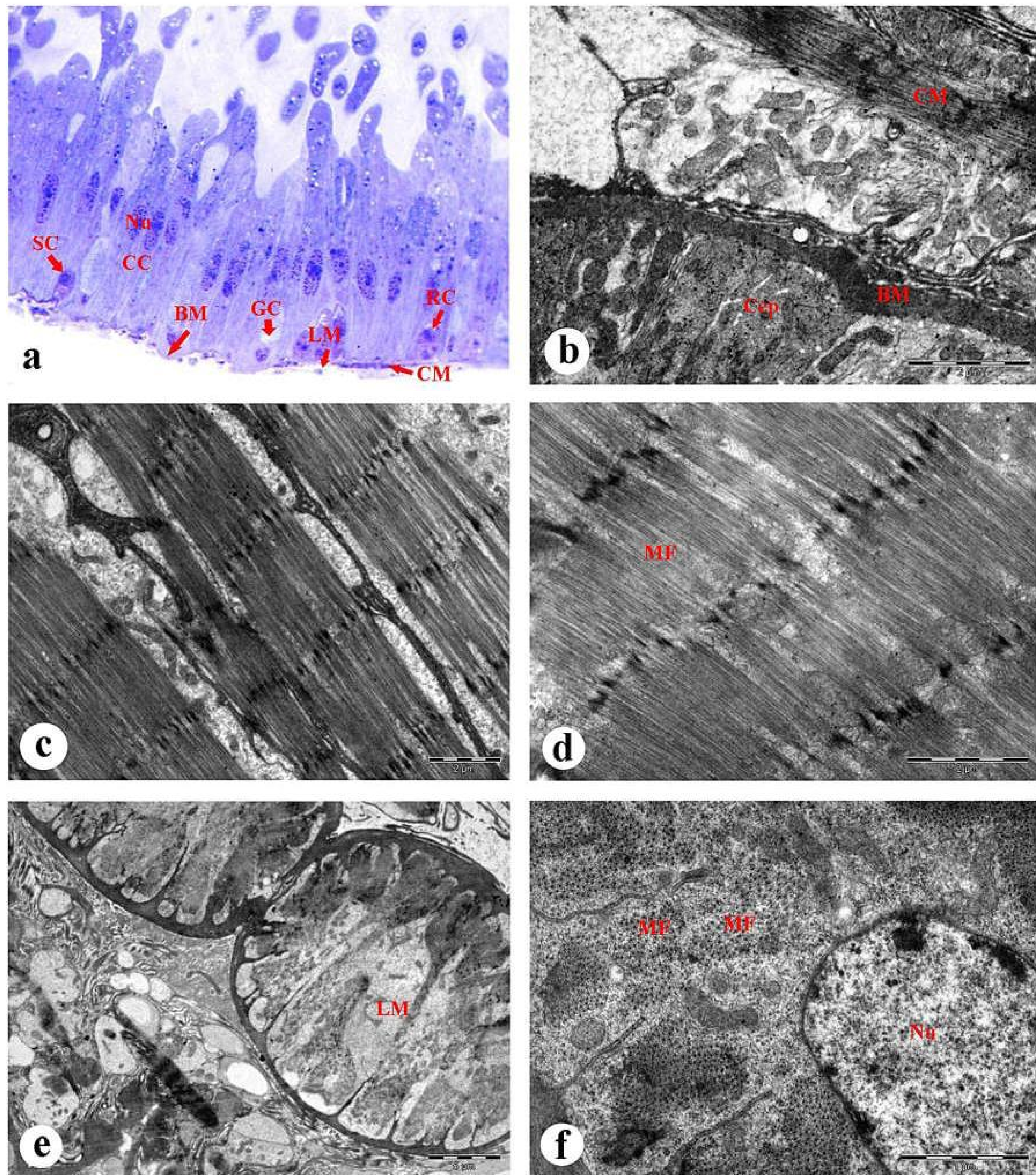


Fig. 1. a. Semithin section showing the common features of the midgut tissue of control sixth instar larva of *O. exvinacea* (1000X); b. ultrastructural view of Basement membrane (BM), Columnar epithelium (Cep) and circular muscle layer (11000X); c. Circular muscle layer (CM), (6800X); d. Magnified view of a part of CM (11000X); e. Basal region with Longitudinal muscle (LM) (2900X); f. Enlarged portion of LM with a part of its nucleus (Nu) and myofibrils (MF) (23000X).

PLATE VI. 5.

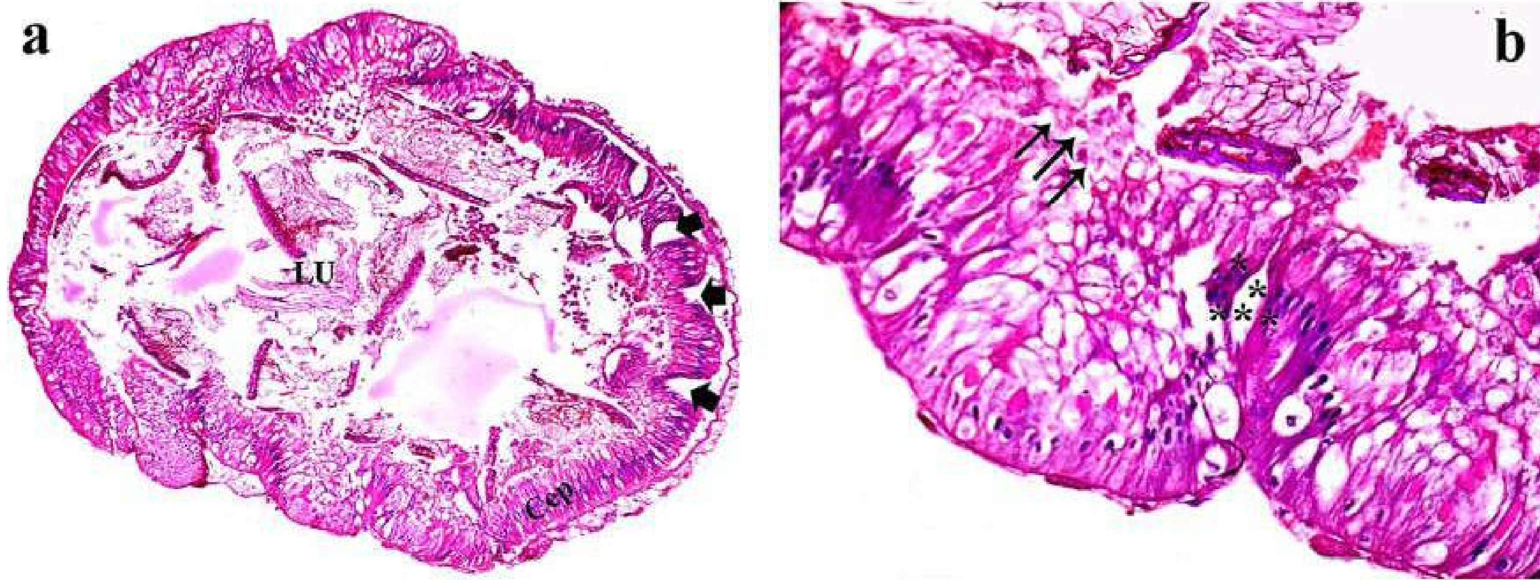
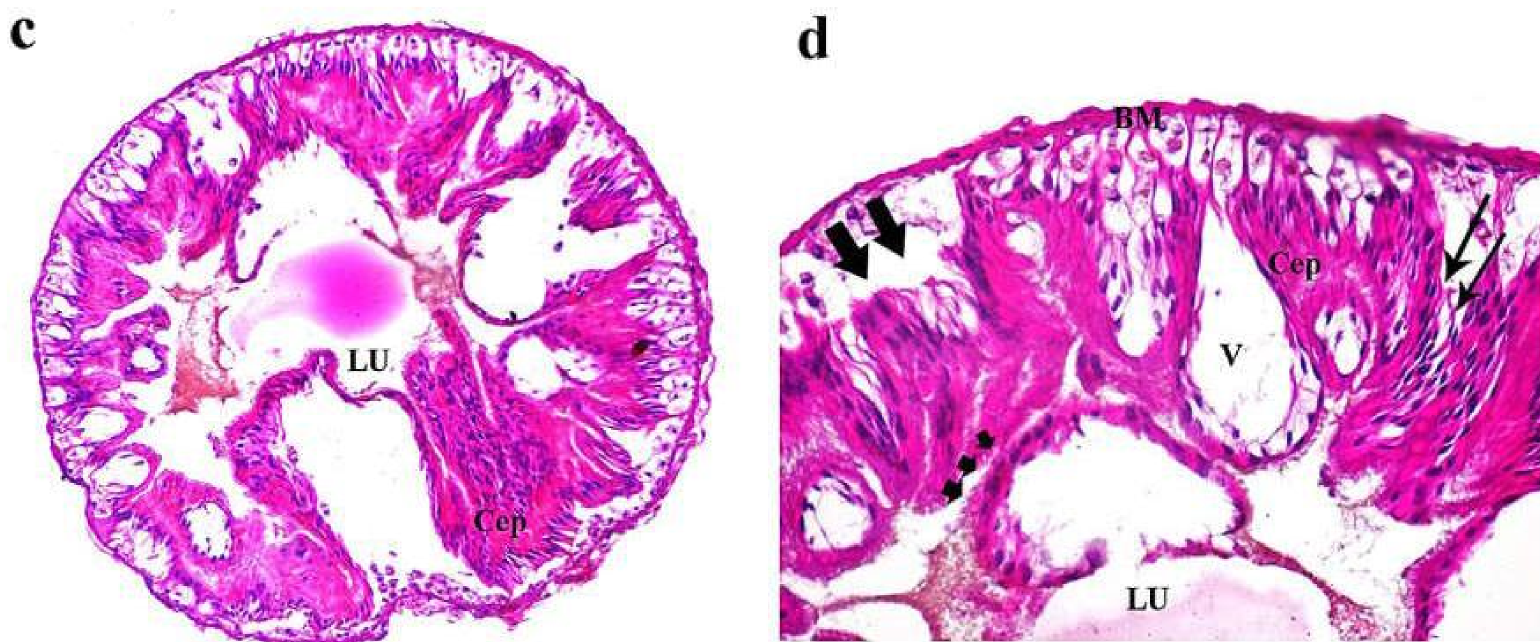


Fig. 5. a. T.S of larval midgut treated with 4% of *V. negundo* (100X); b. An enlarged view (400X). Thick arrows indicate detachment of Cep from BM, asterik-folded, over lapped and congested CC with nuclei, thin arrows-sloughing off.



c. T.S of larval midgut treated with 5% of *V. negundo* (100X); d. An enlarged portion (400X). Thin arrows-elongated nuclei movement towards lumen, thick arrows-detachment of Cep from BM, arrow head-varied fashion of sloughing off. Columnar epithelium (Cep), Basement membrane (BM), Vacuoles (V).

PLATE VI. 4.

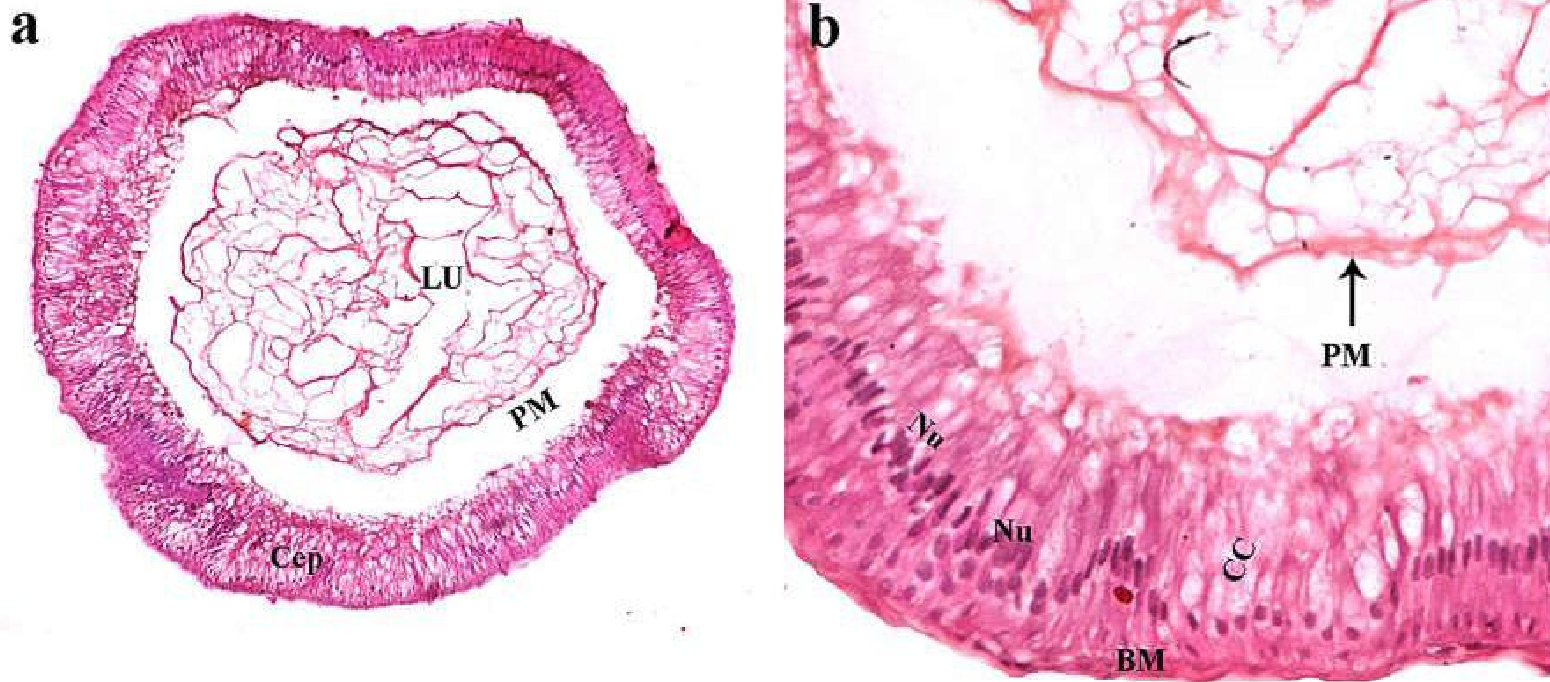
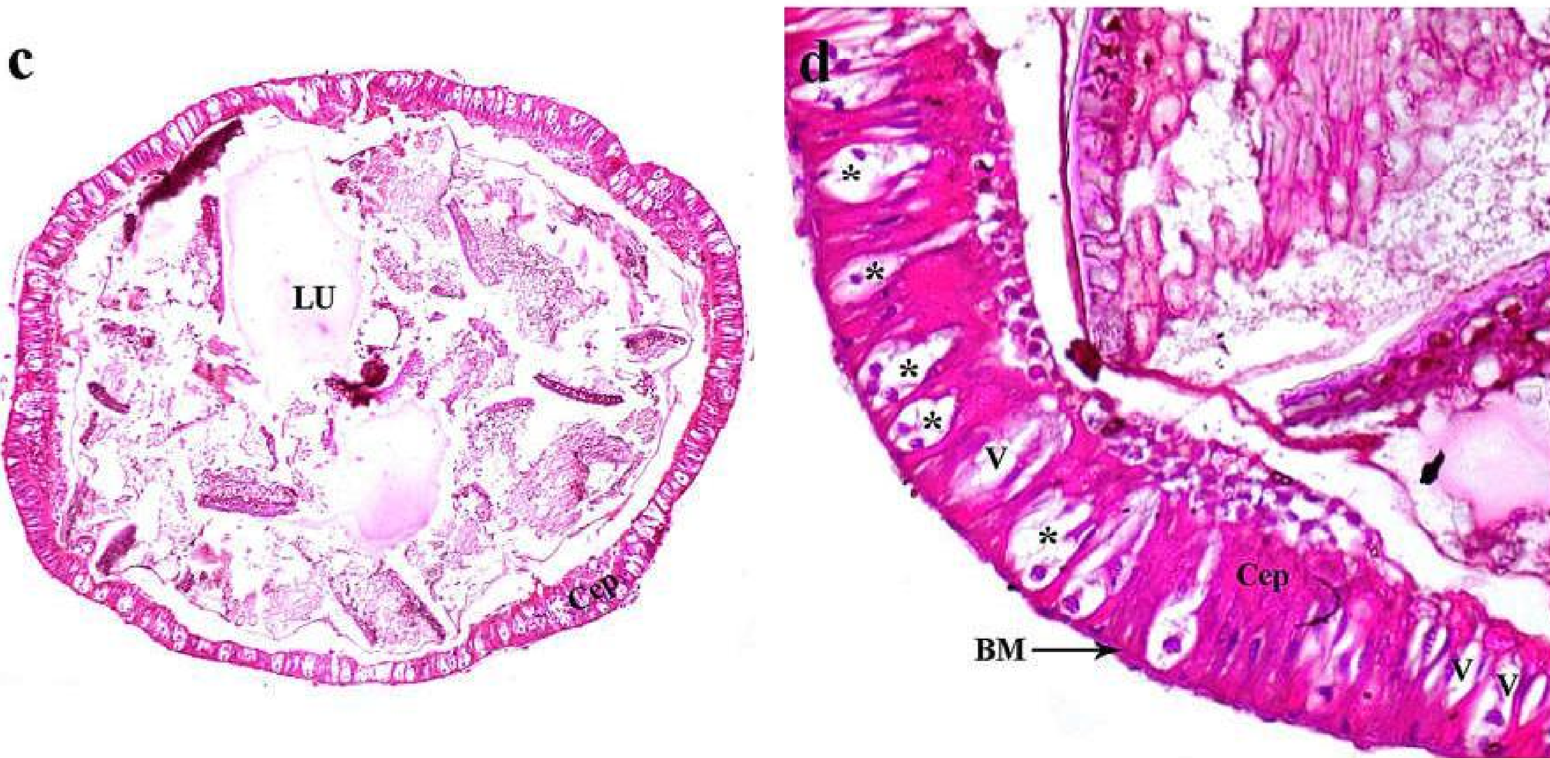
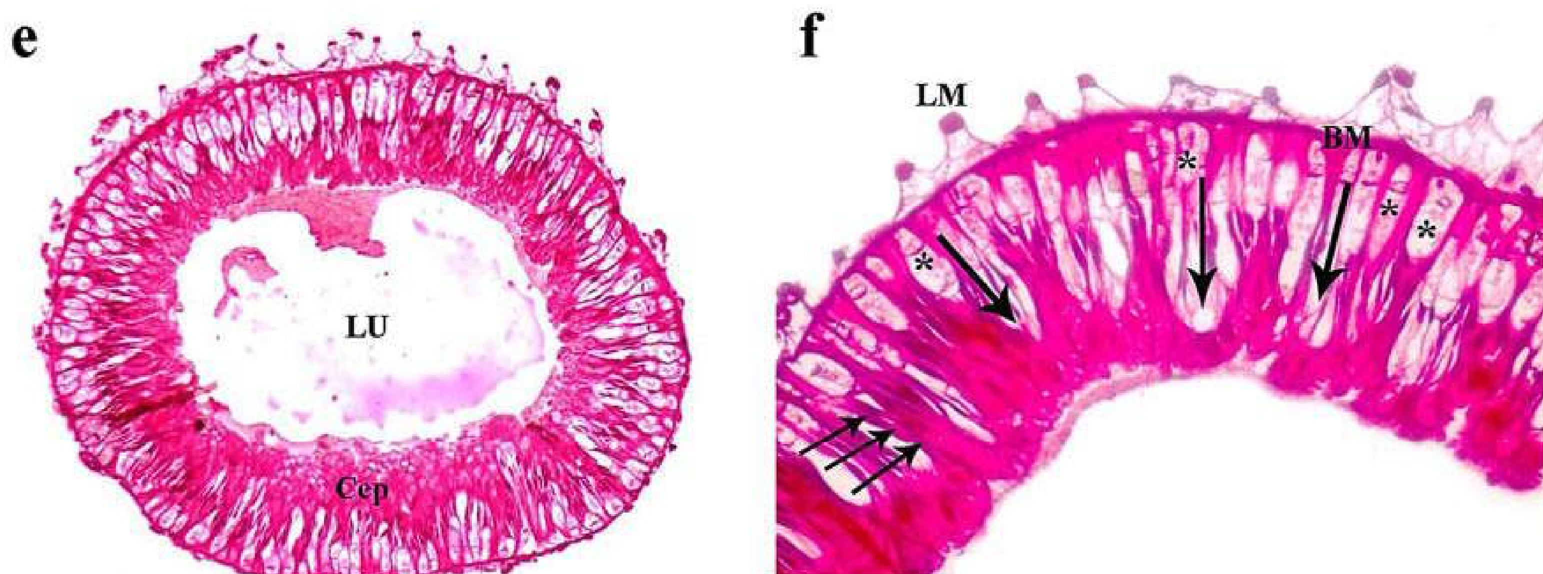


Fig. 4. a. T.S of larval midgut epithelium treated with 1% of *V. negundo* (100X); b. A portion enlarged (400X).

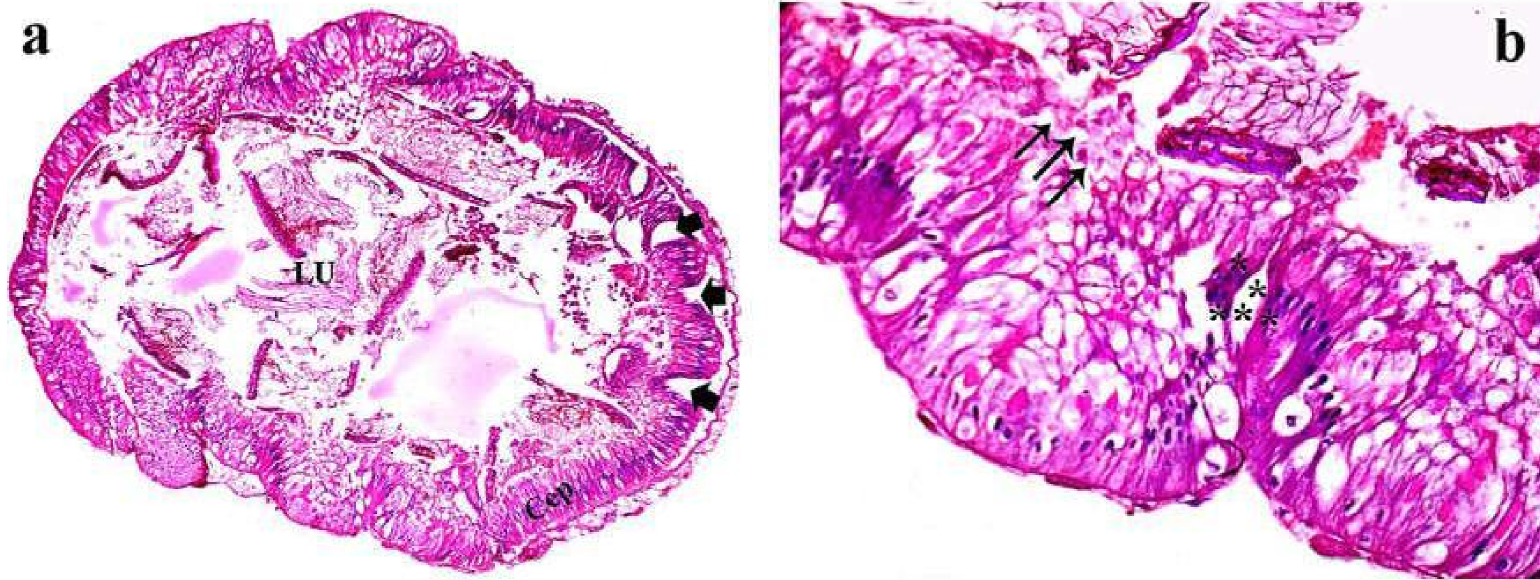


c. T.S of larval midgut treated with 2% of *V. negundo* (100X); d. An enlarged view (400X). Asterik-GC size increased, V-vacuoles.

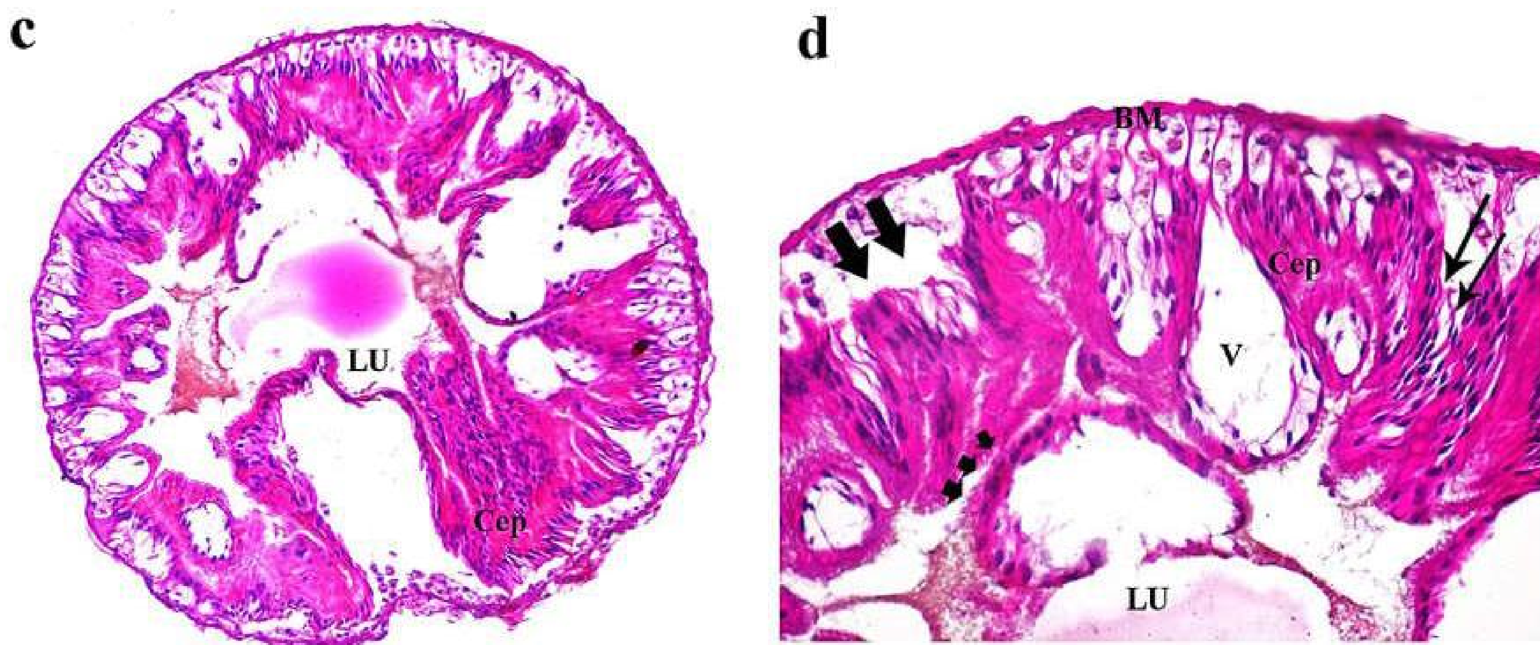


e. T.S of larval midgut treated with 3% of *V. negundo* (100X); f. A magnified view (400X). Asterik-GC size increased, vacuoles (V), thin arrows-congested and over lapped columnar cells with nuclei, downward arrows-elongation of CC. Goblet cell (GC), Columnar cells (CC), Basement membrane (BM), Peritrophic membrane (PM), Lumen (LU), Columnar epothelium (Cep), Longitudinal muscle (LM).

PLATE VI. 5.



ig. 5. a. T.S of larval midgut treated with 4% of *V. negundo* (100X); b. An enlarged view (400X). Thick arrows indicate detachment of Cep from BM, asterik-folded, over lapped and congested CC with nuclei, thin arrows-sloughing off.



c. T.S of larval midgut treated with 5% of *V. negundo* (100X); d. An enlarged portion (400X). Thin arrows-elongated nuclei movement towards lumen, thick arrows-detachment of Cep from BM, arrow head-varied fashion of sloughing off. Columnar epithelium (Cep), Basement membrane (BM), Vacuoles (V).

PLATE VI. 3.

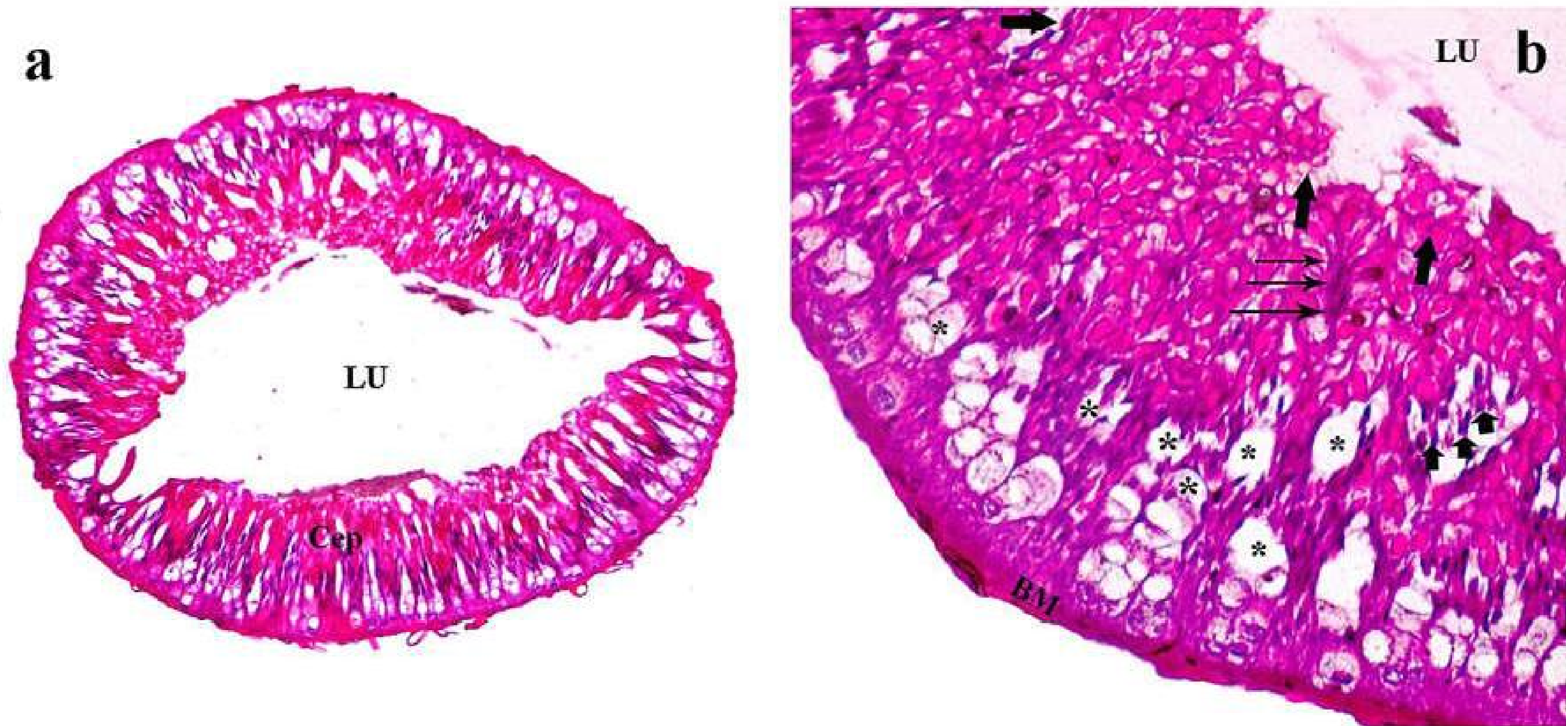


Fig. 3. a. T.S of larval midgut treated with 4% of *H. suaveolens* (100X); b. A portion enlarged (400 X). Asterik indicates prominent vacuolization, arrow head-upward movement of nucleus, thin arrows-overlapped and congested columnar nucleus, thick arrows-sloughing off BB.

c. T.S of midgut epithelium treated with 5% of *H. suaveolens* (100X); d. A portion enlarged (400X). Thin arrows-apical movement of columnar nucleus, asterik-tufty apical arrangement of Brush border (BB) with high secretory activity. Lumen (LU), Columnar epithelium (Cep), Nucleus (Nu).