Investigation on Tissue repair effects and Anti-microbial activity of larval blood extract from *Myrmeleon formicarius*

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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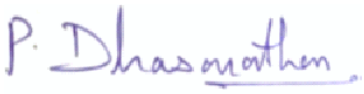

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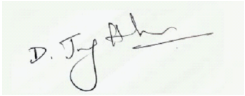

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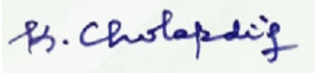

INTERNAL EXAMINER

EXTERNAL EXAMINER

DECLARATION

We hereby declare that the project report entitled "**Investigation on Tissue repair effects, anti-tumor activity and anti-microbial activity of larval blood extract from *Myrmeleon formicarius* "** submitted to the Department of Biotechnology, Prathyusha Engineering College, affiliated to the Anna University, Chennai, in partial fulfillment of the award of the degree Bachelor of Technology in Biotechnology, is the record of the original work carried by us under the guidance of **Ms.D.JOYCE HELLEN SATHYA M.Tech.,** Assistant Professor, Department of Biotechnology, Prathyusha Engineering College and **DR.P.BALASHANMUGAM**, Avanz bio Pvt. Ltd. during the period of December 2019 - March 2020. We further declare that the results of the work have not been previously submitted for the award of any degree or diploma.

Place: Thiruvallur

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TABLE OF CONTENTS

ABSTRACT

Insects form a major part in our ecosystem, insect's body comprises of various bioactive compounds which has therapeutic properties. Ancient people used the insects with medicinal properties to treat various illness. Antlion is an species of insect which feeds on ants and is found in arid and dry areas. A recent study revealed that the insect blood extract of antlion has tissue repair property of liver and kidney in diabetes induced mice model. The present study is focused on investigating the tissue repair effects and the antimicrobial properties of the insect blood extract collected from the larvae of *Myrmeleon formicarius* (antlion). A complete analysis of the insect blood extract was done by GC-MS and SDS-PAGE. The bands observed on the SDS-PAGE show the presence of a specific protein called chaperones in the insect blood extract which is responsible for wound healing activity. The Cytotoxicity of the insect blood extract was studied on VERO African green monkey kidney normal cell line. In-vitro wound healing scratch assay was performed on 3T3 mouse fibroblast normal cell line to determine the tissue regenerative property of insect blood extract. The results revealed that the insect blood extract had tissue regenerative property. Antibacterial activity of insect blood extract was analyzed by well diffusion method and also by statistical method. MIC was found to be 40µg,80µg,60µg on *P.aeruginosa*, *S.aureus*,*E.coli* respectively. Maximum inhibition effect was observed at the concentration of 80µg for all the bacterial strains. From t-Test on comparing the mean zone diameter of three bacterial strains it was evident that insect extract has high inhibition effect on *S.aureus* than on *E.coli* and *P.aeruginosa.* F ratio value was found to be 0.0653 using ANOVA test.

Keywords: Insect blood extract, Cytotoxicity, Chaperones, Apoptosis, Tissue regenerative property, Wound healing property, *Myrmeleon formicarius.*

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

°C	Celsius
µg	Microgram
L	Litre
µl	micro liter
ml	milli liter
min	minute
mm	milli meter
rpm	Revolution per minute
%	Percentage
nm	Nanometer
Da	Dalton
Hrs	Hours
eV	Electron volt
mg	Milligram
Kg	Kilogram
W/w	Weight by weight ratio
KHz	Kilo hertz
V/v	Volume/volume ratio
Cm	Centimeter
P	Probability
pH	Potential of hydrogen ions
ID	Isotopic dilution
df	Film thickness

V	Voltage
U/ml	Unit per ml
KDa	Kilo Dalton
g	Gram
min^{-1}	Minute inverse

LIST OF ABBREVIATIONS

ZOI	Zone of Inhibiton
DMEM	Dulbecco's modified eagle medium
GCMS	Gas chromatography mass spectroscopy
ANOVA	Analysis of variance
IBE	Insect blood extract
NCCS	National Centre for Cell Sciences
SDS	Sodium dodecyl sulphate.
DMSO	Dimethyl sulpho oxide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
DNS	Dinitro salicylic acid
DDH_2O	Double distilled water
DDI	Double de ionized
TLC	Thin layer chromatography
MW	Molecular weight
IC_{50}	Half maximal inhibitory concentration
BW	Body weight

FBS	Fetal bovine serum
CO_2	Carbon dioxide
Hcl	Hydrochloric acid
Rf	Retention factor
NIST	National Institute of Standards and Technology

AIM AND OBJECTIVE

AIM:

To investigate the Tissue repair effects and the Antibacterial properties of the insect blood extract from antlion larvae (*Myrmeleon formicarius*).

OBJECTIVES:

- To collect and acclimatize the antlion larvae in sand tray at laboratory condition.
- To collect the insect blood extract from antlion larvae
- To demonstrate the antibacterial property of insect blood extract by well diffusion method.
- To analyze the level of cytotoxicity expressed by the insect blood extract.
- To prove the tissue regeneration property of insect extract.

CHAPTER 1

INTRODUCTION

This chapter deals with basic information about methods and the sample we have used in our project. It covers various research works of scholars who adapted these methods.

1.1. INVERTEBRATES:

Animals which are void of backbone are called invertebrates. 90% of animal species consists of invertebrates (Erwin, 1983). Invertebrates has many ecological importance and benefits. Invertebrates involve in decomposition of dead organic materials (Wilson, 1990). It also plays role in plant pollination, plant protection, ecosystem stabilization, energy and nutrition transfer (Kellert, 1993).

Invertebrates are precious species but given less importance which is not sufficient for the role they play in our ecosystem (Brussaard *et al.*,1997). Soil quality is indicated by invertebrates. It also helps in maintaining soil porosity along with coordination of plant roots. Nematodes, Microarthropods, and largest Arthropods are three different ranges of invertebrates living in soil based on their sizes. They are actively involved in microbial communities' control and protects plant from pests and diseases, and maintains soil pH, humus content, texture. Thus, they play vital role as soil engineer in ecosystem (Lavelle *et al.*,2006).

1.2. ENTOMOLOGY:

Study of insects and its interaction with other living organisms in the ecosystem is called entomology. Entomologists make great contributions to such diverse fields as agriculture, chemistry, biology, human/animal health, molecular science, criminology, and forensics. The study of insects serves as the basis for

developments in biological and chemical pest control, food and fiber production and storage, pharmaceuticals epidemiology, biological diversity, and a variety of other fields of science. (Borror *et al*.,1989).

The traditional knowledge of the insects based on their medicinal importance is called ethno-entomology, the practices used in ancient times remains unheard to the masses. Manuscripts on the palm leaves which dates back many centuries reveals that many species of insects were found to be used as medical remedy by the various tribal communities and physicians who lived in that period (David, 2001). The practice has been passed on for many generations and it is still in practice, the tribes of Western Ghats in the Tirunelveli district of Tamilnadu, India. Also, traditional medicines are also prepared by village physicians using these medically important insects. Various ailments like skin diseases, hair loss, respiratory syndrome, neurological problems and urinary diseases are said to be cured using these insects. (Ranjit *et al*. 2004)

1.3. CLASSIFICATION:

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Neuroptera
Family	:	Myrmeleontidae
Subfamily	:	Myrmeleontinae
Tribe	:	Myrmeleontini
Genus	:	Myrmeleon

1.4. ANTLION:

Antlions are insect species that belongs to family Myrmeleontidae. They are most diverse family of Neuroptera in Australia and many species remain to be described. Most are found in the arid regions of the continent, where their ground-dwelling larvae occur in litter and sand (Froggatt, 1902). They involve in forming conical pits to catch its prey(Allen *et al.*,1985). They are all natural predators of ants. Myrmeleontidae includes over 600 described species. Like many other members of the order, adult antlions are commonly seen around lights and campfires, particularly during the spring and late summer. They have two pairs of long, narrow, many-veined wings and a long, slender abdomen. Although they greatly resemble small and unrelated dragonflies (viz. damselflies), they belong to an entirely different order of insects.(Hawkeswood, 2006).

Antlion attacks the prey using a toxin produced by itself and its bacterial symbionts. It has discontinuous gut which helps in prevention of solid waste until adulthood (Withycombe, 1925). This helps in their starvation period and it can withstand up to 3 months without food. This accumulation of solid waste restricts the composition of microbial community in their gut. The bacteria present in their gut helps in prey paralysis and digestion. (Dunn *et al.*,2005).

Theories of foraging and intraspecific competition can be studied using ant lion as animal model (Wilson, 1974). They are directly related to foraging because they are reproductively inactive and suffer less predation. These are called sit and wait predators since they don't search for a prey but vigorously attacks the prey when the prey arrives at the conical pit which is a trap constructed by the antlions. (Bernd *et al.*,1984).

Heterozygosity and body size increase follows Bergmann's rule. According to this rule the body size increases towards increase latitude of the habitat (Brown *et al.*,1969). Annual climatic variance is an indicator for predicting heterozygosity of antlions. (Arnett *et al.*,1999).

1.4.1. HABITANT OF ANTLION:

Antlions mostly lives in isolated desert regions. Their pit construction pattern and position within the pit changes according to the temperature of the sand. They need a constant temperature without more fluctuations and thus they live by constructing deep pits. This indicates that temperature fluctuations decrease with increasing depth.(Green, 1955).

1.5. LIFECYCLE OF ANTLION:

The antlions undergo a complete metamorphosis through the four stages of its life cycle (egg, larva, pupa, adult). The life cycle varies and it may take 2-3 years. The female antlions deposit their eggs by sticking their abdomen into the sand after mating. Since the larvae leave spiral patterns on the sand they are locally called "doodlebugs" or "sand dragons". The larvae have a flattened head that is equipped with a pincers-like jaws that can catch hold of the prey in their immediate vicinity. They prey on very small insects, as the name suggests they feed mainly on ants. A fully grown larvae takes up to 2 years for full maturation and during the period it molts thrice. The larvae start to pupate by making a cocoon of sand and silk. These cocoons are then buried deep by several centimeters into the sand. The adult antlions will reach the surface after 4 weeks once after emerging from the pupal case. The adults live for 4 weeks and the life cycle continues. (Hodgson *et al.*, 2008)

1.6. ANTLION PIT CONSTRUCTION:

Antlion pit construction is based on fine sand particles. Angle of repose and drag force are the two physical properties which govern the efficiency of pit structure. Efficiency of prey capture depends on particles of sand. Antlion constructs the pit by digging and throwing away the larger sand particles than smaller ones. Larvae possess a mechanism for circling in order to trace the initial circular groove, they need not possess an intrinsic spiral orienting mechanism (Turner, 1915). As the larva circles and flicks sand along the bottom of a digging groove, the disproportionate collapse of material from the outer wall compared to that from the inner wall of the pit causes the digging groove to shift steadily inward. If the larva simply follows the bottom of this groove and continues to flick sand, an inverted conical pit will be formed. (Lucas.,1982).

M.formicarius reacts according with the substrate vibrations caused by the prey when it falls into the pit constructed by antlions. It tosses the sands in the angle of prey. The presence of prey on Posterior surface results in frequent sand tossing by antlions. (Bojana *et al.*,2008).The pit building depth, pattern of antlion varies with nature of sand particles. The most comfortable pit sand fractions are 0.23 mm and 0.23–0.54 mm. Particle size greater than 1.54 mm is unfit for pit construction. (Devetak *et al.*,2005).

1.7. STUDY ON ANTLION:

1.7.1. STUDY OF GUT MICROBIOTA:

Gut physiology of antlions are unique in nature where the mid gut and hind gut are not connected and gets linked only in adulthood. So they possess a discontinuous hindgut which is a great evolution (Gaumont, 1976). Culture independent 16-s rRNA gene sequencing of bacteria was carried out and the

bacteria associated with tissues of antlion were well characterized. Two families of bacteria were identified and characterized. α-proteobacteria (Enterobacteriaceae) and γ- proteobacteria (Wolbachia) are the families present in gut and head respectively. This is also confirmed by fluorescence in-situ hybridization. (Dunn *et al.*,2005).

1.7.2. BERGMANN'S RULE IN THE ANT LION *Myrmeleon immaculatus*:

Bergmann's rule was studied on antlion based on their pit construction. According to this rule the body size increases towards increase latitude of the habitat (Brown *et al.*,1969). Antlions body size and heterozygosity were keenly observed by on 5 different loci and they were increased with increasing latitude. Heterozygosity was experimented using 5 different enzymes viz; GPI (glucose phosphate isomerase), MDH (malate dehydrogenase), PEP (peptidase), DIA (diaphorase) and SOD (superoxide dismutase). The key indicators of heterozygosity are maximum temperature and precipitation on sand. Diurnal photoperiod which controls the metamorphosis of antlion also varied with latitude.(Arnett *et al.*,1999).

1.7.3. TEMPERATURE RELATIONS OF ANT-LION:

Antlion pit forming strategy varies with temperature of sand. Antlion relationship with temperature was studied by collecting several antlions in square plot of sand. Pit position and antlion position within the pit was observed by varying the surface temperature of sand for every half of an hour. Temperature on the surface was measured using thermocouples. (Wellington, 1950). Temperature fluctuations resulted in increasing pit depth by the antlion.(Green, 1955).

1.8. CYTOTOXICITY STUDY:

Cytotoxicity assay is used to check the cell viability, proliferation and cytotoxicity. Cytotoxicity is the toxic effects of a drug on in-vitro cell lines. This method is also called as MTT assay. This assay uses a specific salt MTT 3-(4,5-dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide which is a tetrazolium dye. The colour of dye is yellow. This dye on presence of cellular oxido reductase enzymes is converted to insoluble formazon which is purple in colour. The absorbance can be measured using microtiter plate reader. The intensity of purple colour indicates the viability and proliferation of cells. (Mosmann *et al*.,1983).

The Cytotoxic activity of hemocytes which was isolated from the blowfly *Calliphoravicina* larvae was tested using the human myelogenous leukemia K562 cells as target. (Hashimoto and sudo, 1971; Rikova *et al.*, 1981). The Cell lines were recognized as non-self by the hemocytes in the single cell, cytotoxicity assays and the hemocytes were able to attach to the cellular surface for inducing the cell destruction (Zachary and Hoffmann, 1973). The cytotoxicity exhibited by the lymphocytes increased tremendously in the later stages of the larval development shortly before the onset of the pupariation, compared to that of the lymphocytes derived from the human peripheral blood or from mouse spleen. The hemocytes in these play a vital role in the defense mechanism against the potential eukaryotic parasites, and to aid in the elimination of its own somatic cells and also during the process of metamorphosis. (Chernysh *et al.*, 2004)

1.9. WOUND HEALING:

Wound healing is a natural tissue repairing process which is involved in the regeneration and reconstruction of damaged tissue. The process takes place in 4 steps.

- Hemostasis and clotting
- Inflammation
- Wound closure: re-epithelialization and wound contraction
- Remodeling of tissue

1.9.1. Hemostasis and clotting:

Wound healing starts with hemostasis and clotting phase. After the wound is created the platelets aggregate in the wounded site and constriction of blood vessels take place to prevent excessive blood loss. A matrix is formed which is involved in infiltration of cells. In this phase prothrombin is converted to thrombin and fibrinogen to fibrin. This fibrin polymerizes to form a blood clot at the wounded site. Vasodilation of blood vessels takes place which helps in infiltration of myeloid cells and neutrophils.(Schmidt *et al*.,2013)

1.9.2. Inflammation:

Inflammation is the second stage of wound healing which involves swelling of wounded tissue to prevent blood loss and bacterial infection. Leukocytes enter the site of wound and create inflammation by engulfing of damaged cells. Platelets release Platelet-derived growth factors which infiltrates macrophages, cytokines, dendritic cells to the wounded site. Macrophages and dendritic cells are actively involved in this phase. These two white blood cells get activated and engulfs the wound cells and release the cytokines.(Schmidt *et al*.,2013)

1.9.3. Wound closure: re-epithelialization and wound contraction:

This phase marks the angiogenesis and granulation tissue formation. Angiogenesis is the formation of new blood vessels using angiogenic factors. Fibroblasts excretes collagen and fibronectin. This involve in provisional matrix

formation which covers the outermost layer. Epithelial cells involves in re-epithelialization. Fibroblasts is converted to myofibroblasts which involves in wound contraction by gripping the wound edges. Collagen I is involved in extracellular matrix formation.(Schmidt *et al*.,2013)

1.9.4. Remodeling of tissue:

Remodeling of tissue takes place of years to resemble to original tissue but leaves a scar. Fibroblasts and endothelial cells involve in apoptosis of damaged cells debris. This phase involves formation of ECM by degeneration and regeneration of ECM molecules. LOX is involved in collagen crosslinking and thus increases wound tensile strength. In this way wound healing takes place in animals. (Schmidt *et al*.,2013)

The whole-body extract of *Lucilia sericata* maggots was used to study the wound healing effects of burns on the skin of rat model. The study includes the investigation of the burn wound healing properties of aqueous extract of maggots on a rat model with second-degree burn. The anti-inflammatory, antioxidative and antibacterial activities in Aqueous extract of maggots were examined. The studies revealed that the treatment of the burn wound with the maggot aqueous extract increased the wound healing to a greater extent and also increases hydroxyproline level in burn-treated rats. The current study of treating burn wounds on rat models successfully demonstrated the beneficial effects of maggot aqueous extract on the burn wounds. (Bian *et al.*, 2017)

1.10. TISSUE REGENERATION:

1.10.1 Antihyperglycemic and tissue-repair effects of antlion species:

Antlion have been consumed as traditional medicine in ancient times. (Narulita,2008). *M. formicarius* (antlion) extract exhibited glucose-lowering effects in streptozotocin induced diabetic mice. This effect could be attributed to the presence of sulphonylurea- and metformin-like compounds in *M. formicarius* (antlion) larvae. The presence of sulphonyl urea compound was verified by the observation of a yellow band on the TLC plate. Mice treated with *M. formicarius* (antlion) extract at 10 mg/ kg showed a significant decrease in blood glucose levels. The doses of *M. formicarius* (antlion) extract required for repairing liver and kidney damage were different, where a dose of 10 mg/ kg was required for repairing liver damage, whereas only 5 mg/ kg was sufficient for repairing kidney damage. (Narulita *et al*.,2019).

1.11. SDS-PAGE for separation of molecular compounds:

Proteins and glycoproteins of peritrophic membranes of larval *Calliphora ervthrocephala* and *Sarcophaga harafa* were separated by SDS-PAGE. Similar band patterns were observed for pure proteins present in both larvae and adults. Larvae showed a variety of protein and carbohydrate bands compared to adults which shown only one fraction of carbohydrate having molecular weight (MW) of 200,000 Daltons. (Stamm *et al.*,1978).

Sodium dodecyl polyacrylamide gel electrophoresis is a technique used in biochemistry, genetics. This technique involves separation of proteins present in the sample according to their electrophoretic mobility in the presence of electric current. The salt gives specific charge to the protein which moves to the opposite charge node in electrophoresis set up. A standard marker is also run along with the sample to compare the molecular weight of protein and identifying the protein respectively. (Sambrook *et al*., 1989).

1.12. BIOMATERIALS:

Biomaterials are materials which are capable of doing tissue function and can able to interact with body fluids when placed inside the body. These materials are used to replace damaged tissue to regain its original function. These materials are either natural or synthetic origin and can be used as a replacement of whole tissue or a part of tissue. Biomaterials has unique surface properties, surface moieties like glycoproteins which helps in catalyzing specific biochemical reactions. Biomaterials should possess good mechanical properties and should have biocompatibility. (Williams, 1987).

1.12.1. DESIGN OF BIOMATERIALS:

Biomaterial design varies according to the tissue function, length of time up it should function and biochemical environment of the damaged tissue. Biomaterials are of various forms viz; polymers, metals, composite materials, and ceramics. These types vary with atomic arrangement and thus possess different mechanical, physical, chemical and biological properties. (Raghavendra *et al.*,2015).

1.12.2. CHARACTERISTICS OF BIOMATERIALS:

Research shows that there are some fundamental characteristics that a biomaterial should possess in order to meet the function of normal tissue (Ratner *et al.,* 2004). They should be nontoxic. Toxicity refers to the adverse chemical reactions caused due to migration of biomaterial substances. Biomaterials should be biocompatible in nature. Good elasticity, tensile strength, young's modulus governs the mechanical strength of biomaterials. (Raghavendra *et al.*,2015).

1.12.3. APPLICATION OF BIOMATERIALS:

Biomaterials can be used in different forms such as solid implants, hydrogels, polymers or composites. Hydrogels, cellulosic materials of biomaterials is used for wound dressing during injury. Synthetic biomaterials are frequently used in dental applications to replace damaged enamel, tooth cavity, tooth gums. Even artificial tooth can be produced using biomaterials (Isa *et al.*,2000).

Research says that pathological processes damages the function and structure of normal healthy tissue. These tissues are physically replaced by biomaterials. Biomaterials is widely used in orthopedic medical field. People who are affected from rheumatoid arthritis, osteoarthritis have damaged knee joints which can be replaced with prostheses. (Scholtz *et al.*,2011).

Implants made from synthetic biomaterials are used to replace damaged arteries, heart valves in cardiovascular applications. Vascular grafts, stents, pacemakers, and complete artificial heart are examples of biomaterials used in this field of medicine. (Varaprasad *et al.*, 2011)

In ophthalmologic field biomaterials are used to make artificial cornea, artificial endothelium, intraocular lenses, and implants for vitreous and glaucoma. This improves the vision of the visually impaired people. Biomaterials differ from normal spectacles, eye lenses by directly contacts with tissues of eye. This leads to their frequent application of biomaterials in this field. (Raghavendra *et al.*,2015).Our present study is focused on investigating the wound healing properties of Antlion blood extract and its application for therapeutic purpose.

The following chapter deals with research conducted by various scholars and their respective findings related to tissue regeneration and insect studies.

CHAPTER 2

REVIEW OF LITERATURE

Our work reported the wound healing properties of antlion on VERO cell lines and protein responsible for it. This work also encloses proof of antibacterial activity present in antlion blood extract and its corresponding statistical analysis.

This chapter provides information we got from literature survey of Green.,(1955) have estimated the relationship between temperature of sand and antlion pit behavior. Ant-lion larvae were collected from a sandy areas of Forest biology division Ontario. He grown the antlion in one square plot and exposed it to direct sunlight all day. The difference in temperature throughout the day was measured using a thermocouple. He observed change in position of antlion within the pit and depth increase in depth of the pit. It was reported that there was an increase in depth of pit when temperature of sand fluctuates. This implies that stable temperature is required for good survival of antlions.

Body size of antlion can be measured by measuring the distance between eyes or by width of the heads according to Lutz *et al.*,(1974).Geographic variation in body size and heterozygosity was analyzed and reported by Arnett *et al.*,(1999).They collected *Myrmelionimmaculatus* larvae from the central and northeastern United States. Geographic variation in body size and heterozygosity were surveyed for collected ant lion. This study was aimed to correlate the relationship between body size and heterozygosity with geographic variations which includes temperature, latitude, longitude, precipitation, elevation of land. Heterozygosity was measured by allozyme survey and protein profiling by gel electrophoeresis. Five different polymorphic loci was found and this was correlated with collected discrete antlion habitats. It was concluded that body size and

heterozygosity positively correlates with latitude and precipitation and maximum temperature.

Yoshida *et al.*,(2001) reported about the toxin present in antlion and its role in prey paralysis. They said that antlion lives by sucking the fluid content of its prey. In this study Endosymbiont *Enterobacter aerogenes* was isolated from saliva of antlion (M. bore) and cultured in laboratory conditions. This species was injected into German cockroaches (*Blattellagermanica*) which was resulted in rapid paralysis. Then they purified one of the insecticidal proteins from culture broth and subjected it to SDS-PAGE to analyse the protein responsible for prey paralysis. They observed a single band at a position corresponding to a relative molecular mass of about 63K which implies that the protein responsible for prey paralysis is GroEL homologue.Partial amino-acid sequencing of this toxin indicated that it was a GroEL homologue. They reported that the toxicity of the GroEL homologue towards insects is similar to the effect of a bacterial extracellular chaperonin on eukaryotic cells. Since insecticidal proteins are produced by *Enterobacter aerogenes* they concluded that there exist an symbiotic relationship between antlion and microbes.

Dunn*et al.*,(2005) investigated the role of microbes in antlion biology. They have performed 16s rRNA sequencing of tissues isolated from gut of antlion. *Myrmelionmobilis* was collected from state botanical garden of Georgia in Athens. They observed the gut physiology of antlion is discontinuous in nature. This implies there exist a microbial community inside the gut of antlion. In their research they subjected the antlion to ventral dissection using scalpel blades and DNA isolation .16s rRNA sequencing of the head ,gut tissue resulted in 222 sequences and reported that the microbiota of gut and head tissues belongs to the

family of α-proteobacteria (Enterobacteriaceae) and γ- proteobacteria (Wolbachia) respectively.

Dunn*et al.*,(2005) has also performed in situ hybridization of homegenized tissue and studied evolutionary relationships using phylip software package,Clustal W tools. He observed it is related to proteobacteria family.Enterobacteriacea has heat shock proteins which is a toxic protein.This proves that microbiota plays main role in prey paralysis of antlion.

Charu (2007) proposed that silk can be as a biomaterial.He reviewed about the mechanical properties of silk produced by silkword and its traditional uses in sutures. In his study he cultured silk based biomaterials both *in vivo* and *in vitro* condition. He observed formation of variety of tissues like bone, cartilage and ligament.

Bojana *et al.*,(2008) studied the pit building behavior of antlion in accordance with substrate (prey) vibrations. They have also recorded the sand tossing angle using a video recorder. They have collected antlions (*Euroleonnostras)* from surroundings of Maribor, Slovenia. The collected antlion larvae were kept in the laboratory at room temperature (22–261°C) in sieved sand. They observed the translocation, opening of jaws, sand tossing by antlion in the pit constructed by antlion by feeding them three different preys namely woodlice (*Trachelipusrathkei)* ,Ant Formica sp., firebug (*Pyrrhocorisapterus)* and mealworm beetle (*Tenebrio molitor)* .

Sanjay (2009) reported cytotoxicity exhibited by *Solanum nigrum* extract on VERO Cell lines and HeLa. He carried out MTT (Microculture tetrozolium) assay to test toxic effects of his extract. He prepared methanolic extract of *Solanum*

nigrum and observed IC50 value on Vero cell was 6.862 .He concluded that it has high toxic effects on HeLa cell lines compared to VERO cell lines.

It was reported that movement of a prey item evoked sand tossing of ant lion. Mirror Video Analysis for Windows is used to trace accuracy of sand tossing response according to vibrations created due to movement of prey. The sand-tossing angle was highly positively correlated with the prey angle. They proved sand tossing of antlion occurred frequently when prey was on the posterior sand surface than anterior surface and tossing of sand by antlion occurred in the direction of a prey item without seeing the prey.It was also noted that antlion translocate if the prey is in anterior surface. This study also confirms that antlion hunting behavior varies with prey.

Agung *et al.*,(2013) have demonstrated combined effects of antlion and bitter gourd extract for antidiabetic effect on insulin resistance rats. Rats were administered with insulin continuously for 15 days. This resulted in developing insulin resistance in rats which ultimately led to Type 2 diabetic condition (hyperinsulinemia). So the blood glucose level was increased in rats. They prepared aqueous antlion extract and ethanolic extract of bitter gourd by cold maceration and combined both for treating the insulin resistance rats.The combination of bitter gourd (60 mg/200 g BW) and ant lion larvae (50 mg/200 g BW) with a ratio of at the ratio of 50:50, 75:25 and 25:75 w/w percentages decreased the blood glucose levels significantly in this study while the standard drug glibenclamide shown comparatively less hypoglycemic activity in insulin resistance rats.

Afidatul *et al.*,(2017) analysed the presence of metmorphin in antlion by performing reverse phase High performance liquid chromatography.They have

prepared methanolic extracts of antlion at various concentrations and subjected them to chromatography. In this experiment standard parameters were set to achieve high resolution and accurate determination of metformin. Optimum wavelength (233nm) and flowrate (0.5 mL/min) were chosen for this study. Four gradient elutions were used to achieve good resolution of peaks. The retention times were calculated and compared with standard peaks. It was found that metmorphin content present in antlion extract was 0.58 mg/g under optimum conditions.

Narayan (2017) reviewed about the medicinal properties of antlion. In his review he said about two important chemical compounds isolated by Nakatani *et al.*,(2006).In her experiment she subjected antlion extract to reverse phase column chromatography,NMR studies and isolated two compounds namely, 4-hydroxyisoindolin-1-one and 2-(2-hydroxyethyl)-4- hydroxyisoindolin-1-one. These two compounds were found to have good medicinal properties.

Marc (2018) studied about the defensive mechanism of antlion to protect itself from its predators. Though antlion itself is a predator it also becomes a prey for bats. This study proved that antlions senses the ultrasonic waves produced by bat and protect themselves. They collected free flying adult antlion *Myrmeleon hyalinus* and exposed them to sound pulses at ultrasonic frequencies which is similar to echolocating bats and observed its response thresholds. They noted abdominal twitches, wing flicks, brief pauses in flight and flight cessation in antlions as a response to sound waves.This research concludes that antlions

responded best to ultrasound between 60 and 80 kHz and have shown response thresholds.

Miler (2018)reported that a trade-off exists between learning ability of larvae and their hunting efficiency. He proved that antlion larvae with greater asymmetry has good learning ability while the less asymmetric ones have high sensing power and relatively quick capture. They conducted training for the antlions collected from Bledowska Desert (Poland) in paper boxes half filled with sand. Training was given by sending vibrational cues 10 secs before prey delivery in order to associate cue with prey. Initial learning criterion was marked when antlion shows movement before the prey arrival but after the cue exposure in 2 consecutive tests. They observed that preparation for hunting was increased only in asymmetric antlions.From this research they concluded that asymmetric ones have high capability of learning than symmetric antlions.

Narulita et.al.,2019 investigated tissue repair effects of antlion extract by using mice as animal model. In this study 24 Male Balb/C mice weighing 20 to 30 g mice were induced with diabetes by injecting streptozotocin intra peritoneally. Untreated diabetes mice served as negative control group. Antlions collected from Mayang village were subjected to cold maceration for making antlion extract. Diabetes were allowed to develop for injected mice over 1 week in liver and kidney of test mice. Then the mice were divided into 4 groups and each group received antlion extract at varying concentrations. They were orally administered with antlion extract once for 5 consequent days. After 20 days histology specimens were prepared. Liver sections from untreated streptozotocin-induced diabetic mice

showed the most severe histological damages while the diabetes induced mice which received antlion extract at 10mg/kg shown less histological damage. This result was attributed to presence of sulphonylureas,metmorphin and chaperone protein present in antlion extract since sulphonyl urea is insulin inducer and metmorphin plays role in reducing blood glucose levels. Presence of sulphonyl urea was confirmed by the yellow band observed in thin layer chromatography of antlion extract.This study concluded that a dose of 10 mg/ kg was required for repairing liver damage, whereas only 5 mg/ kg was sufficient for repairing kidney damage. The presence of chaperone protein was found to responsible for the wound healing property of antlion.

The upcoming chapter describes the methods and materials used in our current study.

CHAPTER 3

MATERIALS AND METHODS

This chapter gives a brief explanation about the materials and methods used in our study.

3.1. INSECT COLLECTION:

The test insect, *Myrmelion formicarius* commonly known as antlion were collected from Prathyusha engineering college campus as shown in Fig 3.1. They were captured using spatula by digging the pits made by antlions. The insects were collected in beaker filled with loosy sand.

Fig 3.1 Antlion pits captured at prathyusha engineering college

Fig 3.2 Antlions collected from sandpits.

3.2.COLLECTION OF INSECT BLOOD EXTRACT:

The collected antlions were pretreated with ethanol (70%) and let to dry as shown in Fig 3.3. The insects were punctured at their ventral side and dorsal side with tweezers and syringe as shown in Fig. 3.4.This allowed the insect blood extract to flow out of the insect's body.

The insects were loaded into a 0.5ml Eppendorf tube. A cut was made in the 'X' pattern at the tip of the 0.5 ml Eppendorf tube. Then the 0.5 ml Eppendorf tube was placed inside the 1ml Eppendorf tube.The sample was centrifuged at 2500 rpm for 15 min as shown in Fig.3.5. The blood extract was collected in 1ml Eppendorf tube and the body remains in 0.5ml Eppendorf tube was discarded.

Fig 3.3 Insects soaked in 70% ethanol

Fig 3.4 Puncturing of the insect using tweezers and syringe.

Fig 3.5 Centrifugation-At Prathyusha Engineering College

The insect blood extract collected was preserved by addition of 50µl phenylthiourea/ascorbic acid as a demelanizing agent and stored at -20ºC.

3.3. THIN LAYER CHROMATOGRAPHY:

Thin layer chromatography was done on silica plate. The extract was prepared by mixing 500µl of crude insect blood extract with 1ml methanol as a solvent. Mobile phase was prepared using hexane, acetic acid and water in the ratio of 5:2:1(v/v) in a 50ml beaker. Two lines were drawn 0.5cm from the top and from the bottom of silica plate. In the bottom line a dot was marked as sample loading point. The extract was loaded using capillary tube for 5 times on sample loading point. The plate was placed inside the mobile phase chamber for 15 min with its top closed.

Fig 3.6 Visible light-NO visualization of compound from TLC

3.4. ANTIBACTERIAL ACTIVITY OF INSECT BLOOD EXTRACT:

3.4.1. WELL DIFFUSION METHOD:

Pure culture of the bacterial strains *E. coli*, *P. aeruginosa*, *S. aureus* were acquired from Billroth Hospitals, Aminjikarai. The pure colonies were isolated and inoculated into nutrient broth and incubated for 24 hrs.

Extract preparation:

The extract was prepared by mixing 0.5ml of crude insect blood extract with 1ml of Dimethyl sulphoxide (DMSO).

Nutrient agar medium was prepared for 150ml and plated on 3 petri plates for *E. coli, P.aeruginosa and S.aureus* respectively. The 3 bacterial culture were spread plated on respective labeled petri plates.4 wells were made using well puncher and wells were marked with different concentrations of extract as 20µl,40µl,60µl and 80µl respectively. A center well was made for gentamycin (20µl) which was used as control. The insect blood extract was loaded in the concentrations of 20,40,60 and 80µl on the respective wells of each plates. The plates were incubated for 24hrs.After incubation the plates were viewed for zone of inhibition (ZOI) and the corresponding was measured.

3.4.2. Statistical Analysis:

The statistical analysis of using t-Test (Hypothesis testing) was done and differences were considered significant when $P \leq 0.05$. The null hypothesis states that there is a significant among the species. Zone of inhibition values were taken as the necessary parameters and the t-test was performed in a pairwise manner. P value was calculated and compared with the level of significance. ANOVA table was created for comparing the effect of insect extract on all the 3 bacterial species.

The level of significance was chosen as 0.05. F value was calculated and compared with the F critical value.

3.5. CYTOTOXIC STUDY:

3.5.1. Cell Culture Maintenance:

VERO African green monkey kidney normal cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator.

3.5.2. Methodology:

The cytotoxicity effect of the sample was tested against VERO cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). The cells were seeded in 96-well microplates (1×10^6 cells/well) and incubated at 37°C for 48 h in 5% CO_2 incubator and allowed to grow to 70-80% confluence. Then the medium was replaced with fresh medium and the cells were treated with different concentrations (3.125, 6.25, 12.5, 25, 50 µg/mL) of sample and incubated for 24 hrs. The morphological changes of untreated (control) and the treated cells were observed under inverted microscope (20X magnification) after 24 h and photographed.

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100.

The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution. To each well (5 mg/mL in PBS) was added. The plates were then allowed to stand at 37ºC in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read spectrometrically at 570 nm. A graph was plotted using the cell viability (%) at Y-axis and concentration of the sample in X-axis. The concentration that inhibited 50% of cell growth referred to as IC_{50} value, was determined.

3.6. BIOCHEMICAL TESTS:

3.6.1. Quantification of Proteins:

Protein present in insect blood extract was quantitatively measured by Bradford assay (1975).

3.6.1.1 Sample Preparation:

0.1ml of insect extract was taken and added with 0.1ml of 40mM of tris HCL. The mixture was subjected to centrifugation at 10000 rpm for 20 min at 4^{o}C. The supernatant was collected and used as assay sample whereas the pellet was stored.

3.6.1.2 Bradford assay

The sample was taken in the volume of 0.2ml,0.4ml,0.6ml in test tube A, B, C respectively. Then 3ml of Bradford reagent was added to all test tubes. The colour change was observed from dark green to blue colour. Absorbance was taken at 595nm using Bradford reagent as blank and graph was plotted to determine unknown protein concentration.The standard graph for Bradford assay was shown in Fig 3.7.

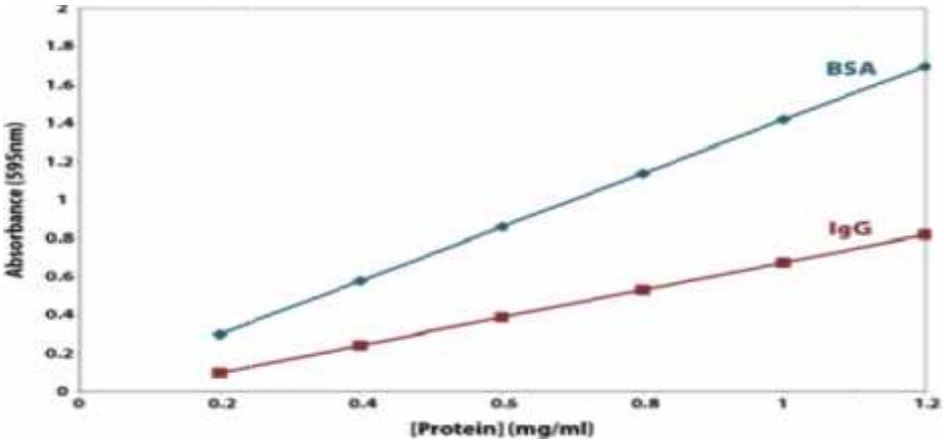

Fig.3.7 Standard graph for Bradford assay (Bradford *et al.*,1975).

3.6.2. Quantification of carbohydrates:

Carbohydrates present in insect blood extract was quantitatively measured by Miller DNSA method (1991).

3.6.2.1. DNS assay:

The sample was taken in the volume of 0.2ml,0.4ml,0.6ml in test tube A, B, C respectively. Each test solution was made to 1ml by adding distilled water. Then 1.5 ml of DNS and 1.5 ml of distilled water was added to all test tubes and the test tubes were subjected to heating in boiling water for 5 min and cooled immediately. Then absorbance was measured at 540nm. The corresponding standard graph was shown in Fig 3.8.

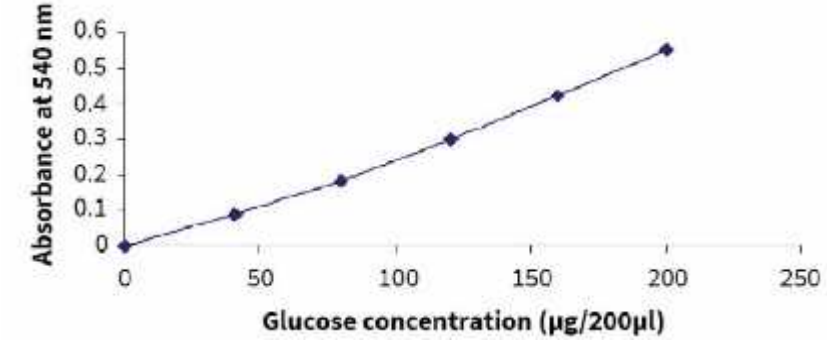

Fig.3.8 Standard graph for Miller's DNSA method (1991)

3.6.3. Quantification of lipids:

Lipids present insect blood extract was measured by Floch's method (1957).

3.6.3.2. Floch's assay:

The sample was taken in the volume of 0.2ml,0.4ml,0.6ml in test tube A, B, C respectively. Each test solution was made up to 1ml by adding distilled water. Then 0.5 ml of sulphuric acid and 1ml of vanillin reagent was added to all test tubes. The pink colour developed was measure at 530nm. The corresponding standard graph was shown in Fig.3.9.

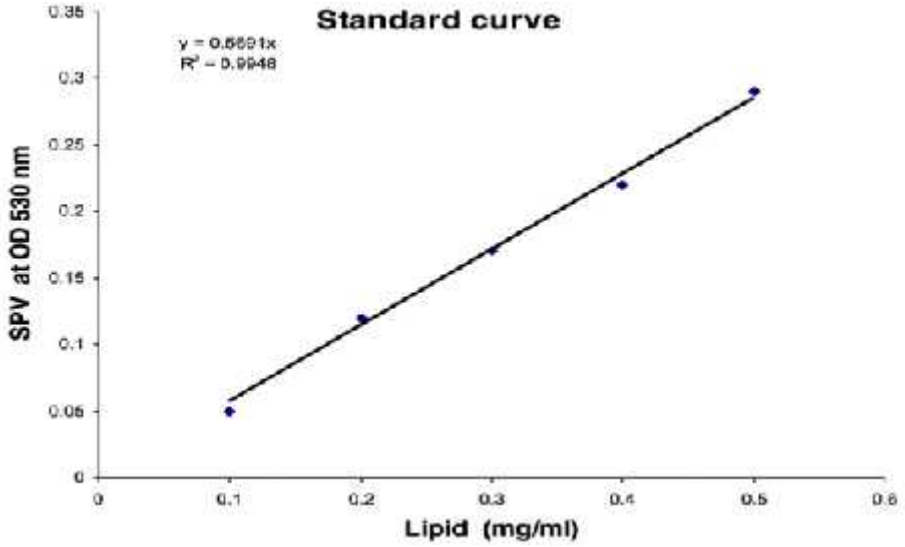

Fig.3.9 Standard graph for Floch's method (1957).

3.7.GC-MS Analysis:

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethyl polysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the

chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min−1; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 230 °C; ion source temperature 230 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST(National Institute of Standards and Technology) (2008) library.

3.8.SDS-PAGE

3.8.1. Casting the Gel:

Glass plates and spacers in gel casting apparatus were assembled. The components for the resolving gel as described in the Mini-Protean II protocol was mixed. The resolving gel mixture into the gel plates to a level 2 cm below the top of the shorter plate was poured. A layer of DDI H_2O over the top of the resolving gel to prevent meniscus formation in the resolving gel was paced. Resolving gel was allowed to stand for 30 min at room temperature. The DDI H_2O from top of the resolving gel was drained. Remaining DDI H_2O was drained away with a Kimwipe. Components for stacking gel were mixed. Stacking gel solution was poured into gel plates (on top of running gel). Then to the top of the spacers the comb was inserted.Gel was allowed to stand for at least 1 hour at room temperature for overnight at 4°C.

3.8.2. Solution Samples

A volume of protein solution (or 1 µl of standard) was placed into a microfuge tube, such that there was 5-10 µg of protein in the solution. Then an equal volume of 2x sample buffer (or 10 µl for standards) was added to it. The

tubes were incubated in boiling water for 5 min. Then the tubes were Centrifuged at 12,000 x g for 30 sec.

3.8.3. Running the Gel

Comb was removed and cast gel was assembled into Mini-Protean II apparatus. Then freshly prepared 1x running buffer (300 ml) was added to both chambers of the apparatus. Prepared sample was loaded into the wells of the gel. Then the gel was allowed to run at 100 V until the dye migrates into the running gel. Then the voltage was increased to 200 V and was allowed to run until the dye front reaches the bottom of the gel.

3.8.4. Staining & Destaining the Gel:

The gel was removed from the apparatus. Then spacers and glass plates were removed. The gel was placed into a small tray containing 20 ml staining solution and was allowed to stain for 30 min with gentle shaking. The staining solution was poured off. Then 5 ml destaining solution was added to the tray and was allowed to destain for 1 min with gentle shaking. The destaining solution was discarded. Then 30 ml of destaining solution was added and the gel was allowed to destain with gentle shaking until the gel was visibly destained (>2 hr). The destaining solution was poured off and discarded. The gel was rinsed with DDI H_2O for 5 min with gentle shaking. The gel was dried on the gel dryer at 60°C for 1 hour with a sheet of Whatmann filter paper below the gel and a piece of Saran wrap over the gel.

3.9. Wound Healing Activity:

3.9.1. Cell Culture Maintenance

3T3 mouse fibroblast normal cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic

phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator.

3.9.2. In-vitro scratch wound healing assay

3T3 cells were seeded in 6-well plates (8×10^5 cells/well) and was allowed to grow until reaching a confluence of 85-90%, in the optimum culture conditions. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed for 25 µg/mL of samples for 24-72 hrs at 37°C in a humidified atmosphere of 5% CO2. The cell grown in extract-free medium were used as control. Scratch wound closure was analyzed under the inverted microscope (Magnus INVI,Noida) equipped with a digital SLR camera, by acquiring digital images at different time 0th day(T0), 24hr (T1), 48hr (T2), 72hr (T3) (static imaging). In the static imaging modality, the closure of the scratch was quantified, as recently described (Felice *et al.*, 2015). Then Difference between the wound width at T0 and T1/T2/T3 was measured using the ImageJ processing software.

The following chapter reveals the results of the current study.

CHAPTER 4

RESULTS

This chapter gives a deep insight about the results observed in our study.

4.1. INSECT COLLECTION:

During insect collection on petri plate all antlions started backward motion in spiral form. Some antlions secreted body liquids and larger ones started hunting smaller antlions. As a result, smaller antlions were dead and the larger ones were considered as fittest amongst the colony. They were then isolated and acclimatized in a sand tray at room temperature as shown in Fig 4.1.

Fig 4.1 Acclimatization of antlion at room temperature in a sand tray.

Fig 4.2 Hunting of smaller ones by the larger ones.

4.2. COLLECTION OF INSECT BLOOD EXTRACT:

The acclimatized antions were taken from their pits in sand tray using spatula. They were ethanol washed and during ethanol wash antlions' death was

observed. Dead antlions were punctured and loaded in eppendorf tubes. The blood extract was collected after centrifugation as shown in Fig 4.3. The colour of insect extract was noted to be brown in colour. Insect debris were properly disposed. Collected insect extract was preserved by adding phenylthiourea and stored at 4°C.

Fig 4.3. Extraction of insect blood at Avanz bio Pvt Ltd.

4.3. THIN LAYER CHROMATOGRAPHY:

After the chromatographic run up to the marked end, the silica plate was taken using tweezers and was observed under TLC viewing chamber. The plate was viewed under short UV, long UV and visible light in the chamber. Different type of results were observed under 3 different conditions. There was no band appeared when silica plate was viewed under visible light and short UV. A red colour band was observed and noted when silica plate was viewed under long UV condition. The red band point was marked with pencil and the retention factor for the red band was calculated using the following formula

$$\text{Retention factor(Rf)} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by solvent}}$$

Retention factor = 1.3/3.8= 0.34.

The retention factor for the compound was found to be 0.34.The compound was found to be pheromones.

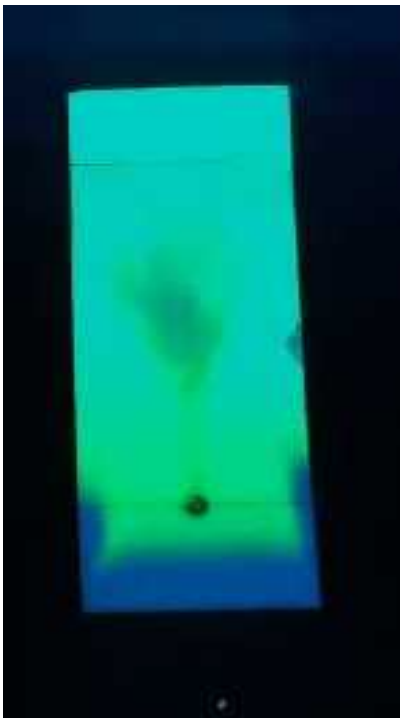

Fig 4.4(a).Observation under Short UV

Fig 4.4(b). Observation under Long UV

4.4. ANTIBACTERIAL ACTIVITY:

Antibacterial activity of insect extract was determined by performing well diffusion method (Sahalan *et al.*,2006). The antibacterial activity of extract was tested against 3 bacterial species namely *P.aeruginosa* , *E.coli* and *S.aureus.* In each agar plate 4 wells were created and insect extract was loaded at four different concentrations such as 20µl,40µl,60µl and 80µl respectively Gentamycin of 20µl was used as positive control and it was loaded in center well. After incubation zone of inhibition in 3 petridishes were observed and the diameter of zone was measured.

4.4.1ZOI in *P.aeruginosa*:

ZOI on *P.aeruginosa* was noted to be 0mm,1mm,2.5mm and 4mm at concentrations of 20µl,40µl,60µl and 80µl respectivelyas shown in Fig.4.5(a). The ZOI of positive control was found to be 10 mm. Minimum inhibitory concentration of insect extract was determined to be 40µg. Maximum inhibition was observed at 80µg and corresponding ZOI was 4 mm.

4.4.2 ZOI in *E. coli:*

In *E.coli* species there was no Zone of inhibition observed in 20µl, 40µl and 60µl respectively as shown in Fig.4.5(c). ZOI was observed at 80µl and corresponding diameter was 7mm. The ZOI of positive control was found to 14mm. Minimum inhibitory concentration of insect extract on *E.coli* was found to be 80µg.

4.4.3 ZOI in *S. aureus:*

ZOI on *S. aureus* was noted to be 0mm,0mm, 2mm and 8 mm at concentrations of 20µl,40µl,60µl and 80µl respectivelyas shown in Fig.4.5(b). The

ZOI of positive control was found to be 13.4 mm. Minimum inhibitory concentration of insect extract was determined to be 60µg. Maximum inhibition was observed at 80µg and corresponding ZOI was 8 mm.

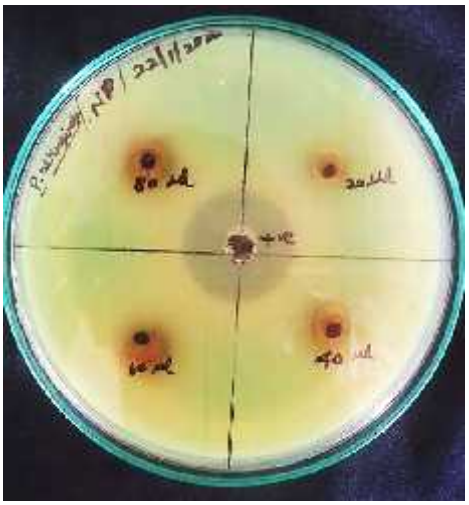

Fig.4.5(a) Antibacterial activity against *Pseudomonas aeruginosa*

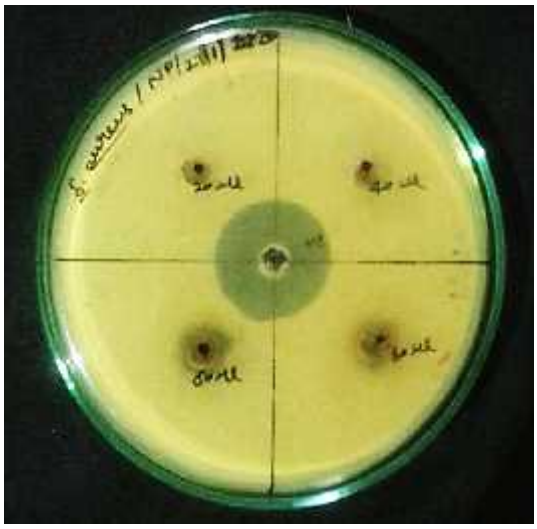

Fig.4.5(*b)*Antibacterial activity against *Staphylococcus aureus*

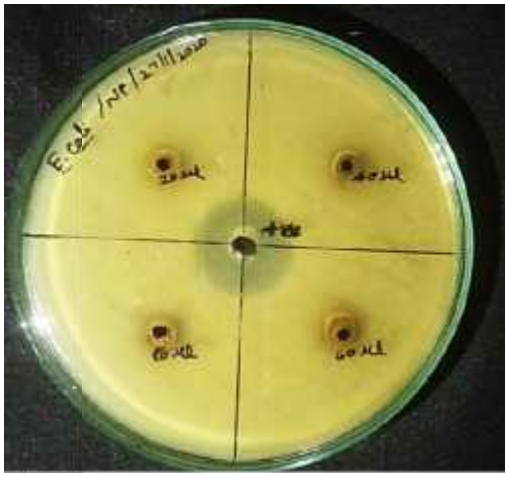

Fig.4.5 (c) Antibacterial activity against *E.coli*

Tabulation 4.1: ZONE OF INHIBITION:

Microbial strain	**20 µl**	**40 µl**	**60 µl**	**80 µl**	**Positive control**
P. aeruginosa	-	1 mm	2.5 mm	4 mm	10 mm
E. coli	-	-	-	7 mm	14 mm
S. aureus	-	-	2 mm	8 mm	13.4 mm

4.5. STATISTICAL ANALYSIS:

4.5.1. t-TEST:

The effectiveness of insect extract on 3 different species were compared by statistical analysis. The statistical analysis was done using t-Test (Hypothesis testing).The inhibition effect of *E.coli,P.aeruginosa,S.aureus* was compared in a pairwise manner and differences were considered significant when $P \leq 0.05$.

Tabulation 4.2:t-Test for *E.coli* and *S.aureus.*

T-test Mean value	**Zone diameter in *S.aureus* (mm)**	**Zone diameter in *E.coli*(mm)**
	0	0
	0	0
	2	0
	8	7
Mean zone diameter	2.5	1.75

P value was found to be 0.390479

Since P>0.05 it is not significant. Mean values of both zone of inhibition were calculated and compared. By comparing mean values it was found that insect extract has high inhibition effect for *S.aureus* than *E.coli*.

Tabulation 4.3: t-Test for *E.coli and P.aeruginosa*

T-test Mean value	**Zone diameter in *P.aeruginosa* (mm)**	**Zone diameter in *E.coli* (mm)**
	0	0
	1	0
	2.5	0
	4	7
Mean zone diameter	1.875	1.75

P value was found to be 0.389286.Since P>0.05 it is not significant. Mean values of both zone of inhibition were calculated and compared. By comparing mean values it was found that insect extract has high inhibition effect *P.aeruginosa* than *E.coli.*

Tabulation 4.4: t-Test for *S.aureus and P.aeruginosa:*

T-test Mean value	**Zone diameter in *P.aeruginosa* (mm)**	**Zone diameter in *S.aureus* (mm)**
	0	0
	1	0
	2.5	2
	4	8
Mean zone diameter	1.875	2.5

P value was found to be 0.475926.Since P>0.05 it is not significant. Mean values of both zone of inhibition were calculated and compared. By comparing mean values it was found that insect extract has high inhibition effect *S.aureus* than *P.aeruginosa*.

4.5.2.ANOVA CALCULATION:

The inhibition effect of blood extract on 3 different species was compared simultaneously using ANOVA table in MS excel. There exist a significant different inhibition effect on all species was considered as null hypothesis. Level of significance was chosen as 0.05%.$F_{observed}$ value was found to be F=0.0653. F critical value (F_{cric}) was obtained from ANOVA table at degrees of freedom(9,2). $F_{critical}$ was noted to be 4.26. It was observed that $F_{cric}>F_{observed}$.

4.6.CELL CYTOTOXICITY:

Cell cytotoxicity was checked on VERO African green monkey kidney normal cell line. Absorbance was measured in microplate reader at 570nm and the same was tabulated as shown in Table 4.5. The insect extract was found to be less toxic at 3.125 and 6.25 µg/mL concentration showing high cell viability of 99.16% and 98.17% respectively. At 50µg/mL concentration, insect extract damaged half of the cell population showing high toxicity and the corresponding cell viability was found to be 58.40%. The IC_{50} value of extract was found to be 60.28 µg/ml.

Tabulation 4.5: CYTOTOXIC EFFECTS OF INSECT EXTRACT ON VERO CELL LINE

Concentrations (µg/mL)	**Absorbance at 570 nm**		**Average**	**Cell Viability (%)**	**IC_{50} (µg/mL)**
	I	II			
Control	0.905	0.898	0.9015	100	60.28
3.125	0.891	0.897	0.894	99.16	
6.25	0.888	0.882	0.885	98.17	
12.5	0.853	0.847	0.85	94.30	
25	0.756	0.763	0.7595	84.25	
50	0.53	0.523	0.5265	58.40	

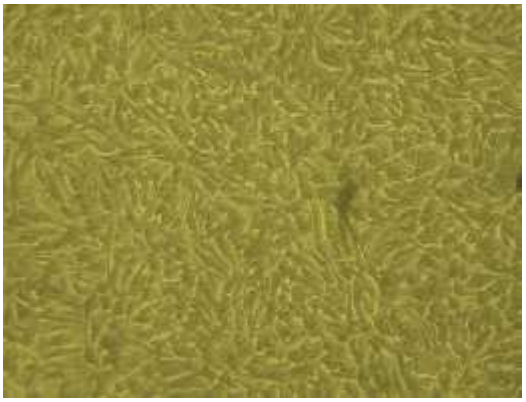

Fig.4.6.a.Control of VERO cell line

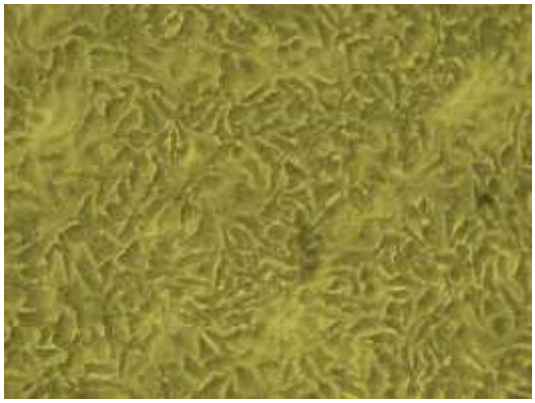

Fig.4.6.b.Cell disruption at 3.12µg/mL

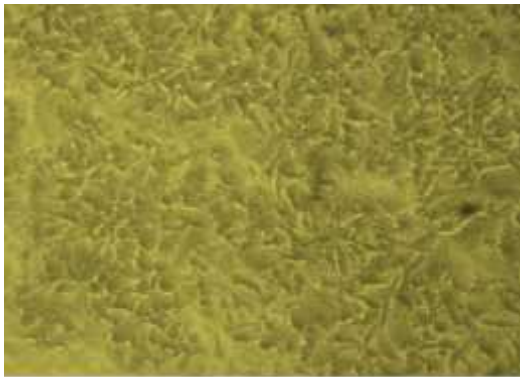

Fig.4.6.c.Cell disruption at 6.25 µg/mL

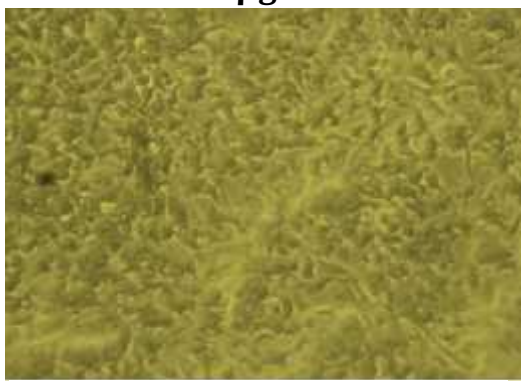

Fig.4.6.d.Cell disruption at 12.5µg/mL

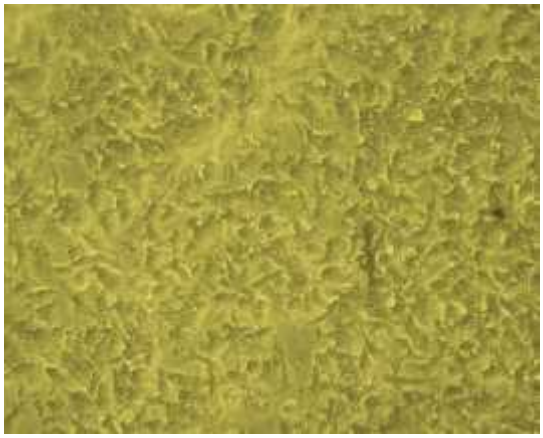

Fig.4.6.e.Cell disruption at 25 µg/mL

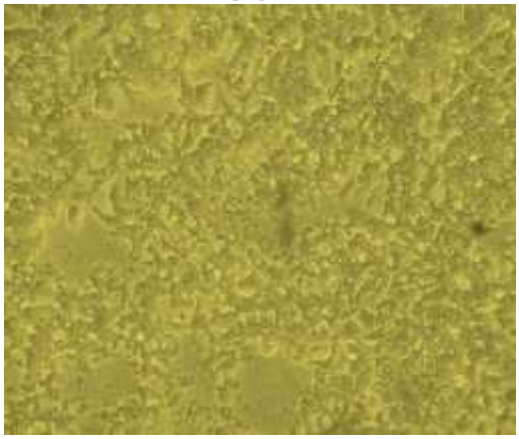

Fig.4.6.f.Cell disruption at 50µg/mL

Fig.4.6.Cytotoxicity on VERO cell lines (Post-insect blood extract administration)

4.7.BIOCHEMICAL TESTS:

4.7.1. Quantification of Proteins:

Protein quantification was carried out using Bradford method (1976). Blue colour change was observed after adding the reagent as shown in Fig.4.7(a). The absorbance of solution was measured at 540nm and tabulated as shown in table 4.6. The protein concentration was found to be high at 0.6mg/mL insect extract with concentration of 0.013 mg/mL.

Fig.4.7(a) Test tubes showing blue color change for Bradford assay (1976).

Table.4.6: Quantification of protein using Bradford assay (1976):

Concentration of Sample(mg/ml)	Absorbance (540 nm)	Protein Concentration (mg /ml)
0.2	1.875	0.010
0.4	2.030	0.012
0.6	2.073	0.013

4.7.2. Quantification of carbohydrates:

Carbohydrates present in insect blood extract was quantitatively measured by Miller's DNSA method(1991). Orange colour change was observed after adding the DNS reagent as shown in Fig.4.7 (b). The absorbance was measured at 540nm and tabulated as shown in table 4.7. The carbohydrate concentration was found to be high at 0.6mg/mL insect extract with concentration of 35 mg/mL.

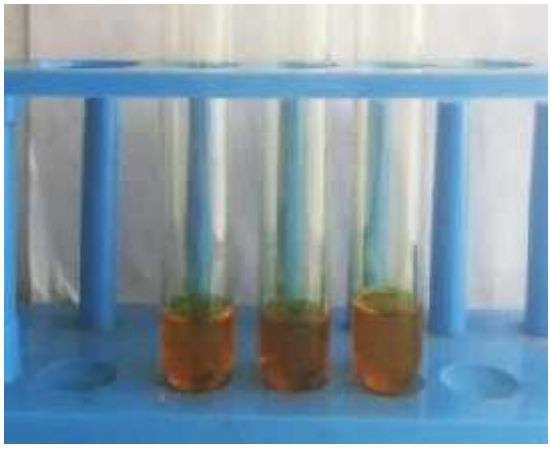

Fig.4.7(b)Test tubes showing orange colour change for Miller's assay (1991)

Table.4.7. Quantification of carbohydrates using Miller's assay (1991).

Concentration of Sample(mg/ml)	Absorbance (540 nm)	Carbohydrate concentration(mg/ml)
0.2	0.264	21
0.4	0.389	32
0.6	0.462	35

4.7.3. Quantification of lipids:

Lipids present insect blood extract is measured by Floch's method (1957). Pink colour change was observed as shown in Fig.4.7 (c). The absorbance was measured at 530nm using glycerol as standard and tabulated as shown in table 4.8.The lipid concentration was found to be high at 0.6 mg/mL insect extract with concentration of 0.33 mg/mL.

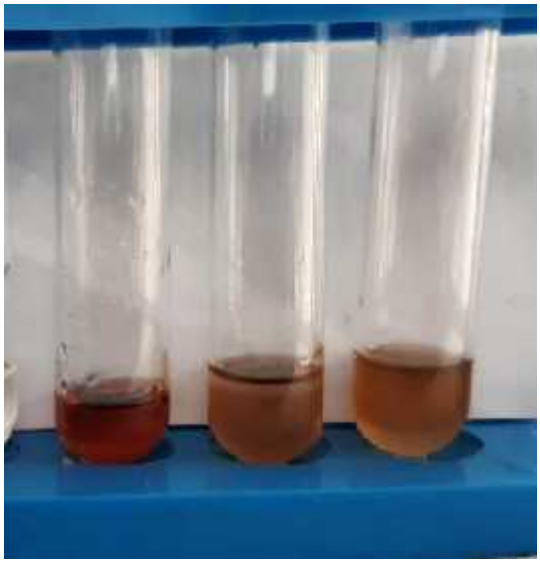

Fig.4.7(c)Test tubes showing pink colour change for Floch's assay (1957)

Table 4.8: Quantification of lipids using Floch's assay:

Concentration of Sample(mg/ml)	Absorbance at 530nm	Lipid concentration(mg/ml)
0.2ml	0.059	0.12
0.4ml	0.141	0.24
0.6ml	0.187	0.33

4.8.GAS CROMATOGRAPHY-MASS SPECTROSCOPY:

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethyl polysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

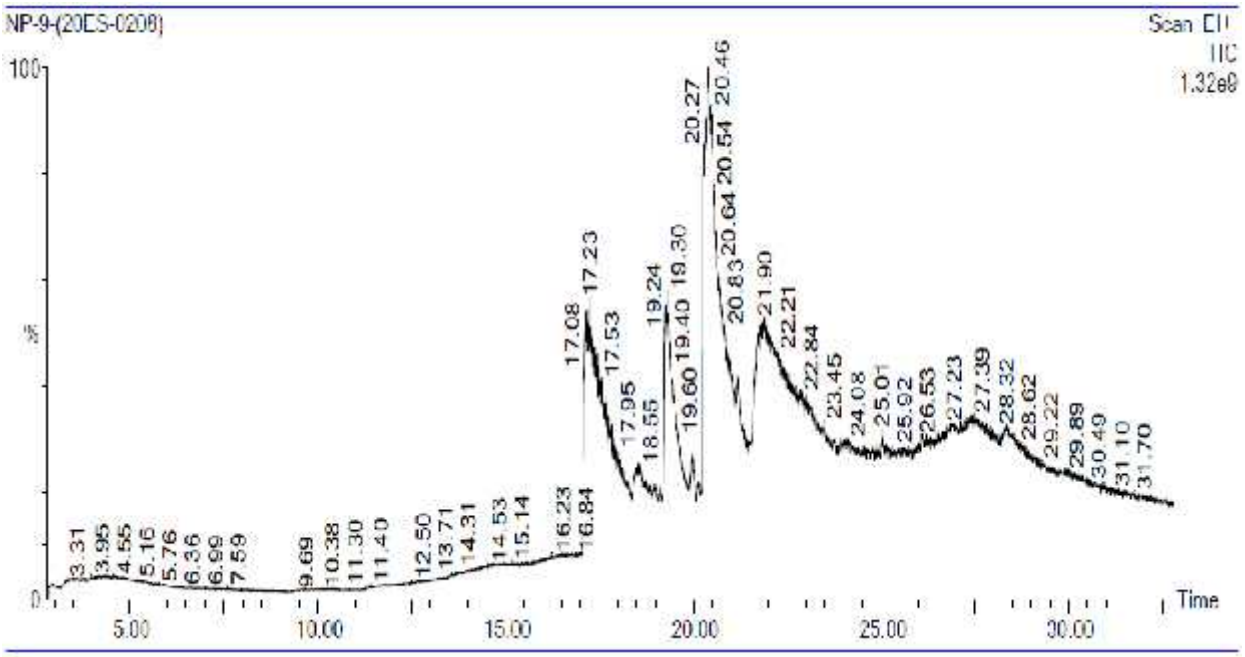

Fig 4.8(a) GC-MS chromatogram of insect extract.

TABLE 4.9: Retention time of various compounds from GC-MS analysis

#	RT	Scan	Height	Area	Area %	Norm %
1	17.234	2886	625,464,576	319,947,008.0	16.811	63.18
2	17.859	3011	248,838,592	63,622,964.0	3.343	12.56
3	18.520	3143	117,369,256	35,438,608.0	1.862	7.00
4	19.300	3299	513,227,712	130,949,784.0	6.880	25.86
5	20.400	3519	1,055,855,040	506,425,280.0	26.608	100.00
6	21.171	3673	274,088,512	58,441,564.0	3.071	11.54
7	21.901	3819	409,909,280	493,942,304.0	25.952	97.54
8	23.922	4223	75,162,312	37,985,128.0	1.996	7.50
9	26.073	4653	68,145,024	24,912,630.0	1.309	4.92
10	27.403	4919	131,269,192	158,614,176.0	8.334	31.32
11	28.324	5103	114,503,792	72,976,920.0	3.834	14.41

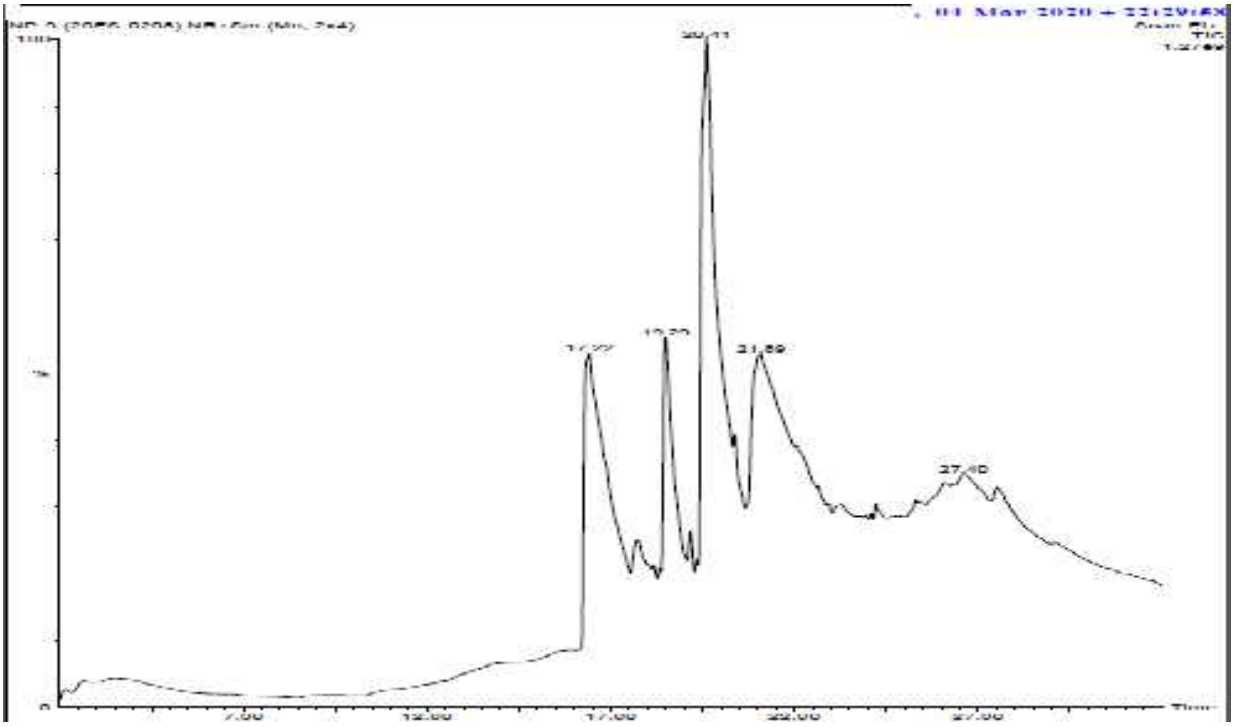

Fig 4.8(b) Retention time for predominant compounds by GC-MS analysis of insect blood extract.

On observing GC-MS chromatogram 4 major peaks have been occurred. The corresponding compounds of four major peaks were diethyl phthalate, hexadecanoic acid, ethyl 9-hexa decenoate, and 11-Tridecen-1-ol. Among these compounds ethyl 9-hexa decenoate was shown highest retention time of 20.46 which indicates this compound is predominantly present in insect blood extract.Yang (2019) reported retention time of ethyl 9-hexa decanoate was found to be 22.04.

4.9. SDS-PAGE:

Sodium dodecyl polyacrylamide gel electrophoresis was carried according to Sambrook and Russel (2006) method. Band observed in gel was compared with standard marker. The molecular weight of the band was found to be 63 kDa.

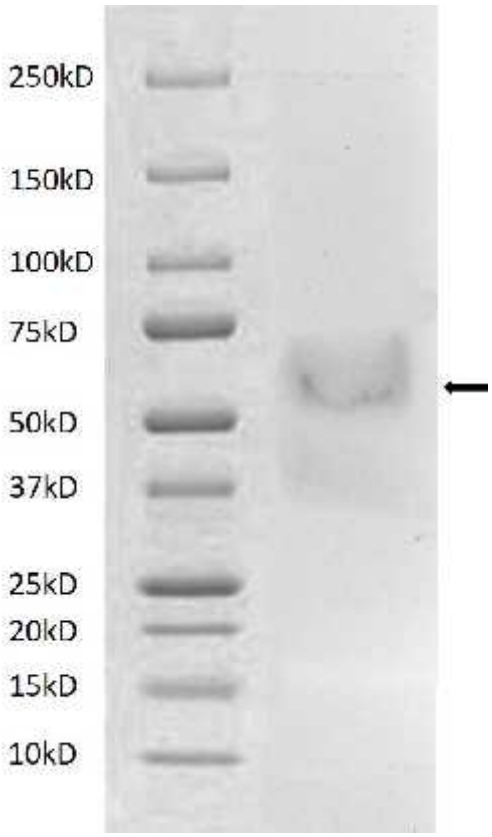

Fig.4.9 Band of Protein observed by SDS-PAGE and compared with standard marker (Precision Plus Protein™)

4.10. WOUND HEALING:

In the vitro wound healing scratch assay which was performed according to (Felice et.al.,2015) using 3T3 mouse fibroblast normal cell lines. Scratch wound closure was analyzed under the inverted microscope (Magnus INVI,Noida) equipped with a digital SLR camera. Digital images at different time 0^{th} day(T0), 24hr (T1), 48hr (T2), 72hr (T3) (static imaging) were acquired. In the static imaging modality, the closure of the scratch was quantified by measuring the difference between the wound width at T0 and T1/T2/T3, using the ImageJ processing software. The % confluence at scratched site was observed at 24hr ,48hr, and 72hr were 10.86%,39.56% and 70.43% respectively.

Fig.4.10.a.Control cells of 3T3 cell line

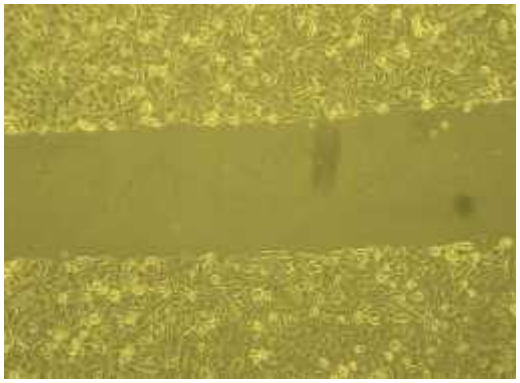

Fig.4.10.b. Wounded 3T3 cell line

Fig.4.10.c.Confluence observed after 24hrs (10.89%)

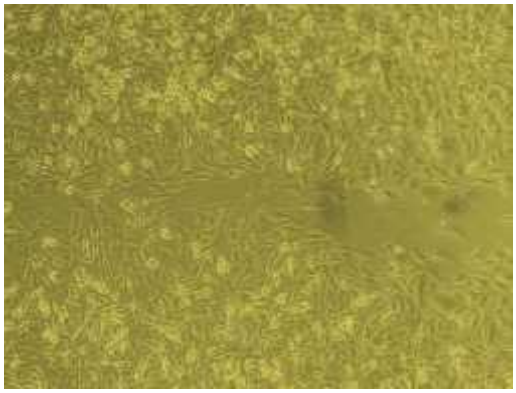

Fig.4.10.d.Confluence observed after 48hrs (39.56%)

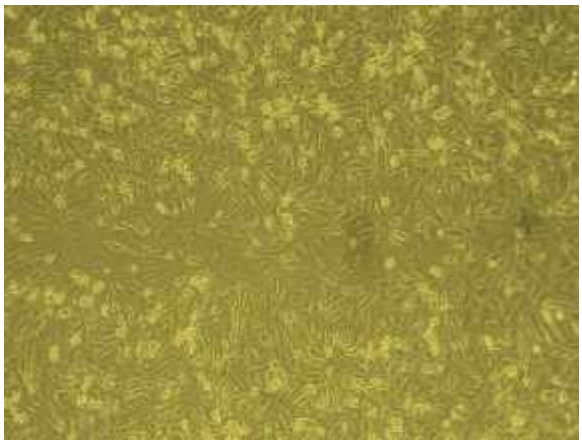

Fig.4.10.e.Confluence observed after 72hrs (70.43%)

Fig.4.10 Wound healing of 3T3 mouse fibroblast normal cell lines.

The following chapter gives a brief discussion about our study by comparing our results with results of previous study carried out by various research scholars.

CHAPTER 5

DISCUSSION

This chapter discusses the main results observed in our study.

Biomaterials revolutionized medical field by making untreatable illness treatable. Natural biomaterials are effective when compared to synthetic alternatives. It is believed that on course of evolution insects adapted themselves to survive the ever changing habitat over millions of years. Insects were traditionally prescribed as medicines by the tribal people. Piek (1986) reported that Chinese people believed antlions as medicines for treating malaria in China. In our present study, we focused on the therapeutic effects of antlion. Our research was aimed to prove wound healing properties and antibacterial properties which are present in the antlion.

The antlion insect collected was acclimatized by providing suitable environment similar to its natural arid habitat at the laboratory. Cannibalism was observed as a predatory behavior among collected antlions. Body secretions were observed from the acclimatized insects when it was triggered.

We adapted the method described by Aneta *et al.,* (2018) to collect antlion extract. Proteins, carbohydrates and lipids present in the antlion blood extract were quantified. Antlion extract exhibited effective wound healing properties.This effect could be attributed to the presence of chaperonin protein in blood extract which was verified by the band observed in SDS-PAGE. It was also found that this extract has good bactericidal effects. Cell cytotoxicity was studied in accordance with method described by Mosmann et.al., (1983). Data collected from antibacterial studies were analysed statistically using software program to perform

t-test and construction of ANOVA table. GCMS was done to determine predominant biological compounds present in insect extract.

From our study we believe that following inferences were allowable. The collected antlion extract colour was noted to be brown in colour. The solvent was standardized and thin layer chromatography was performed. Band was observed only under long condition. This implies that the compound is a UV compound and its corresponding retention factor was calculated to be 0.34.

Antibacterial activity was determined by the method proposed by Boyanova *et al.*,(2005).The effect was analysed against *E.coli*, *P.aeruginosa* and *S.aureus*. Zone of inhibition were measured and compared for the effect of inhibition on 3 different bacterial strains at various concentrations. Minimum inhibitory concentration was found to be 40µg,80µg,60µg on*P.aeruginosa* , *S.aureus,E.coli* respectively. Maximum inhibition effect was observed at 80µg for all the strains namely *P.aeruginosa* , *S.aureus, E.coli* and having a zone diameter 4mm,7mm,8mm respectively.

Similar to the work of Roza *et al.*,(2017) statistical analysis was performed for the antibacterial activity. t-Test and ANOVA was used to check statistical significance of results. The inhibition activity was compared in pairwise manner using t-Test. On observing and comparing the mean zone diameter of three bacterial strains it was evident that insect extract has high inhibition effect on *S.aureus* than on *E.coli* and *P.aeruginosa.*

ANOVA table was tabulated and F ratio value was found to be 0.0653. This value is less than $F_{critical}$ value obtained from the table (0.0653<4.26).From this it was evident that there exists a significant difference between the effects on species.

Toxic effects of insect blood extract was analyzed by the method devised by Mosmann *et al.*, (1983). From the result it was noted that cell viability was high at the concentration of 3.125 and 6.25µg/mL having cell viability of 99.16 and 98.17 % respectively. Increase in cell death was with proportional to concentration of extract. The half maximal inhibitory concentration (IC_{50}) was found to be 60.28µg/mL.

Biochemical test were performed to check the presence of proteins, carbohydrates and lipids according to Bradford's assay (1976), Miller's assay (1991) and Floch's assay (1957) respectively. They were quantified and it was observed that there was a gradual increase in chemical content with increase in concentration blood extract. The concentration of protein, carbohydrate and lipid were found to be high at 0.6mg/mL insect extract and there corresponding concentration were 0.013mg/mL, 35mg/mL, 33mg/mL respectively.

GCMS chromatogram of insect blood extract indicated four major compounds present in blood extract.The four compounds were diethyl phthalate, hexadecanoic acid, ethyl 9-hexa decenoate, and 11-Tridecen-1-ol. It was noted that among these compounds ethyl 9-hexa decenoate has highest retention time of 20.46 .Eller *et al.*,(1984) reported that ethyl 9-hexa decenoate was sex pheromone used by insects to attract opposite sex for mating. Hexadecanoic acid is a palmitic acid and a major food reserve in insects according to Dougherty *et al.*,(1997).

Diethyl phthalate was noted as a insect repellant for flies and mosquitos by Peterson *et al.*,(2001).From this it is evident that antlion extract can be used as pest repellant in agriculture. Mayer (2019) mentioned 11-Tridecen-1-ol as a pheromone of insects.

Insect extract was subjected to SDS-PAGE to seperate proteins based on molecular weight. A band corresponding to molecular weight of 63 kDa was observed. This is similar to the band observed in the work of Yoshida (2001). He reported that this protein is molecular chaperone which is a heat shock protein present in antlion. Kumar *et al.,* (2005) mentioned in his book that chaperone plays main role in cell regeneration. So, we focused to study the wound healing properties of antlion.

In vitro wound healing scratch assay was performed on 3T3 mouse fibroblast cell lines. The % confluence at scratched site was observed at 24hr,48hr, and 72hr were 10.86%,39.56% and 70.43% respectively. From the results we infer that antlion extract has good wound healing properties and it was due to the presence of chaperone protein. In our current study we conclude that antlion extract can be used as substitute in biomaterial and also as pest repellant.

Our study will be extended to analyse anticancer property of antlion extract in future.

CHAPTER 6

SUMMARY AND CONCLUSION

Traditional medicine which has been in practice since the ancient time may not give deep insights regarding the medicinal properties of newly discovered insect species. By the use of modern technology, the therapeutic value of such insects can be discovered. Such discoveries can pave way to the rise of new therapeutic methods derived from Traditional methods. The results may show some positive aspects which can turn out to be very useful in future studies.

In this study, we investigated the tissue repair effects and antibacterial properties of the insect blood extract of *Myrmeleon formicarius*. The thin layer chromatography done for the insect blood extract showed a compound which can only be observed under the Long UV. This compound was then known to have a retention factor 0.34.

The cytotoxicity study on VERO African green monkey kidney normal cell line indicates that the insect blood extract showed cell disruption in the cell line due to the action of haemocytes. The cell disruption increased over increasing the extract concentration, almost half of the cell population was disrupted at 50µg/ml concentration.

The results from experimental trials of In-vitro wound healing scratch assay on 3T3 mouse fibroblast normal cell line indicates that administration of the insect blood extract has significant wound healing property. The static images at the various time intervals reveals that the recovery % was about 70.43% at the end of 72 hrs.

The insect blood extract also exhibited antibacterial activity at different concentrations of insect blood extract. The inhibition was observed on the bacterial strains of *P.aeruginosa, E. coli and S. aureus.* In all the strains the maximum inhibition was observed at 80µl concentration of the insect blood extract. MIC was found to be 40µg,80µg,60µg on*P.aeruginosa*, *S.aureus,E.coli*respectively.

From the various biochemical tests, the level of proteins, carbohydrates and lipids were quantified and observed in the insect blood extract. In order to separate the various proteins SDS-PAGE analysis was carried out, from the results it was evident that the insect blood extract contains molecular chaperones at 63 kDa. To further investigate the lead compound present in the insect blood extract a GC-MS analysis was made. The results from the GC-MS revealed the presence of fatty acids and pheromones in the body of the insect. Further investigation on the compounds was initiated to identify the significance of every single entity present.

APPENDIX

1.Nutrient agar preparation (150ml)

Tryptone	-	1.5g
NaCl	-	1.5g
Yeast extract	-	0.75g
Agar-agar	-	2.75g

Adjust pH to 6.8 before autoclaving.

Autoclave at 121^{o}C for 15 min.

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