STUDIES ON ANTIMICROBIAL AND HAEMOLYTIC ACTIVITIES, PROTEIN PROFILE AND TRANSCRIPTOMES OF *AGELENOPSIS NAEVIA* WALCKENAER, 1842 (GRASS SPIDER)VENOM

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AUGUST, 2016

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DECLARATION

I declare that the work in this dissertation, entitled, "**Studies on antimicrobial and haemolytic activities, protein profile and transcriptomes of *Agelenopsis naevia Walckenaer*, 1842 (grass spider) venom"** was carried out by me in the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University Zaria under the supervision of Prof. I. S. Ndams and Dr. D. M. Shehu. All information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Jamila Ahmed ---------- ----------
Signature Date

CERTIFICATION

This dissertation, entitled STUDIES ON ANTIMICROBIAL AND HAEMOLYTIC ACTIVITIES, PROTEIN PROFILE AND TRANSCRIPTOMES OF *AGELENOPSIS NAEVIA* WALCKENAER, 1842 (GRASS SPIDER) VENOMby Jamila Ahmed meets the regulation governing the award of Master of Science in Biology of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research is dedicated to the entire family of Alhaji Ahmed Ma'aruf for their moral and financial support.

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ABSTRACT

The study was carried out to investigate the antimicrobial and haemolytic activities,profile protein component of *Agelenopsis naevia* venom as well as transcript coding for protein in *A. naevia* venom. Venom was collected from the spiders by micro-dissection after homogenization of the venom gland and the concentration of venom was determined in a nanodrop spectrophotometer. Antimicrobial activity of venom against*Bacilus subtilis, Candida albicans,* and *Salmonella typhi* was carried out by disc diffusion and well diffusion assay. Haemolytic activity was carried out using purified 1% human erythrocyte. Crude venom was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a precasted 4-20% gel. Gel was stained with commassie blue and destained to reveal the protein bands. Two dimension gel electrophoresis was also carried out in 13-cmimmobilized pH gradient(IPG) strip with a linear range of pH 3 to 10. Electrofocusing was carried out and the gel was rehydrated and subjected to a second dimension electrophoresis using a 15% SDS gel. The spots were visualized using commasie blue and detected using dynamic spot detector software. The messenger RNAs (mRNA) were isolated from venom gland and the first and second complimentary DNA (cDNA) strand were synthesized using ThermoScientific kit following manufacturer's instructions. The cDNA library was constructed and purified. Pair-end sequencing was carried out using illumina NextSeq 500. The transcriptomes derived were searched against public databases using BLASTx alogrithm. Multiple sequence alignment andphylogenetic analysis was achieved using ClustalX and MEGA softwares respectively. Antimicrobial activity data were subjected to ANOVA to compare means, where significant, Duncan multiple range test was used to separate means. Effective concentration of crude venom was calculated using probit analysis. The crude venom of *A. naevia* showed significant activity against *Bacillus subtilis*(23.5±0.5) when compared to the Controls and no activity against *Candida albicans* and *Salmonella typhi* on the disc diffusion assay. However, the venom did not show activity against the three micro-organisms using well diffusion assay. The crude venom also showed haemolytic activity on human erythrocytes with activity within the first 1hr (42.40%) for all the three concentrations (0.579mg/ml, 2.843mg/ml and 4.044mg/ml) used. Percentage haemolysis ranged between 42.40%-52.52% within 1-6hr using three different concentrations. The venom has an EC_{50} of 2.07mg/ml. Six bands were evident on the SDS-PAGE gel with molecular weights ranging from below 6-64kDa. The crude venom showed both protein (>10kDa) and peptide (<10kDa) resolutions. Over 300 spots were detected on the 2D gel with molecular weight ranging from below 14kDa to 94kDa. Twelve transcriptomes homologous to sequences in databases were identified. Transcript from *Agelenopsis naevia*venom gland clustered with that of *Stegodyphus mimosarum*. It was concluded that *A. naevia* venom have both antimicrobial and heamolytic activities. Its venom is made up of both proteins and peptides that are both cationic and anionicwhich could be harnessed as a potential bioinsecticide and antimicrobial agent.

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LIST OF ABBREVIATIONS

2D-gel:	Two dimension gel electrophoresis
ABS:	Absorbance
BLAST:	Basis local alignment search tool
cDNA:	Complementary deoxyribonucleic acid
dNTPs;	Deoxynucleoside triphosphate
DTT:	Dithiothreitol
IPG:	Immobilized pH gradient
MEGA:	Molecular evolutionary genetic analysis
mRNA:	Messenger ribonucleic acid
ORF:	Open reading frame
PBS:	Phosphate buffer saline
pI:	Isoelectric point
qPCR:	Real time polymerase chain reaction
RBC:	Red blood cells
RNA:	Ribonucleic acid
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl polyacrylamide gel electrophoresis

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Among various venomous animals, spidersare the mostsuccessful, the most geographically distributed and consumed the most diverse prey with an estimated 120,000 species (Agnarsson *et al*., 2013). Majority of spiders employ a fatal mixture of toxins that they use to subdue their preys, which are, often, larger than their size. Even though these creatures have fearsome reputation, only about a handful of these arthropodsare harmful to humans (Isbister and White, 2004; King, 2004). Nonetheless, the few medically important species prompted scientists, more than half a century ago to begin exploring the pharmacological complexity of spider venoms. Though various other animals, such as scorpions, snakes, bees and ants amongst others employ venom for prey capture as well as defence, spiders are the most successful. Most spiders feed on other arthropods while few species feed on other animals like small fish, reptiles, amphibians, birds, and mammals (Rash and Hodgson, 2002). Consequently, spider venoms contain a diverse amount of toxins which targets vertebrate and invertebrate species (Escoubas *et al*., 2004).

Spider body is divided into two main parts; the cephalothorax (prosoma) and the abdomen (ophistosoma) joined by a pedicel. Extending from the rear end of the prosoma are the chelicerae whilefrom the rear end of the ophistosoma are the spinnerets (Foelix, 2011). Anterior to the cheliceral basal segmentlies the venom glands. The well-developed venom glands are still found in the basal segment of the chelicerae in Mygalomorph spider which produce venom that is injected via the cheliceral fangs into the victim. However, in Araneomorphae, the venom glands have

become much longer and reach into the prosoma where they take up considerable proportion of their body part (Sutter and Stratton, 2013).

Despite the key role that venom gland plays in the survival of spiders, some spiders have significantly reduced or lost their venom glands. The Uloboridae evolved a unique technique to compact the living prey with extensive wrapping and by wetting the entire prey with a fluid containing digestive enzymes that kill insects quickly (Weng *et al*., 2006). Forster and Platnick, (1977) reported that the only two species of the genus *Holarchaea* have very long and slender cheliceral fangs without a venom gland opening on the cheliceral top. In spitting spiders (Sycotidae), the venom glands are highly modified to produce a gluey fibrous glycoprotein which is used to fix their prey to the ground from a distance (Kovoor, 1987).

Spider venom consists of components with different types of biological activities. On a conservative estimate of 100,000 species, there are 200 peptide toxins per venom with more than 10 million bioactive peptides in the spider venoms (King *et al*., 2008). Some spider toxins have been used pharmacologically to probe the structure and function of ion channels and receptors (Liang, 2004; Siemens *et al*., 2006; Bohlen *et al*., 2010). Roerig and Howse (1996) have reported on the effect of ω-agatoxin IVA, a toxin isolated from venom of American funnel web spider (*Agelenopsis aperta*), against thermal stimulation in the tail flick test, when co-administered with morphine intrathecally. Also, intrathecal injection of ω-agatoxin IVA (0.2nmol/kg) in mice was reported to decreased the licking time in both the early and late response phases in a dose-dependent manner in formalin test (Murakami *et. al.*, 2004). This peptide could be of particular benefit if used as an analgesic in patients tolerant or opioid-dependent, since this compound exhibits selectivity for the Ca^{2+}ion channels (Rajendra *et al*., 2004). Other spider toxins are being developed as environmentally-friendly

bioinsecticides (Windley *et al*., 2012; King and Hardy, 2013) andas potential drug leads (Escoubas and King, 2009; Saez *et al*., 2010). Proteomicsand peptidomics have become very powerful tools for exploring the complexity of the spider venom (Liao *et al*., 2007; Yuan *et al*., 2007; Liang, 2008; Tang *et al*., 2010). In particular, transcriptomics which is the study of complete collection of messenger RNA in a cell at a particular time (Jeremy *et al*., 2012) has been widely used to discover the molecular diversity of venom toxins from spider venom glands (Chen *et al*., 2008; Jiang *et al*., 2008;Diego-Garcia *et al*., 2010; Tang *et al*., 2010; Zhang *et al*., 2010). Despite various reports on spider toxin, only 916 peptide toxins from 85 spider species have been characterized and deposited in the database of ArachnoServer 2.0 (Herzig *et al*., 2011).

In most studies, RNA extracted from the venom glands of multiple spiders were used to generate the pooled cDNA library (Chen *et al*., 2008; Jiang *et al*., 2008;Gremski *et al*., 2010; Zhang *et al*., 2010). However, Escoubas *et al*.(1999) reported that sex was a substantial factor in the intraspecific variation of spider venom using reversed-phase chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In Korea-sourced spider,*Araneus ventricosus*, Avtox-1 and Avtox-2 toxins were identified from the cDNA library of the venom gland. However, these were not found from the venom gland cDNA library of the China-sourced spider *A. ventricosus* (Duan *et al*., 2013). Thus, spiders of the same species from different natural environment may show variation in venom composition.

Steiner *et al*.(1981) identified the first antibacterial peptides, cecropins, from the *Hyalophora cecropia* moth. This led to a search for antimicrobial agents in animals peptides presenting antibacterial activity were isolated throughout the entire animal

kingdom, ranging from different insect orders to amphibia, humans and other mammals (Boman, 1991; Haeberlia *et al*., 2000). Gomesin was the first peptide isolated from a spider exhibiting antimicrobial activities. This highly cationic peptide is composed of 18 amino acid residues including four cysteines that form two disulfide linkages (Mandard *et al*., 2002). Similarly, Kuhn-Nentwig *et al*. (2002) have investigated the antimicrobial activity of the venom of*Cupiennius salei*. They isolated several antimicrobial peptides from it, synthesized their analogs and reported that these peptides had lytic activity on human red blood cells and also insecticidal effect on *Drosophila melanogaster*. In another study, Corzo *et al*. (2002) reported five amphipathic peptides – called oxyopinins which exhibit antimicrobial, haemolytic and insecticidal activities which were isolated from the crude venom of the wolf spider (*Oxyopes kitabensis)*.

1.2 Biologyof *Agelenopsis naevia*

Agelenopsis naevia belongs to the Family Agelenidae which is a large family of Araneomorph spiders commonly referred to as grass spiders (Platnick, 2007). *A.naevia*share the same family with the hobo spider (*Tegenaria agrestis*) whose venom may or may not be harmful to human (Binford, 2001; Gaver-Wainwright *et al*., 2011). The number and arrangement of spider's eyes are often helpful in determining to which family it belongs. Agelenids (like spiders in many other families) have eight eyes and in most genera, these are arranged in two procurved transverse rows, i.e., two rows of four, each of which has lateral eyes set farther forward than the median eyes. Many Agelenids have light brown bodies with paired darker longitudinal stripes on the cephalothorax (and sometimes on the abdomen as well). They have long and conspicuous posterior spinnerets that extend well beyond the tip of the abdomen. The extension of spinnerets beyond the tip of the abdomen is a

distinguishing feature of Agelenids. Their legs are relatively long and they are able to run very quickly (Bennett, 1987).

Agelenids build a flat sheet of web that has a funnel-shaped retreat in the middle. They usually stay in their retreat awaiting prey to blunder by.Agelenid spiders typically trap their prey on the sheet-like portion of their webs, often with the aid of barrier of silk spun a few centimeters above the sheet. Flying insects are trapped to this sheet, where their legs become ensnared in the lattice works of silk. Within an eye blink, the grass spider shoots out of its funnel, grabs the insect, envenomates it and brings it back to the safety of the funnel where it feeds. The spider does not become caught in its own web because it places its tarsi in an almost vertical tip-toe poise that avoids entanglement as it runs across the sheet. In contrast, insects generally place their tarsi in a flat splayed fashion that is easily caught in silken. While some other spiders in the Agelenidae family produce stick silk to wrap insects in their sheet web, *Agelenopsis naevia* always use purely mechanical ensnarement (Bennett, 1987).

1.3Statement of Research Problem

Over, 10,000 arthropod species are currently considered to be pest organisms. They are estimated to contribute to the destruction of approximately 14% of the world's annual crop production and transmit many pathogens. Presently, arthropod pests of agriculture and health significance are controlled by chemical insecticides (Windley *et. al.*, 2012). However,insects are becoming more resistant to existing pesticides and other pesticides are deregistered due to established ecological and human health risks associated with their use. Thus, there is an urgent need for new insecticides that can be used in insect control such as bioinsecticides. Bioinsecticides are natural organisms or their metabolic products that can be employed to control insect pests (King and

Hardy, 2013).Various microorganisms have developed resistance against antibiotics (Ang *et al*., 2004). Widespread misuse of broad-spectrum antibiotics has led to the emergence of drug-resistant pathogens, both in medicine and in agriculture (Benli and Yigit, 2008). The first case of envenomation by *Agelenopsis aperta* was reported in Southern California (Vetter 1998). Victims showed several envenomation symptoms such as severe lesion, acute pain and itching among others at the site of bite.*Agelenopsis* bite in defense when they feel threatened without an option to escape (Whitman-Zai*et al*., 2015).The effect of the venom of *A. naevia* has not been characterized.

Spider venom peptides are produced in a combinatorial fashion, which leads to an estimated total of about1.5 million spider venom peptides (Escoubas *et al*., 2006). Consequently, spider venoms are a rich source of interesting novel compounds that have received increased attention from pharmacologists and biochemists recently which could be applied in pharmacological and agro-allied industries. However, during the past decades, only a few spider venoms have been studied in sufficient details, and therefore less than 0.01% of spider venom peptides have been identified so far (Escoubas and Rash, 2004; Tedford *et al*., 2004; Sollod *et al*., 2005).

1.4Justification

The potential medicinal uses of spider venom are largely due to their selectivity and affinity for ion channels and other receptors. This makes them suitable for studying cell function and for designing new therapeutics (King and Hardy, 2013). Antimicrobialpeptides are present in both vertebrates (Nicolas and Mor, 1995; Fathi, *et al*., 2010) and invertebrates (Liu*et al*., 2009; Umair*et al*., 2012) and have broad-spectrum activity against awide range of micro-organisms, including viruses, Gram-

positive and Gram-negative bacteria, protozoa, yeasts and fungi, and may also be haemolytic and cytotoxic to cancer cells (Larrick and Wright, 1996; Hancock and Lehrer, 1998).Gomesin was the first peptide isolated from a spider exhibiting antimicrobial activities. This highly cationic peptide is composed of 18 amino acid residues including four cysteines that form two disulfide linkages (Mandard *et al*., 2002). Since then, several antimicrobial peptides have been isolated from spider venom.Protein profiling, antimicrobial and haemolytic activity as well as transcriptomic studies provide information which contributes to the discovery of novel potential drugs and bioinsecticides from spider venom and a better understanding of evolutionary relationship of spider toxin (Liang, 2008; Liu *et al*., 2009;Jiang *et al.*, 2013). Due to their immense importance, spider venoms have recently garnered most attention from several research groups worldwide. Despite the uses of spiders' venom and their abundance, there is dearth of information on spiders' especially their venom in different geographical regions like the Tropics. This study serves as baseline information on *A. naevia* venom transcriptomes, proteins, antimicrobial and haemolytic activities which could be engineered towards pharmacological and/or agro-allied applications.

1.5Aim of Research

The aim of this research is tostudy theantimicrobial andhaemolytic activities, protein profile and transcriptomes coding for protein/peptides of*A. naevia* venom.

1.6Objectives of the Research

The specific objectives include to determine;

i) Antimicrobial activities of *A. naevia* venom on*Bacillussubtilis*, *Salmonella typhi* and *Candida albicans*.

ii) Haemolytic activity of *A. naevia* venom on human erythrocytes.

iii) Molecular weight of proteins/peptides of*A. naevia* venom.

iv) Transcriptomes encoding proteins/peptides of*A. naevia* venom.

v) Phylogenetic relationship of toxins in *A. naevia* venom.

1.7Research Questions

i) Does *A. naevia* venom have antimicrobial activity on *Bacillus subtilis, Salmonella typhi* and *Candida albicans*?

ii) What is the effect of *A. naevia*venom on human erythrocytes?

iii) What are the molecular weights of proteins/peptides found in *A. naevia* venom?

iv) What are the transcripts that codes for *A. naevia* venom proteins/peptides?

v) What is the phylogenetic relationship of toxins in *A. naevia* venom?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Spiders

Spiders are an ancient group with the oldest known fossil record dating 300 million years from the carboniferous period. They (Araneae) are an Order of Arachnida (Chelicerata) with more than 45,000 described species (World Spider Catalog, 2015) distributed among 3,958 genera and 114 families.Excluding insects, spiders are the most successful invertebrate occupying nearly allhabitats from the most arid deserts to the extreme Arctic, from seashores to alpine region owing to broad physiological adaptations and a diverse behavior (Platnick, 2012).Spiders and human have been sharing habitat since the existence of man. They elicit numerous and various responses from people such as fascination, wariness and terror in arachnophobias.Various cultures all over the world use spiders including modern popular culture from nursery rhymes to horror movies. Spiders can be grouped into two major clades: the Mygalomorphs (Orthognatha)containing tarantula-like spiders that are ground dwelling, having chelicera that projects forward from the cephalothorax and fangs move downwards, which are sit-and-wait predators that subdue passing prey by ambushing or grasping with fangs.The other clade is the Araneomorphs (Labidognatha) that are greatly diversified to occupy all types of above-ground habitats with chelicera position vertically and together with fangs move laterally, who evolved different typesof capture webs to intercept flying insects (Dimitrov *et al*., 2012). The significant ecological impact of spiders as predators in the control of insects and pests in natural and managed ecosystems (Schmitz *et al.*, 1997; Langellotto and Denno, 2004) is tightly linked to the use of lethal venom to

rapidlysubdue prey, which in combination with the production of silk webs, enables efficient prey capture involving minimum energetic cost. These adaptations allow spiders to catch prey as much as seven times their own body weight (Nyffeler and Knornschild, 2013), an amazing ratio for predators across all taxa. Majority of spiders feed mostly on insects and other arthropods with few feeding on birds, lizards, snakes, rodents etc. (Rash and Hodgson, 2002). Naturally, most spiders are not aggressive and are harmless to humans; nonetheless, several species of spiders bite humans in defence or accidentally. Spider venom attracts wide interest because of its biochemical and structural properties (Rash and Hodgson, 2002; Lee and MacKinnon, 2004), pharmacological applications (Escoubas *et al*., 2000) and the pathophysiological impact on humans following bites from species such as the redback, black widow and brown recluse spiders. Venom neurotoxins target specific types of insect ion channels and receptors and have wide applied potential as insecticides in pest control (King and Hardy, 2013).

2.2 Spider Venom

Venom can be broadly defined "as a secretion, produced in a specialized gland in one animal and delivered to a target animalthrough the infliction of a wound, which contains molecules that disrupt normal physiological or biochemical processes so as to facilitate feeding or defense by the producing animal"(Fry *et al*., 2009).Venoms have evolved on numerous occasions throughout the animal kingdom (Casewell*et al*., 2013). It is possible that the use of venom for prey capture in spiders was developed very early when their age as an evolutionary group and morphology over time are being considered (Bettini and Brignoli, 1978). Spiders use venom mainly for prey capture, however it has been suggested that some spiders do not use venom when subduing their prey. Other uses of spider venom include defence and might play a role

in pre-digestion (Khun-Nentwig *et al*., 2011). Generally spider venoms are colourless liquid that are easily soluble in water (Savel- Neimann, 1989) with a ρH ranging from neutral to alkaline with some being acidic as found in *Antrax robustus, Dugesiella hentzi* and *Eurypelma californicum* with ρHs of 4-5.5 (Savel- Neimann, 1989). When lyophilized, the spider venom range in colour from whitish to whitish-pink, yellow or grey (Weiner, 1961;Brucherl, 1971). For Araneomorphs, the dried venom is reportedly stable for years in vacuum with that of Mygalomorph being more hygroscopic and will liquefy in air. However when kept in vacuum, it may last a few months (Buchrel, 1971).

2.3 Components of Spider Venom

Similar to other venomous animals like snake, snail, lizard; spider venom is a multicomponent substance with both intra- and interspecific variation in venom components. Studies on the venom of *Atrax robustus*more than 600 peptides, however a similar study on a related species*Hadronyche versuta* revealed around a thousand peptides in the mass range 1–10 kDa (Escoubas *et al*., 2006). Many studies have been conducted to shed light on the evolutionary processes leading to this venom complexity (Sollod *et al*., 2005; Fry *et al*., 2009), especially considering the rarity of such extreme functional redundancy in most other systems. Some studies have linked the complexity of venoms to a molecular“armsrace”in which the processes of toxin mutation and countermutation of the toxin target result in strong directional selection (Kordis and Gubensek, 2000).

The major components of spider venoms are protein, polypeptide and polyamine neurotoxins, enzymes, nucleic acids, monoamines, inorganic salts and free amino acids (Jackson and Parks, 1989). On the basis of their toxic activity, these components

can be divided into neurotoxic and necrotic although these effects can be exhibited simultaneously (Vassilevski*et al.*, 2009). Spider venom components can be grouped into three major chemical classes: low molecular mass organic molecules (Mr <1 000 Da), polypeptides (Mr 3 000—10 000 Da) and high molecular mass proteins (Mr >10 000 Da) (Windley *et al*., 2012).

2.4 Neurotoxins

Spiders produce venom primarily to paralyze their prey; as such their venom contains a variety of toxins which affect the nervous systems. Acylpolyamines, proteins, and peptides are the most relevant and studied compounds representing the main toxic arsenal of spider venom (McCormick and Meinwald, 1993; Olivera *et al*., 1994). Two important characteristics of nervous tissue are the excitability of the cell membrane and the ability to transmit the electrical signal action potentialacross a synapse. As such, the majority of spider toxins are therefore targeted at neuronal receptors, neuronal ion channels or presynaptic membrane proteins involved in neurotransmitter release. On the basis of target action, neurotoxins can be grouped into the following:

i) Toxins affecting glutamateric transmission involving Calcium (Ca^{2+}), Sodium (Na^{+}), Potassium (K^{+}) and Chloride (Cl^{-}) channels.

ii) Toxins that stimulate neuro-transmitter release and

iii) Toxins blocking postsynaptic cholinergic receptors.

2.4.1 Proteins and Peptides

A large number of peptidesand polypeptides found in spider venom are highly characterized by several disulfide bridges and possess modified amino or carboxy termini or both. These function to increase their invivo stability(Norton and Pallaghy,

1998). A fold, presumably largelydictated by the gaps between the half-cystine residues andtheir pairing pattern, is crucial for the toxin's activities. Itappears to be an ideal compact globular scaffold for thepresentation of functional groups, generating a range of peptides acting on diverse biological targets. Manyion channel toxins have a consensual sequence known as the cystine knot. Cystine knot is reticulated by a triple-stranded, antiparallel-sheet stabilized by internal disulfide bridges. The molecular scaffold is highly stabilized by disulfide bridges with Cys_{III-VI} bridge going through the ring formed bridges Cys_{I-IV} and Cys_{II-V} to form the knot (Phallaghy *et al*., 1994). It is believed that these groups play a role in interactions between the toxin, the vestibule and the pore of the targeted ion channel. The robust nature of this motif makes it an excellent scaffold for the design and bioengineering of novel polypeptides showing enhanced activity against existing targets, or activity against new ones (Omecinsky *et al*.,1996; Norton and Pallaghy, 1998). Venom proteins include both high-molecular mass neurotoxins and enzymes. However, with the exception of highly necrotic venoms such as *Loxosceles*, in which enzymes have been well characterized, the presence of enzymes in spider venoms should be viewed with caution. This is because venom may be contaminated with digestive fluid during extraction via electro-milking. This may be the reason why published results are contradictory, and the presence of such enzymes was not detected by some authors. Proteases, hyaluronidases, sphingomyelinases, phospholipases and isomerases have been reported in spider venom (Schanbacher *et al.*, 1973). Hyaluronidases (45kDa) have been identified in various spiders such as *Loxosceles reclusa,L. laeta,A. robustus,Vitalius dubius*etc. (Wright *et al*.,1973; Geren *et al*., 1976; Sutherland, 1983; Sutti *et al*., 2014). Hyaluronidase substrate is mucopolysaccharide, hyaluronic acid which is the main constituent of the extracellular matrix (Kreil, 1995). Proteases have

also been reported in *A. robustus* (Kaire, 1963), *Pamphobetus roseus* (Mebs, 1972) etc. Two metalloproteasess (28kDa) from the venom of *Loxoceles intermedia* have been reported and characterized. They had both fibronectinolytic and fibrinogenolytic activity (Feitosa *et al.*, 1998). A peptide isomerase (29kDa) was also isolated from *A. aperta* which is assumed to help in envenomation process (Shikata *et al.*, 1995).

Several other peptides which are non-neurotoxic with novel activities have been described. A necrotoxic peptide of 6.7 kDa has been found to be the predominant toxic component of female *Dugesiella hentzi* (Lee *et al*., 1974). It was found that this toxin is capable of histological changes in the site of injection and also in the heart. The lycotoxin I and II isolated from *Lycosa carolinensis* have been found to cause complete loss of cell membrane potential and blockade of neurotransmitter in insect, haemolysis in rabbit, antimicrobial activity in both prokaryotes and eukaryotes etc. The ability to form pores in biological membranes can account for all the observed actions of lycotoxins I and II (Yan and Adams, 1998). Several authors have reported different sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) band from spider venom. Liu *et al*. (2009) reported proteins /peptides within the range of 4-31kDa from *Lycosa singoriensis* venom. Lopes *et al.*(2013) reported major band with molecular weight of 32-35kDa, in the venom of *Sicarius* and *Loxeceles.*

2.4.2 Acylpolyamines

Acylpolyamine are a class of neuroactive compounds, containing a polyamine structure that mainly contains an aromatic acyl endmoiety (indolic or phenolic) at its end, with molecular masses between 350 and 1000 Da (Khun-Nentwig *et al*., 2011).Acylpolyamines,are potent small molecules, often specific blockers of ion channels or receptors. Their targets are receptors that recognize excitatory amino

acids in the mammalian and insect nervous systems.Their blocking activity on cationic-selective ion channels is associated with acylpolyamines having positive charges on the free and secondary amine of their polyamine chains (Khun-Nentwig *et al*., 2011). They act as glutamate receptor blockers. L-Gultamate is the principal excitatory transmitter in the mammalian central nervous system (Greenamyre and Porter, 1994). Acylpolyamines can be grouped into two: the amino acid-containing acylpolyamines, and the non-amino acid-containing acylpolyamines.

The amino acidcontaining acylpolyamines, the first to be discovered, possess one, two, or three basic amino acids between the acyl end group and the polyamines chain (McCormick and Meinwald, 1993; Blagbrough *et al*., 1994). Various toxins (acylpolyamine) have been isolated from spider venom. These include Joro spider toxin (JSTX) isolated from *Nephila clavata*, Nephila spider toxins (NSTX) isolated from *Nephila maculata*, argiopine from *Argiope lobata*etc which were found to be closely related (Kawai *et al*., 1982, Usmanov *et al*., 1983; Grishin *et al*., 1986). Amino acid containing acylpolyamide isolated from Araneidae spiders have the acyl head group connected to the polyamine tail by one or two of the amino acids asparagine, ornithine and ω-methyllysine (McCormick and Meinwald, 1993; Schulz, 1997). Generally amino acid containing polyamine amide appear to cause an open channel, irreversible or slowly reversible, non-competitive block of quisqualate sensitive glutamate receptors at invertebrate neuromuscular junction (Jackson and Parks, 1989).

Non-amino acid containing acylpolyamine isolated from spiders include α-agatoxin isolated from*Agelenopsis aperta*which are selective and reversible, non-competitive antagonists of N-methyl-D-aspartate (NMDA) receptor in mammalian brain (Parks *et al*., 1991); toxins isolated from *Hololena curta* which blocked non-NMDA mediated

transmission in chick central nervous system. Others include those isolated from *Hebestatis theveniti, Aphonopelma chalcodes, Harpactirella sp.* (Rash and Hodgson, 2002).

2.5 Peptide Nomenclature

Peptide toxins are venom peptides with molecular mass less than 10kDa (King *et al*., 2008). Exponential increase in the number of spider toxin in recent time (Wood *et al.*, 2009) has led to the need to develop a rationale for peptide nomenclature to avoid confusion. Peptide name should include information about the biological origin of the peptide as well as its molecular target and/or biological function. The name should be in such a way that it facilitates rapid searching of electronic database for toxins from different venomous animals that act on the same or similar molecular target. It should also reflect evolutionary relationship.A peptide toxin's name should include the following:

a) Begin with a Greek letter or symboldenoting its biological activity or molecular target.

b) This should be followed by a subscript denoting channel/receptor subtype specificity. It should follow the international union of pharmacology recommendations for vertebrate receptor and ion channel subtypes.

c) Generic toxin name

d) Genus and species name in upper and lower case respectively for proper identification of organism from which the toxin was isolated. Where the initials of two species are similar, a second letter should be added to the species name.

e) A numeral to distinguish different toxins with similar pharmacology and

f) A letter to denote paralogs (King *et al*., 2008).

2.6 Sex and Geographical Location as Factors for Intra-specific Variation in Spider Venom

Spider venoms are heterogeneous in terms of their chemical composition and mode of action (Shulz, 1997). There are various evidences that points to inter-specific and intra-specific venom variability (Barbaro *et al.*, 1996; Escoubas *et al*., 1997, 1999). Variability in symptoms following envenomation by many spiders can be due to the site of envenomation, species of the spider, susceptibility of the victim and quality and/or quantity of the venom injected. Within species variation in venom composition can be due to geographical origin, size, age and sex of the spider (Rash and Hodgson, 2002). Evidence of variability in venom composition between geographically isolated populations and between sexes of *Tegenaria agrestis* was reported (Binford, 2001).In a study carried out by Duan *et al*. (2013), Avtox-1 and Avtox-2 were identified in the cDNA library of Korea-sourced spider *Araneus ventricosu*s which were absent in China-sourced *A. ventricosus*.

In *Latrodectus mactans* (black widow), the venom from the female spider was lethal to rats while that from males was significantly non-toxic (D' Amour *et al*., 1936). The same was reported for *Steatoda paykulliana*. Guinea pigs bitten by female *S. paykulliana* and not male developed symptoms analogous to those of lactrodectism (Maretic *et al*., 1964). Venom from female African tarantula (*Scodra griseipes*)bred in captivity was of higher toxicity in mice and contained three more electrophoretic bands than venom from male spider (Celerier *et al.,* 1993).

However, for Sydney funnel-web spider (*A. robustus*) and *M. bradleyi,* reverse is the case. Envenomation by male *A. robustus* was responsible for 14 human fatalities (Gray and Sutherland, 1978), while envenomation by the females seems to lack

efficient amount of the lethal neurotoxin, robustin (Sheumack *et al*., 1984). The female venom was also found to be 19 times less potent than that of the male in toxicity assays in new born mice. In *M. bradleyi,* the male contained a potent neurotoxin with activity in avian and mammalian nerve muscle preparations. This seems to be absent in female *M. bradleyi* venom (Rash *et al*., 2000). Male, *S. griseipes* have higher protein content than the female (Celerier *et al.,* 1993). High protein content in venom of male spider as compared to the female was also obtained in *Cheiracanthium furculatum* (Croucamp and Veale, 1999).

Despite various reports on venom variation based on sex, it has been reported that, the venom from both male and female *Hadronyche versutus* were found to be very similar at LD_{50} in new born mice and HPLC profile (Sheumack *et al*., 1984). In *L. cylindrata venom,* similarity in SDS-PAGE patterns was found in male and female with differences existing in biogenic amine content and adenosine receptor activity (Rash *et al*., 2000). Escoubas *et al*. (1999) carried a study to observe variability in venom from several tarantulas using HPLC and MALDI-TOF-MS and found both qualitative and quantitative differences in six of the eight species examined. This reflects the effect of sex as a substantial factor in intraspecific variation of spider venom. Kent *et al*.(1984), reported that although the venom composition of male and female spider of the same species may be the same, the relative abundance may vary which is tightly linked to venom toxicity.

2.7 Spider Peptides as Potential Bioinsecticides

Insects constitute major agricultural pest with some serving as vectors for many medically important disease while others constitute domestic and industrial nuisance. Due to perceived health concerns resulting from the use of chemical pesticide as well

as rapid development of resistance to these insecticides by insects, researches are going on at various level on the search for novel biopesticide,with emphasis on insect-specific neurotoxins from plants, fungi, bacteria, sea anemone and arachnid venoms (Nicholson, 2007).

During evolution, spiders have develop a combinatorial peptide library of neurotoxins, enzymes, antimicrobial and cytolytic peptides in their venom gland to diversify their toxin pool. This was postulated to be achieved through hypermutation in mature peptide region while conserving the basic disulphide framework (Sollod *et al*., 2005). These peptides are mostly translated as propeptides that undergo post-translational modification to yield mature toxin (Sollod *et al*., 2005).These neurotoxins target various insect preys with some being phyla-selective while others have broad spectrum of toxicity; although most are generalist predators (Nentwig, 1987; Possani *et al*., 1999). Frontali and Grasso (1964) were the first to note the existence of distinct toxic component in *Lactrodectus tredecimgattatus*venom with specific effects on mammals and insects. An insect specific toxin is a toxin that has the ability to modify insect, but not vertebrate ion channel (Tedford *et al*., 2001). The use of proteinaceous toxins that are highly toxic against insect crop pest as a means of augmenting biological control was first suggested in the early 1990's (Maeda *et al*., 1991; McCutchen *et al*., 1991; Stewart *et al*., 1991). The focus of these studies was to find peptide toxins that target insects but no other animals. Other's focused on finding an appropriate vector for delivering the insecticide to its site of action e.g. the insertion of gene encoding a toxic peptide or protein into the genome of a plant or baculovirus (McCutchen *et al*., 1991; Khan *et al*., 2006).

About 800 peptides have been described in the Arachnoserver 2.0 database. 136 of these peptides are insecticidal with 38 being insect-selective, 34 non selective and 64

are of unknown phyletic selectivity (Windley *et al*., 2012). Various insects of the Order Dictyoptera, Lepidoptera, Orthoptera, Spodoptera and Diptera have been used for analysis of insect toxicity (Quistat *et al*., 1991; Ali *et al*., 2001; Maggio and King, 2002; Manzoli-Palma *et al*., 2003; Castro *et al*., 2004; Rajendra *et al*., 2006). Insecticidal spider venom toxins have been found to target the Na_v channels, Ca_v channels, lipid bilayer, calcium-activated potassium channel (Kca), presynaptic nerve terminals and N-methyl-D-aspartate receptors. Most insecticidal toxins from arthropods seemto affect sodium ion channels (de Lima *et al*., 2002; Bosmans *et al*., 2005) and calcium ion channels (Wang *et al*., 2001; Liang, 2004). A few have been reported to affect potassium channels and other receptors (Maggio and King 2002; Tedford *et al*., 2004).

2.7.1Spider venom peptide targeting sodium(Na_V)channels

During evolution, toxins targeting Na_V channels are expressed in most families of araneomorph and mygalomorph. These spider toxins regulate neuronal excitability, resulting in paralysis and death of insects. Some spider venom toxins have high affinity forNa_V channels in insects (Table 2.1) and as such have potential to be developed as bioinsecticide (Windley *et al*., 2012).

Three neurotoxin binding sites (site-1, site-3 and site-4) which are called neurotoxin receptor site that binds to spider venom peptides have been identified in insects (Catterall *et al*., 2007; Nicholson *et al*., 2007; Stevens *et al*., 2011). Spider neurotoxins affecting Na_V channel can be grouped according to their target. Neurotoxins affecting Na_Vchannelsite-1 are referred to as pore blockers. µ-Theraphotoxin-Hhn2b, an abundant component of the *Haplopelma hainanum* crude venom (Liang *et al*., 1999) block insects channel with high affinity (Li *et al*., 2003). It

manifests 15-fold selectivity for *Drosophila* para channel ($DmNa_V1$) as compared with that of rat $Na_V1.2$, with no effect on $Na_V1.1$ and $Na_V1.4$-1.8 (Li *et al*., 2003). Another toxin in terms of structure and function is HWTX-iv from *Ornithoctonus huwena* venom (Xiao and Liang, 2003).

δ-Ctenitoxin-Pn1a toxin which was isolated from *Phoneutria nigriventer* venom targets the insect Na_V channel site-3. Peptides targeting this site are also known as gating modifiers of inactivation because they block or slow Na_V channel inactivation which lead to excitatory effect due to increased activity of Na_V channels (Cestele and Catterall 2002; Catterall *et al*., 2007). This toxin was found to have significant selectivity for Dipterans and Blattarians with no neurotoxic effects in Lepidopterans and Coleopterans (Figueiredo *et al*., 1995). It also had no effects on the mammalian $Na_V1.2$ and $Na_V1.4$ channels (de Lima *et al*., 2002). This toxin causes excitation resulting in immediate knockdown with trembling and uncoordinated movement (Figueiredo *et al*., 1995). µ-Hexatoxin-Mg1a from *Macrothele gigas* (Japanese funnel web), a toxin targeting site-3 Na_V channel, demonstrate high selectivity and affinity for insect Na_V channel resulting in flaccid paralysis of insect larva. When compared with effects on mice, it shows no symptoms (Corzo *et al*., 2003).

Other spider neurotoxins such as δ-amaurobitoxin (δ-AMATX-P11a to δ-AMATX-P11d) and µ-agatoxin-Aa1 (µ-AGTX-Aa1a to µ-AGTX-Aa1f) are reported to target insect Na_V channel site-4 (Adams *et al*., 1989; Quistad *et al*., 1991; Corzo *et al*., 2000; Kozlov *et al*., 2005). Neurotoxins targeting this site are referred to as modifiers of activation. This is because they alter the threshold for action potential generation (Windley *et al*., 2012). δ-amaurobitoxins isolated from the venom of *Pireneitega luctuosa* demonstrate high selectivity for insect Na_V channels with slow channel inactivation and without any significant shifts in the voltage dependence of channel

activation (Corzo *et al.*,2000). This activity was not found in mammalian $Na_V1.2$ channel at concentration up to 10µM (Ferrat *et al.*, 2005) and mice (Corzo *et al.*, 2000). µ--agatoxin-Aa1 toxins isolated from *Agelenopsis aperta* (Adams *et al.*, 1989; Skinner *et al.*, 1989) are insect selective causing convulsion paralysis in insects. These toxins demonstrate high potency in Dipterans (LD_{50} of 30-1380 pmol/g), moderately potent in Orthopterans (LD_{50} of 944-4875 pmol/g) and weakly active in Lepidopterans (LD_{50} of 6565-18258 pmol/g) (Adams *et al.*, 1989).

2.7.2 Spider venom peptide targeting calcium(Ca_V)channels

Calcium channels are present in many tissues throughout the body where they play a key role in the release of neurotransmitters, enzymatic activities and patterns of gene expression (Catterall *et al.*, 2007). Calcium channels are of three types: Ca_V1, Ca_V2 and Ca_V3. These channels in insects are divided into two broadfamilies based on their voltage-dependence of activation: low voltage activated (LVA) Ca_V and high voltage activated (HVA)Ca_V channels. HVA Ca_V channels are more sophisticated in structure as compared to LVA Ca_V channels which have simple structure comprising of only α_1 subunit (Catterall *et al.*, 2005; Bourinet and Zamponi, 2005). So far, no spider toxins have been found to target Ca_V3 channels. Structural differences existing between the Ca_V channels of insect and that of human as well as differences in their pharmacological sensitivities (Witcher *et al.*, 2001) makes calcium channels an ideal target for the development of bioinsecticide. Various toxins isolated from spiders, which were found to target Ca_V channels are as follows:

ω-Hexatoxin-Hv2a, a family of peptide neurotoxin (Table 2.1) isolated from *Hadronche versuta* displayed selectivity for blocking insect voltage gated calcium channels (Wang *et al.*,1999). These toxins have 10,000 fold preferences for insect

over vertebrate Ca_V channels(Wang *et al*., 2001). Another toxins ω-Hexatoxin-Hv1a isolated from Australian funnel web spiders has high affinity and specificity for insect Ca_V channels (Atkinson *et al*., 1996; Fletcher *et al*., 1997; Wang *et al*., 1999). Theses toxins were shown to have ED_{50} values in Orthoptera, Hemiptera, Dictyoptera, Diptera, Acarina and Lepidoptera (Atkinson *et al*., 1996;Fletcher *et al*., 1997; Bloomquist, 2003). However no effects were observed in vertebrate with concentration 10,000 fold higher (Atkinson *et al*., 1996, Khan *et al*., 2006; Chong *et al*., 2007). It appears that ω-Hexatoxin-Hv1a has high affinity for insects Ca_V1channels and moderate affinity for Ca_V2channels as suggested by various studies (Chong *et al*., 2007; Tedford *et al*., 2007). Further, transgenic expression of ω-Hexatoxin-Hv1a in tobacco plants results in protection from *Helicoverpa armigera* and *Spodoptera littoralis* larvae while topical application of recombinant thioredoxin-ω-Hexatoxin-Hv1a also demonstrate lethality to these caterpillar species (Khan *et al*., 2006). ω-theraphotoxin-Hh2a isolated from Chinese tarantula *Haplopelma schmidti* (Zhang *et al*., 2003) was found to induce a reversible paralysis when injected into locusts and cockroaches which had no effect when injected in mice at higher doses (Zhang *et al*., 2003).

2.7.3 Selection Critera for Bioinsecticide Leads

For a toxin to be considered as a bioinsecticide lead, various critera has to be considered. These are:

i) Potency on insect target

ii) Selectivity for insects

iii) Low molecular mass

iv) Target only a narrow range of pest species

v) Orally or contact active

vi) Sufficiently stable under field conditions and permit complete degradation to innocuous metabolite overtime to avoid adverse impacts on the environment.

Table 2.1: Spider Peptide Toxins suitable as Insecticidal Leads

Toxin Name	Source	Insect Target	Acute toxicity test species (Order †: Genus species)	ED50 or PD50 (pmol/ g)	LD50 (pmol/ g)	Paralogs/ orthologs
δ-CNTX-Pn1a	*Phoneutria nigriventer*	Na_V channel	B: *Periplaneta americana* D: *Musca domestica*	95 ‡ 36		2
Γ-CNTX-Pn1a	*Phoneutria nigriventer*	GluR	B: *Periplaneta americana* D: *Musca domestica* O: *Acheta domesticus*	48 ‡ 10 ‡ 29 ‡		0
κ-HXTX-Hv1c	*Hadronyche versuta*	BKCa channel	D*: Musca domestica* D: Musca domestica D: *Lucilia cuprina* L: *Heliothis virescens* L: *Spodoptera frugiperda* O: *Acheta domesticus* O: *Acheta domesticus*		91 319# 117# 3195# 3070# 167 1022#	6
μ-AGTX-Aa1d	*Agelenopsis aperta*	Na_V channel	D: *Musca domestica* L: *Manduca sexta*		30 9524	11
μ-DGTX-Dc1a	*Diguetia canities*	Na_V channel	L: *Heliothis virescens*	380		3
ω-HXTX-Hv1a	*Hadronyche versuta*	Ca_V channel	D: *Musca domestica* L: *Heliothis virescens* O*: Acheta domesticus*	 250#	77 89	27

B = Blattaria, D = Diptera, L = Lepidoptera, O = Orthoptera;

‡ Not ED50/PD50 values-Neurotoxic effects noted immediately after intrathoracic injection at this concentration;

#Recombinant toxin(Windley, 2012)

2.8 Spider Peptides as Potential Therapeutics

Due to susceptibility to proteolytic degradation *invivo* and limited penetration of intestinal mucosa, peptide are generally considered poor candidate for human therapeutics (Hamman *et al*., 2005; Nestor, 2009). The presence of an inhibitor cystine knot (Pallaghy *et al*., 1994; Norton and Pallaghy, 1998; King *et al*., 2002) in most spider toxins provides these peptides with extraordinary stability. As defined by most authors (Pallaghy *et al*., 1994; Norton and Pallaghy, 1998; King *et al*., 2002), the inhibitor cystine knot is an antiparallel β sheet, stabilized by cystine knot into hyperstable mini proteins making them chemically, biologically and thermally table. It also provides them with the ability to resist proteases lasting several days in human serum with half-lives in stimulated gastric fluid of >12 hours (Fu *et al*., 2002).

2.8.1 Spider venom toxins as antiarrhythmic drugs

Atrial fibrillation results from stretching of the atrial chamber which has been associated with activities of mechanosensitive channels (MSCs). MSCs, which are sometimes referred to as stretch activated channels, are found in all cells (Martinac and Kloda, 2003). M-TRTX-Gr1a, a spider toxin isolated from tarantula *Grammostola rosea* locks mechanosensitive channels. The activity of this toxin explains why it suppresses atrial fibrillation in dilated rabbit heart (Bode *et al*., 2001). Thus suggest MSC as a novel target for antiarrhythmic agents. Even though M-TRTX-Gr1a is unlikely to be a useful therapeutic agent since it does not interact with MSC directly (Bowman *et al*., 2007), it is likely to be a useful tool for determining MSCs potential as a therapeutic agent for the treatment of pathologies such as arrhythmic, spinal cord damage, muscular dystrophy and gliomas (Bowman *et al*., 2007). Another toxin isolated from *G. rosea*is K-TRTX-Gr2a (Oswald *et al*., 2002)

which is a low affinity locker of MSCs with a K_d of 6µM in rat astrocytes. However, this toxin potentially inhibits $K_V4.2$ and $K_V4.3$ channels with IC_{50} values of 34 and 71nM respectively (Oswald *et al*., 2002).

2.8.2 Erectile dysfunction treatment using spider toxins

Symptoms in humans who have been bitten by *Phoneutria nigriventer* in South America include priapism (a condition in which erect penis does not return to its flaccid state) among others. Toxin responsible for this effect, δ-ctenitoxin-Ph2a was isolated in 1992 (Cordeiro *et al*., 1992). Upon injection of this toxin intracerebroventricularly in mice, symptoms such as scratching, hypersalivation, lachrymation, sweating and agitation were visible including spastic paralysis of the anterior and posterior extremities and eventually death (Cordeiro *et al*., 1992). However, subcutaneous injection of δ-CNTX-Pn2a into rats induced erection. Similar erection was observed in rats with severely depressed erectile function when toxin was administered subcutaneously(Nunes *et al*., 2008). In mice a minimum dose of 0.006µg/kginjected directly into the corpus cavernosum causes erection with no collateral toxic effect. Erection was lost after 120-140min (Andrade *et al*., 2008). The potency of this toxin makes it suitable as a drug lead for treating erectile dysfunction.

2.8.3 Spider Toxins as an Antimicrobial Agent

About 40 membrane-acting antimicrobial peptides (MAMPs) have been isolated from four different families of Araneomorphs. Surprisingly, none have been isolated from the Megalomorphs. Various MAMPs have antimicrobial activities both in gram positive and gram negative bacteria with others affecting fungal pathogens such as *Candida albicans* (Yan and Adams, 1998). Some membrane acting antimicrobial peptides also have anti-trypanosomal activity (Khun-Nentwig *et al*., 2002). These

peptides differ in their mode of action as compared to inhibitor cysteine knot peptides. They are α-helical amphiphatic peptides that interacts with and disturb cell membrane thereby causing antimicrobial effects (Khun-Nentwig, 2003; Nomura and Corzo, 2006; Pukala *et al*., 2007). Due to their cytolytic activities, MAMPs might not be useful candidate in therapeutic (Vorontsova *et al*., 2010). Adao *et al*. (2008), in a study showed that this cytolytic activity can be minimized by truncation without significantly disrupting their antimicrobial activity. Gomesin is a potent antimicrobial peptide isolated from *Acanthoscurria gomesiana* (Rodrigues *et al*., 2008).

2.8.4 Antimalarial Spider Toxins

Due to resistance developed by *Plasmodium* speciesagainst antimalarial drugs, there is need to search for potential drug leads in treating malaria. Interestingly two of such leads have been isolated from the venom of Trinidad Chevron tarantula, *Psalmopoeus cambridgei*. These are U_1-TRTX-Pc1a and U_2-TRTX-Pc1a ICK peptides which are effective against intra-erythrocyte stage of *P. falciparum* (Staines *et al*., 2005). Though, these toxins do not demonstrate antimicrobial activity, they inhibit intra-erythrocyte development of *P. falciparum* with ED_{50} values of 1.1-1.6µM (Staines *et al*., 2005).

2.8.5 Spider Toxins in treating Cancer

Even though most spider toxin demonstrates anti-tumor potential, very few studies have been published using spider venom as anti-tumor agent (Heinen and Gorina da Veiga, 2011). Notably is a report by Gao *et al*. (2005) where they studied the effects of *Macrothele raven* venom on proliferation and cyto-toxicity on human cervical carcinoma cells (HeLa). At doses of 10, 20, 40 mg/l, the venom significantly decreased cell proliferation in HeLa cells in dose and time dependent manner. Cyto-

toxicity was significantly increased, arrest of cell cycle and activation of caspase-3 in treated cells leading to apoptosis. Using the same venom on human breast cancer cells, Gao *et al*. (2007)discovered that the venom affected cell viability in a dose and time dependent manner. At the same doses as above, cell death occurred both by necrosis and apoptosis. Another toxin isolated from *Pancratium littorale*, Pancratistatin (PST) was shown to have apoptotic effects on human neuroblastoma cell, human lymphoma and breast cancer (McLachlan*et al*., 2005; Kekre *et al*., 2005; Siedlakowski *et al*., 2008). This venom had no effect on normal cells as compared to other drugs employed in clinical treatment of cancer. Rodrigues *et al*. (2008) tested the effect of gomesin on melanoma breast cancer and colon cancinoma. Gomesin which was isolated from *Acanthoscurria gomesiana*displayed cytotoxic effect on these cancer cells as compared to the Control.

2.8.6 Spider Toxin as an Anticonvulsant

Various potential anticonvulsant toxins from spider venom have been reported. Several studies have demonstrated the anticonvulsant effects of polyamine toxin when injected in mice and rat. Himi *et al*., (1990) showed that jorotoxin injected intracrebroventricularlly in mice (4.7nmol/mouse) specifically antagonizes AMPA-induced convulsion but not NMDA or KA induced seizures with no behavioral toxicity in the effective doses. Further polyamine-containing fraction of the North American funnel web spider *Agelenopsis aperta* suppresses behavioral convulsions at 435nmol/animal (Jackson and Parks, 1990). The specific potential of polyamine toxins as drug candidates lies in their properties as selective inhibitors of Ca^{++} permeable glutamate receptors (Stromgaard and Mellor, 2004).

ω-AgatoxinIVA from *A. aperta* was reported to have potent anticonvulsant effect on intense sound stimulation at dose of 0.09µg/animal (Jackson and Scheideler, 1996). Anticonvulsant assays with venoms of *Scaptocosa raptorial* and *Parawixia bistriata* have been performed by Cairrao *et al*. (2002). It was reported that intracerebroventricular injections of denatured venom abolished rat tonic-clonic seizures induced by picrotoxin and bicuculline. It also inhibited GABA uptake in synaptosomes from rat cerebral cortices.

2.9 Potential Therapeutics from other Venomous Animals

Several antimicrobial and haemolytic activities have been reported from snakes, scorpion, wasp and Echinoderms. Umair *et al*. (2012) reported antimicrobial activity of the venom of *Heterometrus xanthopus.* The venom showed antimicrobial activity on *Bacillus subtilis, Salmonella typhimurium, Enterococcus faecalis* and *Pseudomonas aeruginosa* with the highest activity on *B. subtilis*. Fathi *et al*. (2010) reported antmicrobial activity of *Echis carinatus venom*on *Escherichia coli,* and *Staphlococcus aureus*. Jalaei *et al*. (2014) also reported antimicrobial activity of wasp venom (*Vespa orientalis*) on *B. subtilis, E. coli, S. aureus* and *Klebsiella pneumonia.* Several authors have reported more antimicrobial activity on gram positive bacteria as compared to gram negative bacteria which is related to their cell wall structure (Schwarz and Reiter, 2001: Singleton, 2004).

Furthermore, Lee *et al.* (2013) reported a rapid and a dose-dependent response curve of the haemolytic activity of *Acanthaster planci*, a venomous Echinoderm. Snake venom from *Bothrops lanceolatus* causes indirect haemolysis on sheep erythrocytes (Martins *et al*., 2009). Seyedian *et al*. (2014) also reported a dose dependent curve of haemolytic activity of venom from *Hemiscorpius lupterus* (scorpion).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of Site of Spider Collection

The study was conducted in Ahmadu Bello University Samaru, Zaria (11° 9'N and 7° 39'E)in Kaduna state, Nigeria. Zaria is located at an altitude of 199m above sea level withinthe Northern Guinea Savannah ecological zone of Nigeria (Google Earth, 2015). The wet season starts from May to Octoberwhile the dry cool season is between mid-October to February. This is followed by hot dry weather from March to April. Zaria has an annual rainfall of approximately 9.63-20.65mm/year with relative humidity that ranges from 63-80% during wet season and about 17-35% in dry season. Daily temperature ranges between 14^{O}C-32^{O}C during harmattan and from 19^{O}C -36^{O}C during hot season (Buvanendran *et al*., 1981).

3.2 Collectionof *Agelenopsis naevia*

Adults of *A. naevia* were collected within Ahmadu Bello University, Samaru,Zaria. Funnel webs of *A. naevia* were searched for in open lands and around houses within the main campus. Water was used to create disturbance which made the spiders came out of their funnel web. *Agelenopsis naevia* was caught by hand-picking using forceps into cleaned specimen bottles which were taken to the laboratory for identification and venom extraction. They were identified using morphological features by Whiteman Zai et al., (2015). A total of100 spiders were caught and used for this study (Plate I).



a



b

Plate I:*Agelenopsis naevia*, **a-** funnel web, **b**- Anaestatized Adult (Spinneret extending from the abdomen, a distinguishing feature of *Agelenopsis*)

3.3 Determination of Sex of*Agelenopsis naevia*

Agelenopsis naevia sex was determined by examining their reproductive organs and spinnerets. The males have extra spinnerets which they use to spin sperm web. The special silken glands (epiandrous glands), which are hardly noticeably, open with minute spinnerets in the area of epigynum, anteriorly of the epigastric furrow. These minute spinnerets are situated in 2-4 rows and have semicircular or triangular form (Marpels, 2008). The distal end of the male pedipalp is modified into a club structure made up of fixed and movable sclerites that are used to transfer sperm into the female seminal receptacles during mating (Plate II), this feature is absent in the females (Foelix, 2011).

3.4 Extraction of Venom

*Agelenopsis naevia*collected were anesthetized by freezing at −20°C for 5mins. The dissection area and equipment was cleaned with 1M NaOH solution to avoid contamination by RNases. A petri dish was placed on the microscope stage and filled with 1X saline sodium citrate buffer (pH 7.0). The anesthetized spider was placed under the microscope and the cephalothorax was separated from the abdomen. The cephalothorax was held under the dissecting microscope with forceps and the chelicera was positioned into field of view.The cuticle joining the carapace to the lateral aspects of thechelicerae was cut with forceps. The chelicerae weregrasped using the second pair of forceps, and gently tugged back and forth until the venom glands were pulled out. The glands were then separated from the chelicerae (Garb, 2014). The glands were then transferred into 500µl of cold 1X phosphate buffer saline (PBS) and homogenized to elute the venom. This was followed by centrifugation at 12000rpm at 4^{O}C(Nagaraju and Kemparaju, 2013). The supernatant was separated from the pellet and quantified for proteins using a NanoDrop 2000c

spectrophotometer (Thermo Scientific). Venom glands for RNA extraction were placed in RNAlater and stored at 4^{O}C for further use.



a



b



c

Plate II: Photomicrograph of *Agelenopsis naevia,* **a-** male pedipalp, **b-** femalepedipalp, **c-** venom gland

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3.5 Antimicrobial Assay of *Agelenopsis naevia* Venom

Two methods were employed to determine antimicrobial activity of *A. naevia* venom; the well diffusion and the disk diffusion methods. Three organisms where use for this study. Thesewere*Bacillus subtilis* (gram positive), *Salmonella typhi* (gram negative)and *Candida albicans* (fungi). Cultures of microorganisms grown for 24hr,from Department of Microbiology, Ahmadu Bello University Zaria,were used and diluted with sterile physiological saline solution to form 0.5 X 10^7 cfu/ml. 100µl of test organisms were inoculated on the surface of solid medium agar (potato dextrose agar for *C. albicans* and Muller Hinton agar for bacteria). 6mm diameter sterilized filter papers were placed on the surface of the medium and 10µl of venom was added to filter paper. PBS was used for negative Control. Three agar plates were punctured with 4mm diameter borer to create well in the plates. The wells were filled with 10µl crude venom and 10µl PBS as negative control.Multiple disk containing 30µg of ceftazidine, cefuroxime, gentamicin, cefixime, ofloxacine, augmentin and ciprofloxacine served as positive Controls for bacteria while econazole served as positive Control for*C. albicans*. The bacteria cultureswere incubated at 37^{O}C while *C. albicans* was incubated at room temperature for 12hr (Benli and Yigit, 2008). Following incubation, all plates were observed for inhibition zones. This was repeated once.

3.6 Haemolytic Assay of *Agelenopsis naevia* Venom

This was done according to the methods of Seyedian*et al*. (2014) with slight modification. 5ml of fresh blood samples was collected and transferred into EDTA bottle. This was subjected to centrifugation at 1500xg to separate serum from red blood cells (RBC). 0.5ml of the RBC was transferred into 1.5ml eppendurf tube and rinsed 3 times with 1ml 1X phosphate buffer saline. During each rinse, PBS was

added to blood and rocked for effective mixing. This was then followed by centrifugation at 3000xg for 5min. The supernatant was collected and discarded. 0.5ml of rinsed blood was now transferred into 49.5ml of distilled water to form 1% red blood cells. Twenty eppendorf tubes were filled with 1ml 1% RBC. 10µl of crude venom at three concentrations (4.04mg/ml, 0.58mg/ml and 0.138mg/ml) were added to 12 tubes containing RBC (4 tubes for each concentration). The remaining eight were used for positive and negative controls where 10µl of PBS (negative) and 10µl of 1% SDS were added to tubes. This was followed by incubation at room temperature with gentle agitation at intervals for 1, 2, 4, and 6hrs. After each time lapse a set will be centrifuged at 1500xg at $4^{O}C$ for 5mins. The supernatant was collected and its absorbance (ABS) at 490nm wavelength was taken using a UV spectrophotometer (JENWAY 6405UV/VIS). This experiment was repeated once. Percentage haemolysis was calculated using the following formula

$$\% \text{ Haemolysis} = \frac{\text{ABS of sample} - \text{ABS of PBS}}{\text{ABS of SDS} - \text{ABS of PBS}} \times 100$$

Where, ABS = Absorbance at 490nm wavelength
SDS = Sodium dodecyl sulphate
PBS = Phosphate buffer saline

3.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis(SDS-PAGE) Analysis of Crude Venom

This was carried out according to the methods of Nagaraju and Kemparaju, (2013) with slight modification. Precasted GenScript SDS-PAGE gel of (4-20%) was used. 10µl of venom sample was added to 10µl of loading dye. This was heated at $70^{O}C$ for 10mins in a water bath. The gel was placed in SDS-PAGE tank which has been filled with SDS running buffer (Tris-MOPS-SDS). 20µl of samples and 10µl of 1X Seeblue prestained standard were loaded in the wells. The electrophoresis tank was covered

and connected to the power supply appropriately and ran at 90V for 1½ hr until the dye front reached the end of the gel.

3.7.1 Staining and destaining

After electrophoresis, the gel wasfixed with 25% ethanol and 8% acetic acid according to Nagaraju and Kemparaju, (2013). This was followed bystaining with 0.1% Coomassie brilliant blue (R-250) for the detection of the protein bands and after which it was detained using distilled water. The protein bands on the gel were then captured using a digital camera (SONY)

3.8Two Dimension Gel Electrophoresis (2D gel)

The method of Vejayan *et al*. (2010) was adopted. A 13-cmimmobilized pH gradient(IPG) strip with a linear range of pH 3 to 10 was rehydrated overnight with 200µL of rehydration solution. The venom proteins were dissolved in 100µL of rehydration solution containing 8M urea, 2% CHAPS (w/v), 20 mM DTT (dithiothreitol), 0.5% IPG buffer (v/v), and 0.002% bromophenol blue (w/v) after which the protein samples were applied to the IPG strip. Electrofocusing was carried out at 30 kVh using an IPGphor at 20°C according to the manufacturer's instruction. Before the second dimension, the IPG strip was equilibrated in two steps: reduction buffer with 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), bromophenol blue and 1% DTT(w/v) for ten minutes under agitation; alkylation buffer with 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), bromophenol blue and 2.5% iodoacetamide (w/v) for ten additional minutes. The equilibrated strip was loaded and run on 15% polyacrylamide gel (m/v) (18 x 16 cm) using a vertical system with a programmable power control, initially at 90V for 40 minutes, followed by 250V for 1½hr. The two dimensional gel proteins were stained

with Coomassie brilliant blue and visualized and the image taken. Spots were detected with Dynamic software detector.

3.9Messenger RNA (mRNA) Isolation

Total RNA was isolated from venom gland using GeneJET^{TM} RNA purification kit following manufacturer's guide. MagJET mRNA enrichment kit was used to isolate mRNA from total RNA according to users' guide.

3.9.1 Complementary DNA (cDNA) synthesis and sequencing

The construction of cDNA library was accomplished according to the method of Luna-Ramírez*et al*. (2015) with slight modification. Isolated mRNA was subjected to polyA enrichment followed by fragmentation with fragment buffer treatment. The first strand was synthesized by random hexamer using mRNA as templates. Buffer, dNTPs, RNAase and DNA polymerase 1 were used to synthesize the second strand. This was followed by adenylation of the 3' ends, adapter ligation and DNA fragment enrichment. The double strand cDNAs were subjected to real time PCR (qPCR) quantification using KAPA SYBR fast qPCR kit (following user guide). Pair-end sequencing was carried out using illumina NextSeq 500.

3.9.2Sequence analysis

Adaptor fragments were removed from the raw reads to yieldthe clean read required for the analysis in FASTQ format. This was subjected to quality control analysis using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Andrews, 2010). De novo transcriptome assembly paired end reads was performed using Trinity RNA-seq software (http://trinityrnaseq.sourceforge.net/) (Grabher *et al*., 2011). Raw reads were mapped to the assembled contigsusing the Tophat suite

(http://tophat.cbcb.umd.edu/) on galaxy (Blankenberg, *et al*., 2010) online software package (http://usegalaxy.org). TopHat is a fast splice junction mapper for RNA-Seq reads. It uses Bowtie (ultra-high throughput short read aligner) to align RNA-Seq reads to mammalian-sized genomes then analyzes the mapping results to identify splice junctions between exons (Trapnell *et al*., 2012).

The resulting contigs were searched against public data base containing toxins from spiders (http://www.arachnoserver.org/spiderP.html)and NCBInon-redundant (nr) database using the BLASTx algorithm with the expect value (e-value) cutoff set to$<10^{-5}$ to determine similarity between sequences and to mine genes with homologous toxins or proteins.Nucleotide sequences weretranslated to obtain precursor peptides using ExPasy-Translate tool program (http://web.expasy.org/translate).The theoretical monoisotopic molecular mass of putative mature peptide was obtained using ProtParam (http://web.expasy.org/protparam). The signal peptide, the propeptide and the mature toxin was determined using SpiderP 1.0 program (http://www.arachnoserver.org/spiderP.html) (Herzig *et al*., 2011; Petersen *et al*., 2011).

3.9.3 Phylogenetic analysis of toxins

Multiple alignments were performed with CLUSTALW v2.0. Multiple alignment gap opening penalty and gap extension penalty was set at 3 and 1.8 respectively.The phylogenetic analysis of spider toxin families was conducted by the MEGA 6.0 software package. Maximum likelihood method was used running 1000 bootstrap replicates (Kumar *et al.,* 2004).

3.10Data Analysis

Descriptive statistic was used to report data from haemolytic activity of venom. Effective concentration (EC_{50}) was determined using probit analysis (Liu *et al*., 2009). Analysis of variance (ANOVA) was used to analyze data from antimicrobial activity of venom to compare means of inhibition zone ($P<0.05$). Where significant, Duncan multiple range test (DMRT) was use to separate the means and ranked using SPSS version 20.

CHAPTER FOUR

4.0 RESULTS

4.1 Antimicrobial Activity of *Agelenopsis naevia* Crude Venom

The results showedactivity of *Agelenopsis naevia*crude venom with significantly higher activity against *Bacillus subtilis* with 23.5±0.5mm zone of inhibition at 40µg using disc diffusion assay when compared with the controls (Table 4.1). The venom did not show activity on*Salmonella typhi* and *Candida albicans*with 0.00±0.0mm zone of inhibition (Table 4.1). The crude venom did not show activity on the three microorganisms in the well diffusion assay (Table 4.1).

4.2 Haemolytic Activity of *Agelenopsis naevia* Crude Venom

The crude venom disrupted human erythrocytes. The haemolysis increased with increase in time with rapid haemolysis in 1hr (42.40%) at 0.579mg/ml which proceeded slowly with time (Figure 1). Its effective concentration of 50% inhibition (EC_{50}) at 6hrwas 2.07mg/ml (Table 4.2).

4.3 Protein Profile of *Agelenopsis naevia* Venom

The protein profile of the venom afterSDS-PAGE gel electrophoresis revealed six bands of proteins with molecular weights ranging between6kDa-64kDa. Accordingly, the gel separated proteins whose molecular weights were greater than 10kDa and peptides less than 10kDa resolution. Similar banding patterns were observed for both male and female grass spider (Plate III). Further resolution, using Two Dimension (2D) gel, revealed over 300 spot and over 50 spots being detected using visual count (Plate IV) that vary between14kDa-94kDa and isoelectric points of between 3-10.

Table 4.1: Antimicrobial activity of *Agelenopsis naevia* crude venom

	Zone of inhibition(mm)					
	Bacillus subtilis		*Salmonella typhi*		*Candida albicans*	
Treatment (µg)	DA	WA	DA	WA	DA	WA
Venom (40)	23.5±0.5[a]	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Econazole (30)	Na		Na		20.0±1.0	
Ceftazidine (30)	21.5±0.5[b]		19.0±1.0		Na	
Cefuroxime (30)	13.5±0.5[e]		13.0±1.0		Na	
Gentamicine (30)	15.5±0.5[de]		15.5±0.5		Na	
Cefixime (30)	13.5±0.5[e]		12.5±0.5		Na	
Ofloxacine (30)	16.5±0.5[d]		14.5±0.5		Na	
Augmentin (30)	18.0±0.0[c]		19.0±1.0		Na	
Ciprofloxacine (30)	20.5±0.5[bc]		14.5±0.5		Na	
PBS (Control)	0.0±0.0		0.0±0.0		0.0±0.0	

Means with different superscript within column differ significantly (P<0.05), Na-not applicable, WA- well assay, DA- disc assay

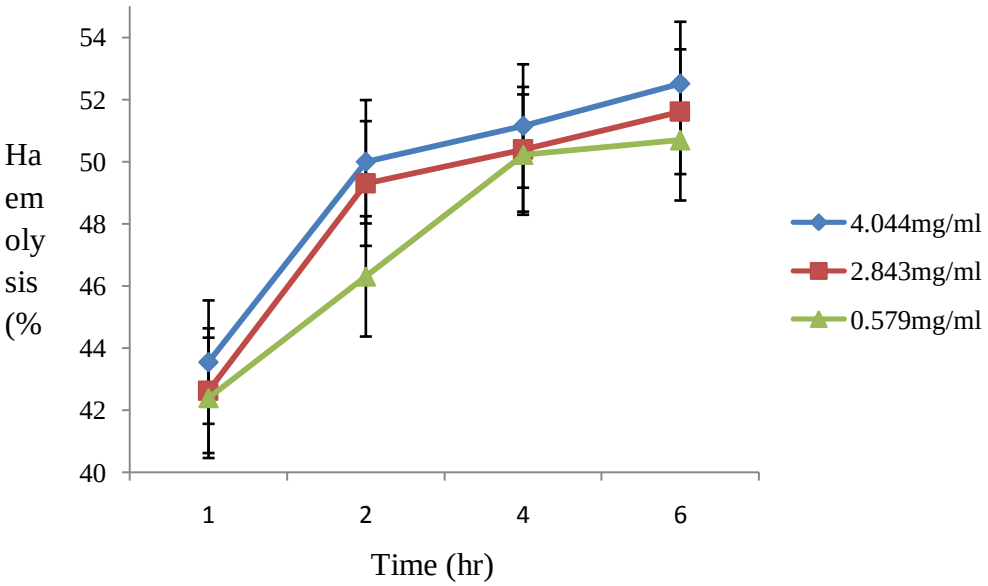


Figure 1: Haemolytic activity of *Agelenopsis naevia* crude venom

Table 4.2: Determination of Effective Concentration (EC_{50}) of crude venom

Concentration (mg/ml)	Haemolysis (%)	Log of concentration	Probit	EC_{50}(mg/ml) (y=0.05x+5.0397) R^2=0.7968
0.579	50.69	-0.23	5.03	2.07
2.843	51.61	0.50	5.05	
4.044	52.52	0.60	5.08	

KDa Marker Female Male Female

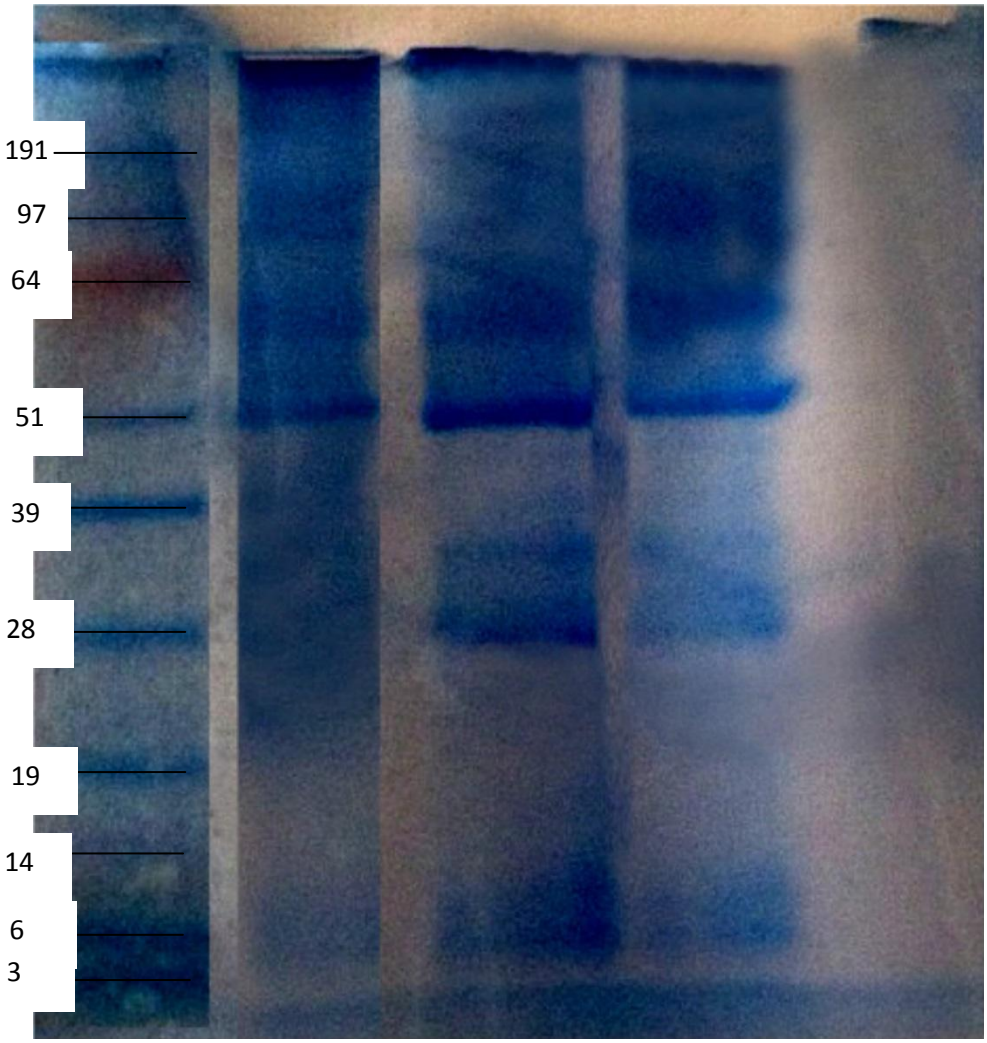


Plate III: Sodium dodecyl sulphate polyacrylamide gel electrophoresis electrophoregram of *A. naevia* crude venom

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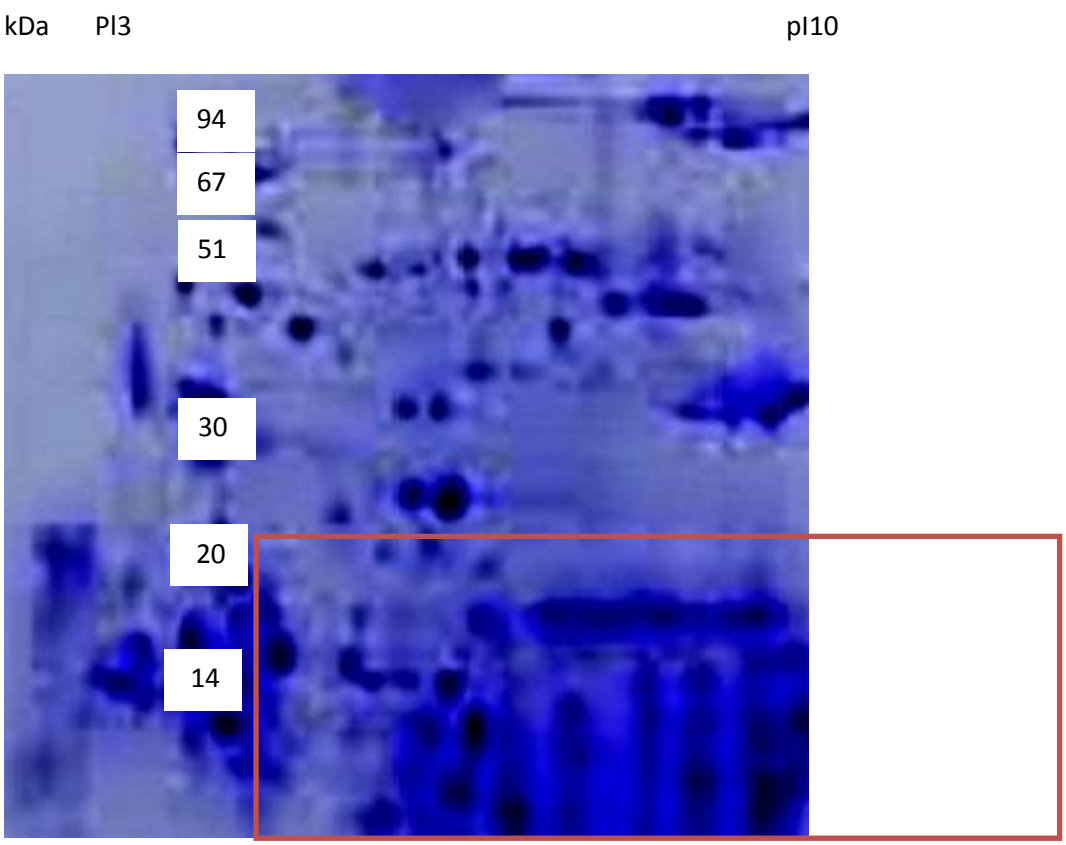


Plate IV: Two dimensional gel electrophoresis of crude *Agelenopsisnaevia* venom

The highlighted region is the expected region of resolution for protein/peptides whose transcripts were identified in this study.

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4.4 Transcriptomes of *Agelenopsis naevia* Venom Gland

11167123 paired end reads were generated a nucleotide length that ranged between35-76. Average percentage GC content was 44.5%. 90.1% right reads and 90.8% left reads were mapped to assembly transcriptomes with 35.3% right reads and 35.4% left read having multiple alignment (Table 4.3). Twelve putative protein/peptides which include toxins and enzymes were identified from assembled transcriptomes. Their open reading frame (ORF) sequences, theoretical molecular weight (MW) and isoelectric point are (pI) as presented in Table 4.4. While none of the sequences has propeptide four proteins/peptide sequences had signal peptides (Table 4.4).

4.5 Phylogenetic Relationship of *Agelenopsis naevia* venom toxins

Agelenopsis naevia venom gland transcript showed homology with those from other spiders, scorpions, tick, insect, carb and nematode. Each sequence clustered with their respective mined sequences to form distinct clades. *Agelenopsis naevia*transcript clustered with *Stegodyphus mimosarum* to form sister groupings for metalloproteases, calmodulin, cystatin, tachylectin and acetylcholinesterase with 99%, 65%, 68%, 42% and 73% node reliability, respectively (Figure 2).

Table 4.3: Mapped Reads of *Agelenopsis naevia* venom transcriptomes

Measure	Right Reads	Left Reads
Input	11167123	11167123
Mapped	10063923	10144990
Percentage mapped	90.1	90.8
Multiple alignment	3551626	3593126
Percentage multiple alignment	35.3	35.4

Table: 4.4 Identified protein sequences encoded by transcripts

Sequence Name	Fasta Sequence (ORF) amino acid	Sequence Description (BLAST)	Theoretical Molecular Weight (Da)	Theoretical Isoelectric Point (PI)
TRINITY_DN 15486	**M T D V F G F F G E K D P Q I N R T F G V S I I K R Q F K G F T A P C A V V R Q Y L G G I P E D D Y D A I R Q Q V F T A T G D F R R T C P A T Y F A E S F A K K G A R A Y Y Y F F S H R P S D S P F A P W M L A S H F E E T P F V F G Y P L R Y G S N Y T L R E R I L S R R M I Q A W S T F A K T G I P V Y G V W P R Y S R E N P**	Acetylcholi-nesterase-1	18701.2	9.54
TRINITY_DN 14565	**M C K S V E C P C G K Y L D Q C G CC E Y C Y K C P G E E C L T M A N E L C T E G Y K C E H A K G T S G P D Y W N K P G R C I KME**	U_{11}-Scytotoxin-Sth1a	7420.5	5.27
TRINITY_DN 8738	*MK T I C C I A A F* **S V C L V L V M A K T D C E E H R E R E L K S N T K V K L V P K C D A N G E Y E A L Q C F Q G S P F C M C W R K D G T H I T E P S I H L K S C A C I V H R D N V A Q K K L I G N Y H P Q C E A D G T Y S R T Q C H G G M G Y C W C V D E H G E K V S N N L N D C**	U_{24} Ctenitoxin	15507.8	6.63
TRINITY_DN 11185	*M K L F V A L L* **V L G S V I S S K V V V S D I Y D P E K S Q N V T K Y S R D S F I V S L L N D A E S L I A R A K K H T S A G I G H Y D I H S T H E K P M D C F E I Y K R N P A A K S G I Y K I W P R S R V L D T G I G V Y C D M D D D G G S W T V L Q R R G E F N S S E D N F F R D W A A Y K T G F G D L M K D F W L G N D N I F A L T N Q R A N S V K F I L A D W E G N T A Y A T Y E D F W I D D E I N N Y T**	Tachylectin-5A	22816.5	5.09

Table: 4.4 Identified protein sequences encoded by transcripts cont'd

Sequence Name	Fasta Sequence (ORF) amino acid	Sequence Description (BLAST)	Theoretical Molecular Weight (Da)	Theoretical Isoelectric Point (PI)
TRINITY_DN18	*M Q I I I L L A* **Y I S L A V G Y N E C G Q N E H Y D P C G T V C S L T C D N Y K D A P L P C V K M C N P G C Q C N S D Y V R S T D G I C V K P E E C S T G S P R L R T K R S S S Y I C E L P A T S G**	U_{32} Aranetoxin	10652.1	5.18
TRINITY_DN6597	**M T P L Q H A A Y K G D Y N L C K L L L D C G A D V N L T R H T S Q Y S A L T F A G L S G K N D V V S L L L E H G**	δ- Lactroins-ectotoxin	6165.0	6.22
TRINITY_DN12147	**M V D Y G L T E E Q V A E F K E A F L L F D K D S D G M I T A A E L G V V M R S L G Q R P S E M E L R N M V H M V D K D G N G T I E F D E F L S M M S K K L Q D S D S E T E L R E A F R V F D K N G D G F I S P E E L R H V M T N L G E K L T D E D V E D M I R E A D L D G D G L V N Y S E F V F I L T S A K**	Calmodulin	17137.1	4.22
TRINITY_DN14130	*M W K S L V I F G F L* **L V A C Y A L T G A Y E K V D V N E E D V V R A A N E A T R Q L S K T F A N K Y H H R L T K V A K A R R Q V V A G M N Y Q M D I I V G M T K C R K D E V E F D D A Q D C E F Q D N I S T Y K K C T V T V Y R D L N D K H K L T F S G C I L A S K K D L**	Cystatin-1	15370.7	8.56
TRINITY_DN26101	**M V D E L V N A E A D I N A A D D H G K T A L H W A A A V N N V D A V R V L I M H G A N K D A Q D N K E E T P L F L A A R E G S Y E A A K I L L E N L A N K E I T D H M D T L P R D V A C D R M H M**	α-Lactrotox-in	10795.1	4.60

Table: 4.4 Identified protein sequences encoded by transcripts cont'd

Sequence Name	Fasta Sequence (ORF) amino acid	Sequence Description (BLAST)	Theoretical Molecular Weight (Da)	Theoretical Isoelectric Point (PI)
TRINITY_DN 14150	**M W L Y K R D T C I R F V P R T N E A N Y I R I F P G Q G C Y S H V G L T L V G A Q P V S L G Q G C G Y M G T V I H E L A H A L G F Y H E Q N R S D R D D W L T I Y W D N I K E G M E P Q F M K L K P D Q N Q L L T P F D Y D S I M L Y G S Y T F S K E Y G K L K T M E G K G G M F L K D V I R K Y F M S K S D I Q R I K M L Y K C T N**	Astacin like metalloproteases	19174.1	8.80
TRINITY_DN 16498	**M S S S S Q A E S L R Q A A G Q G D A D Q V K A L L E D G A R I E P D L E G R T A L H L A S A N G H V N T V K T L I N G G A K I N A L D A A G Y S P L H Q A A T E G H E E V V K L L L K S G C H V D T**	α-Lactrocru-stotoxin	10227.3	5.38
TRINITY_DN 9301	**M S K C L L S S L A K S S G I H N E V L S A L A D E T L L Q N L F L D Q S A I E I A Y E T A K L A A G S S K S L A G L E L S D E K M F F L S Y A Q S Q C E A D L Q L Q K S K H I P T R K R L N S I L Q N F D S F N Q V F S C A S S T H C Q I W A**	Neprilysin	13206.0	5.85

Bold: Mature peptide, *Italics: Signal Peptide*

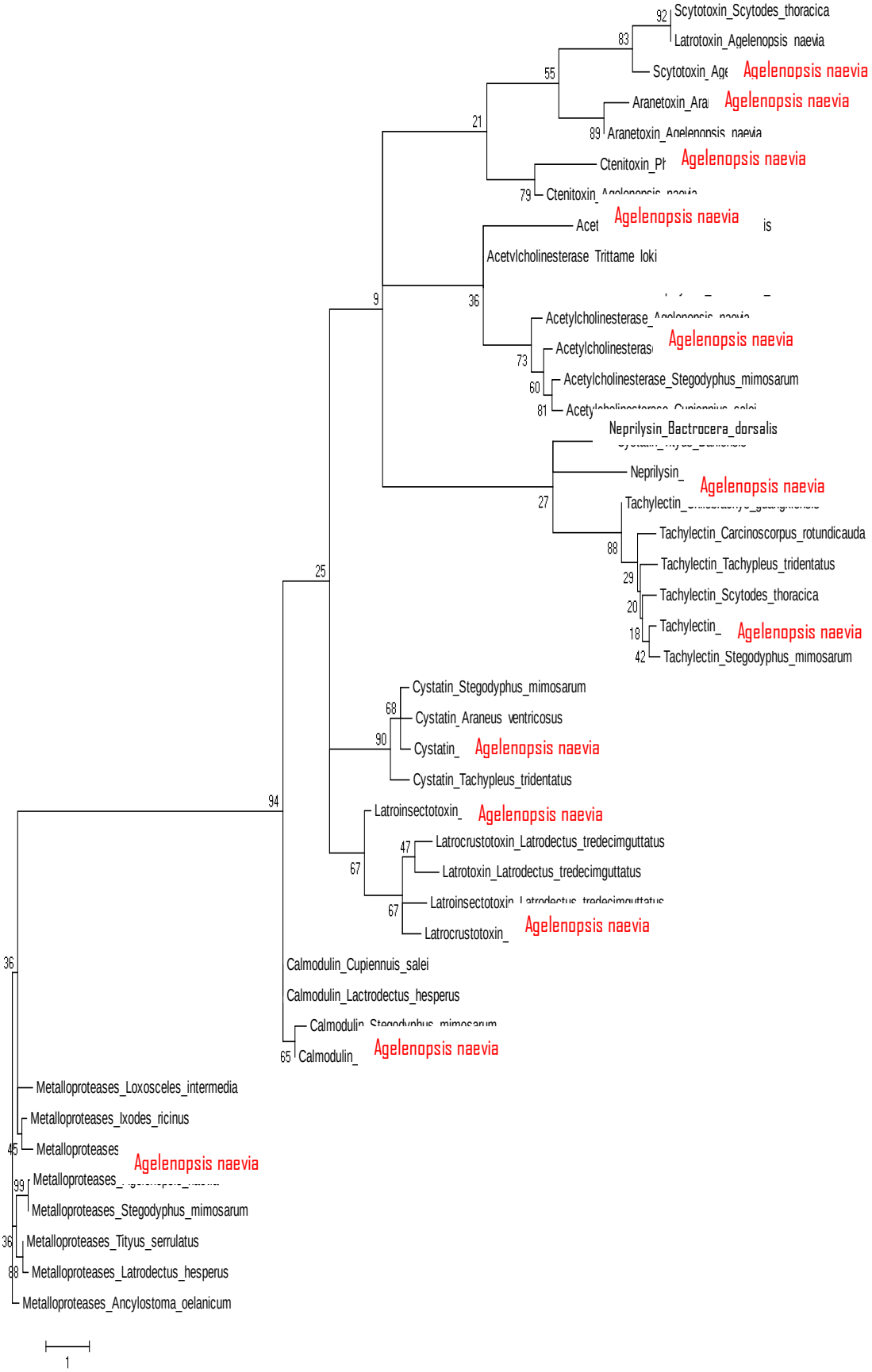


Figure 2: Phylogenetic tree of *Agelenopsis naevia* venom gland transcripts

CHAPTER FIVE

5.0 DISCUSSION

5.1 Antimicrobial Activity of *Agelenopsis naevia* Venom

The observed antimicrobial activity of *A. naevia*venom against *B. subtilis* is in line with the report of Umair*et al*. (2012) who reported similar activity of scorpion venom (*Heterometrus xanthopus) against B. subtilis.* Benli and Yigit (2008), and Liu *et al*. (2009) also reported activity against *Bacillus subtilis* with venom of *Agelena labyrinthica* and *Lycosa singoriensis* at low concentrations of 3mg/ml and 1/100 respectively. In contrast, Fathi *et al*. (2010) reported that*Echis corinatus* venom had no activity against *B. subtilis* and *Salmonella typhimurium* but showed activity against *E. coli* and *S. aureus*. The authors concluded that, the venom contains various substances like proteins and enzymes with various biological activities which may interact with specific molecules of some bacteria while not affecting others.In this study *A. naevia* venom did not show activity against *C. albicans,* however, Liu *et al*. (2009) reported weak activity against *C. albicans* at high concentration of 12mg/ml (60µg) with*L. singoriensis* venom. Activity against *C. albicans* was not observed in this study probably due to the concentration (40µg) of venom used. Difference in antimicrobial activity might also be due to venom composition as there are interspecificvariations in venom components (Rash and Hodgson, 2002). Antimicrobial activity against *S. typhi* was not observed with *A. naevia* venom which might be due to the organism being gram negative bacteria.Jalaei *et al.*(2014), reported effective activity of wasp venom (*Vespa orientalis*) against gram positive bacteria as compared to gram negative bacteria which is attributed to the difference in cell envelope structure. The cell wall of bacteria comprises a complex structure that is fundamentally different between gram- positive and gram-negative bacteria. It

consists of a polymer of disaccharides cross-linked by short chain peptides, forming a type of peptidoglycan. The outer membrane is more hydrophobic in gram-negative than in gram-positive bacteria (Schwarz and Reiter, 2001; Singleton, 2004).However, Umair*et al*. (2012) reported activity of *H. xanthopus* venom against *S. typhimurium*. Scorpion venom has been reported to contain antimicrobial peptides that have activity against multi-drug resistance pathogen (Cociancich *et al*., 1993; Ehret-Sabatier *et al*., 1996; Corzo *et al*., 2001).Antimicrobial activity of *A. naevia*was not reported in well diffusion assay probably because venom peptides do not diffuse easily through agar gel (Yan and Adams, 1998).The observed antimicrobial activity of *A. naevia* venom is similar to previous reports indicating that gram-positive bacteria may be less resistant to antimicrobials, whilst gram-negative bacteria and fungi are more resistant (Wong*et al*., 1997; Yan and Adams, 1998).

5.2 Haemolytic Activity of *A. naevia* Venom

Increase in concentration of *A. naevia* venom result in increase in percentage haemolysis which proceeds at a rapid rate within one hour (Figure 1). This is in line with the reportfor *L. singoriensis* (Liu *et al*., 2009) where haemolytic activity was observed in one hour using *Lycosa singoriensis* venom. However, the effective concentration of 50% haemolysis (EC_{50}) of *L. singoriensis* was 1.25mg/ml while that of the present study was 2.07mg/ml. Difference in effective concentration might be due to difference in venom composition (Rash and Hodgson, 2002). Similar findings have been reported by several authors; Tambourgi *et al*. (2000) reported peak activity of *Loxeceles intermedia* venom within one hour with dose-dependent activity; Lee *et al*. (2013) reported a rapid and dose response curve for the haemolytic activity of *Acanthaster planci* which is a venomous Echinoderm. *Bothrops lanceolatus*venom causes an indirect haemolysis on sheep erythrocytes at a concentration between 5.0-

13.3µg/ml (Martins *et al*., 2009) and a dose-dependent curve haemolytic activity. In contrast to rapid haemolytic activity that was reported in this study, *Hemiscorpius lupterus* (scorpion) venom had a gradual activity with peak haemolytic activity between 12-18hrs. Theseauthors also recorded a dose dependent activity (Seyedian *et al*., 2014).

5.3 Protein Profile of Crude *A.naevia* Venom

Six bands were evident within the range of 6-64kDa. This indicates the presence of proteins (>10kDa) and peptides (<10kDa). Based on the classification by Windley *et al.*(2012), using molecular mass, *A. naevia*venom contains disulphide rich neurotoxins and cytolytic peptides (1-10kDa) and high molecular mass proteins (<30kDa) made up of enzymes and neurotoxins.This is in line with the findings of Liu *et al*. (2009) who reported proteins/peptides within the range of 4-31kDa from *L. singoriensis* venom. Bands within the range of 6-8kDa indicating the presence of agatoxins were observed in this study. Agatoxins are toxins isolated from different species of *Agelenopsis* which act on calcium and sodium channels of insects with µ-Agatoxin-Aa1d isolated from *A. aperta* been considered as a suitable insecticidal lead against *Musca domestica*and *Manduca sexta* (Windley *et al*.,2012). The presence of 28kDa band in this study might indicate the presence of metalloproteases which have fibronectinolytic and fibrinogenolytic activity (Feitosa*et al*., 1998) as observed in *Loxosceles intermedia*. The band above 28kDa band in *A. naevia*gel might indicate the presence of peptide isomerase (29kDa). This enzyme was also isolated from*A. aperta* and is assumed to aid in envenomation process (Shikata *et al*., 1995).

Hyaloronidase (45kDA) which contributes to the diffusion of venom from inoculation site (Sutti*et al*., 2014) isolated from *Vitalius dubius* and phospholipase A, were not observed in the gel. With *Sicarius*sp and *Loxeceles* sp venom, Lopes *et al*. (2013)

reported major band with molecular weight of 32-35kDa which shows the presence of shingomylase D. Such a band was not seen in SDS-PAGE of *A. naevia* venom. The major band in *A. naevia* venom has a molecular mass of 51kDa. The difference in protein/peptide bands between different spiders might be due to interspecific variation in venom component (Rash and Hodgson, 2002).

Furthermore, an SDS-PAGE protein molecular weight pattern of male and female *A. naevia* venom is similar. Similar pattern was reported for male and female *Lycosa cylindrata* venom (Rash *et al*., 2000) indicating similar composition. However similar pattern of bands does not imply similar relative abundance of component as this is tightly linked to venom toxicity as reported by Kent *et al*. (1984) when they carried out a study on *Lycosa reclusa*.

The 2D gel electrophoresis shows spots that corresponded to the molecular mass of bands observed in SDS-PAGE gel. It also showed better peptide resolutions with spots evidently present below 10kDa. It is also evident that the venom of *A. naevia*has both cationic and anionic proteins/peptides. Vejayan *et al.* (2010) and Richardson *et al*. (2006) have described the use of 2D gel as a better tool for profiling proteins while working with Malaysian snakes venom and *Phonoturia* species respectively.

5.4 Transcriptomes of *Agelenopsis naevia* venom gland

Sequences reported in this study do not have propeptide (Table 4.4). Toxin precursors that have no propeptides usually occur in scorpion but rarely in spiders (Luna-Ramirez *et al*., 2015). Similar findings have been reported by Zhang *et al*. (2010) from transcripts of *L. singoriensis* venom gland. Absence of propeptide sequence has also been reported in latrotoxins from *Lactrodectus* spp (Windley *et al*., 2012). Three sequences in this study homologous to latrotoxins also lack propeptides. Signal

peptides are also absent in some of the sequences in this study which is in agreement with the findings of whole transcriptomics of *Uradacus yanschenkoi* (Luna-Ramirez, *et al*., 2015).Sequences in this study have 1-15 cysteine residues. Cysteine is used to form several disulphide bridges (1-7) in venom peptides known as cystine knot (Windley *et al*., 2012). This knot creates highly stable proteins which are able to resist extreme pH, organic solvents, proteases and high temperature (Colgrave and Craik, 2004; Fry *et al*., 2009)). Cysteine residues in spider transcripts have been reported by several authors (Zhang *et al*., 2010; Jiang*et al*., 2013; Undheim *et al*., 2013).

A sequence homologous to calmodulin was identified in this study. Calmodulin functions in regulating the process of protein transport and secretion and have been reported from transcripts of *L. singoriensis* (Zhang *et al*., 2010).Calglandulin which is very similar to calmodulin have also been reported from several snake species (Junqueira-de-Azevedo *et al*., 2003; St. Pierre *et al*., 2005).

Sequences homologous to Astacin-like metalloproteasess, acetylcholinesterase and neprilysin were also identified. Astacin are a family in the metzincin clan of metallopeptidase zymogens which become proteolytic upon activation (Cerda-Costa and Gomis-Ruth, 2014) Astacins were first characterized in spider venom of *Loxosceles* by da Silveira *et al*. (2007). In *Loxosceles* venom, astacin degrade matrix protein suggesting the role it plays in spreading of venom components. Paralogs from the same species vary in specificity of gelatinolytic function (Trevisan-Silver *et al*., 2010). The activity of metalloproteases results in haemoglobin efflux leading to haemolysis (Seyedian*et al*., 2014). These metalloproteases might have been responsible for the haemolytic activity observed in this study (Tambourgi *et al*., 2000) Astacin-like metalloproteasess have been reported by several authors from different

spider species (Nagaruju *et al*., 2007; Zobel-Thropp *et al*., 2014a; Zobel-Thropp *et al*., 2014b).

Acetylcholinesterase and neprilysin have been reported from the venom gland transcripts of *Trittameloki* (Undheim *et al*., 2013). Acetylcholinesterase reduces the amount of acetylcholine at synapses while neprilysin play a role in degradation of extracellular matrix (Undheim *et al*., 2013). These enzymes are likely to play roles in envenomation process; neprilysin aiding the action of acetylcholinesterase and other neurotoxins by facilitating their access to synaptic targets (King and Hardy, 2013).

Tachylectin-5A and cystatin-1 homologous sequence were identified. Tachylectin-5A was first reported from horse-shoe crab (*Tachypleus tridentatus*). It was found to exhibit Ca^{2+} dependent haemaglutination activity against human A, B, and O types of erythrocytes and also had activity against gram positive and gram negative bacteria (Kairies *et al*., 2001). Lectins that agglutinate human erythrocytes have also been reported from the venom of *Ornithoctonus huwena* (Jiang *et al*., 2009). This toxin might have been responsible for the antimicrobial activity observed in this study. In contrast C-lectin was reported in transcripts of *Loxosceles laeta* (de Fernandes-Padrosa*et al*., 2008). Difference in lectin component of spider venom might be attributed to interspecific variation (Rash and Hodgson, 2002).

Cystatin are characterized as inhibitors of cysteine proteinases having activity against papain and legumain. It plays an important role in the control of intracellular protein degradation (Wan *et al*., 2014). Cystatin have been reported from venom gland transcripts of tarantula, *Chilobrachys jingzhao* (Chen *et al*., 2008), *L. laeta*(de Fernandes-Padrosa*et al*., 2008) and *A. ventricosus* (Wan *et al*., 2014)

Agelenopsis naevia venom gland transcripts were found to be homologous to U_{32}-aranetoxin, U_{24}-ctenitoxin,U_{11}-Syctotoxin-Sth1a, α-latrotoxin, δ-latroinsectotoxin and α-latrocrustotoxin.U_{24}-ctenitoxin is a toxin from *Phoneutria nigriventer* with weak homology to cysteine proteinase presumed to be a protease inhibitor. It has two thyroglobulin type-1 domains but its biological activity and function is not known (Richardson *et al*., 2004). U_{32}- aranetoxin, is a toxin isolated from *Araneus ventricosus* but its molecular target and function is not yet known (Duan *et al*., 2013). Similarly, U_{11}-Scytotoxin-Sth1a is a toxin from spitting spider, *Scytodes thoracica* with an unknown molecular target and function (Zobel-Thropp*et al*., 2014b). α-latrotoxin is the main component of*Loxosceles* venom toxic to vertebrate which is responsible for human envenomation (Ushkaryov, 2002). This toxin binds to neurexin 1α, latrophilin1 and receptor-like protein tyrosine phosphatase to induce neurotransmitter exocytosis.It is said to act through two calcium-dependent mechanism and a yet to be identified calcium-independent mechanism (Rohou *et al*., 2007). δ-latroinsectotoxin and α-latrocrustotoxin are toxins from *Loxoceles* venom that induces neurotransmitter release in insect neuromuscular junction (Rohou *et al*., 2007) and neurotransmitter exocytosis in crustacean (Volynskii *et al*., 1999) respectively. Both toxins are presumed to induce neurotransmitter release similar to α-latrotoxin (Rohou *et al*., 2007). Sequences homologous to toxins from other spider venom have been reported by several authors which is an indication of phylogenetic relationship among spider species (Zhang *et al*., 2010; Jiang *et al*., 2013; Undheim *et al*., 2013).

5.5 Phylogenetic Relationship of *Agelenopsis naevia* venom toxins

Agelenopsis naevia venom gland sequence clustered mainly with that of *Stegodyphus mimosarum* indicating close relationship. *Stegodyphus mimosarum* is a social spider

belonging to Eresidae family whose venom is not very toxic to humans which is similar tothe venom of *A.naevia* as observed in the haemolytic activity in this study. Both Eresidae and Agelenidae belong to entelegynes group of spiders (Khun-Nentwig *et al*., 2011). Putative astacin-like metalloproteases sequence clustered with that of *Stegodyphus mimosarum* with 99% identity indicating that they are orthologs. *Lectrodectus hesperus* (black widow spider) which causes serious clinical symptoms in humanclustered with *Tityus serrulatus* which is the most piosonous scorpion in Brazil (Maruo *et al*., 2002; Rohou *et al*., 2007). This is in agreement with the work of Da Silveira *et al*. (2007) who reported phylogenetic studies based on protein sequence analysis of astacin-like metalloproteases of *Loxoceles intermedia* with the highest sequence similarity (35.0%) to *Caenorhabditis elegans* (nematode). Similarly, in tachylectin clade, *A. naevia* clustered with *S. mimosarum* with 68% node reliability. The sequence also present homology with that of *T. tridentatus* which was the organism from which tachylectin was first characterized. Similar finding was reported by Rego *et al*. (2005) from *Ornithodoros moubata* and *Ixodes ricinus* which clustered with *T. tridentatus* sequence to form a clade.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The study was carried out to investigate the antimicrobial and haemolytic activities, profile protein of *A. naevia* venom as well as transcript coding for protein in *A. naevia* venom. Venom was collected by micro-dissection after homogenization of the venom gland and the concentration of venom was determined in a nanodrop spectrophotometer. Antimicrobial activity of venom against*Bacilus subtilis, Candida albicans*, and *Salmonella typhi* was carried out by disc diffusion and well diffusion assay. Haemolytic activity was carried out using purified 1% human erythrocyte. Crude venom was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a precasted 4-20% gel. Gel was stained with commassie blue and destained to reveal the protein bands. Two dimension gel electrophoresis was also carried out in 13-cmimmobilized pH gradient(IPG) strip with a linear range of pH from 3 to 10. Electrofocusing was carried out and the gel was rehydrated and subjected to a second dimension electrophoresis using a 15% SDS gel. The spots were visualized using commasie blue and detected using dynamic spot detector software. The messenger RNAs (mRNA) were isolated from venom gland and the first and second complimentary DNA (cDNA) strand were synthesized using ThermoScientific kits following manufacturer's instructions. The cDNA library was constructed and purified. Pair-end sequencing was carried out using illumina NextSeq 500. The transcriptomes derived were searched against public databases using BLASTx alogrithm. Multiple sequence alignment andphylogenetic analysis was achieved using ClustalX and MEGA softwares, respectively. Antimicrobial activity

data were subjected to ANOVA to compare means, where significant, Duncan multiple range test was used to separate means. Effective concentration of crude venom was calculated using probit analysis. The crude venom of *A. naevia* showed significant activity against *Bacillus subtilis*(23.5±0.5mm) as compared to the controls and no activity against *Candida albicans* and *Salmonella typhi* on the disc diffusion assay. However, the venom did not show activity against the three micro-organisms using well diffusion assay. The crude venom also showed haemolytic activity on human erythrocytes with activity within the first 1hr (42.40%) for all the three concentrations (0.579mg/ml, 2.843mg/ml and 4.044mg/ml) used. Percentage haemolysis ranges between 42.40%-52.52% within 1-6hr using three different concentrations an EC_{50} of 2.07mg/ml. Six bands were evident on the SDS-PAGE gel with molecular weights ranging from below 6-64kDa. The crude venom showed both protein (>10kDa) and peptide (<10kDa) resolutions. Over 300 spots were detected on the 2D gel with molecular weight ranging from below 14kDa to 94kDa. Twelve transcriptomes homologous to sequences in databases were identified. Transcript from*Agelenopsis naevia*venom gland clustered with thatof *Stegodyphus mimosarum*.

6.2 Conclusions

In conclusion, *Agelenopsis naevia* venom has a significant antimicrobial activity against *B. subtilis* 40µg and has no antimicrobial activity against *Salmonella typhi* and *Candida albicans* at 40µg. The crude venom did not show activity on the three microorganisms in well diffusion assay. Disk diffusion assay is a better sensitive antimicrobial test for the spider venom.

Agelenopsis naevia venom has haemolytic activity on human erythrocytes within 1hr and increases with time and concentration. The EC_{50} of the crude venom is2.07mg/ml.

Molecular weight of *A. naevia* venomproteins range between14kDa-94kDa and peptides ranges between 5-8kDa. The venom contained both cationic and anionic proteins/peptides (3-10PI).

Agelenopsis naevia venom gland produces protein/peptides of which twelve transcriptomes were identified, which includes metalloproteases, tachylectin, acetylcholinesterase, neprilysin, latroinsectotoxin, latrocrustotoxin, latrotoxin, aranetoxin, cytotoxin, ctenitoxin, calmodulin and cystatin.

Agelenopsis naevia clustered with *Stegodyphus mimosarum* to form a sister group.

6.3 Recommendations

- Disc diffusion assay should be used to study antimicrobial activity of spider venom.
- *A. naevia* venom peptides could be harnessed as a potential antimicrobial and bioinsecticide agent.
- 2D gel electrophoresis should be used to study venom proteins of spiders.
- Microsequencing of venom proteins to enable proper identification.
- X-ray crystallography to enable identification of molecular structure of proteins.

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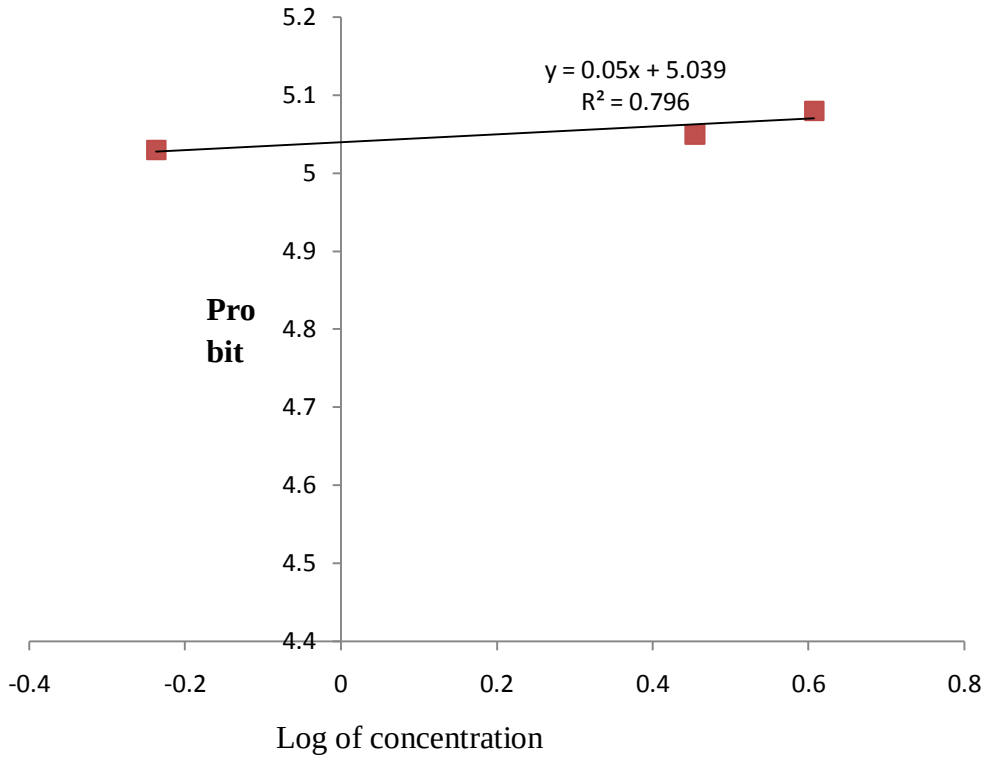
Appendix I

Quality control analysis of *Agelenopsis naevia* reads

Measures	Right Reads	Left Reads
Total sequences	11167123	11167123
Sequence flagged as poor quality	0	0
Sequence length(n)	35-76	35-76
%GC	44	45
Per base N content	0	0
Adapter content	0	0
Per tile sequence quality	Blue	Blue

Appendix II

Effective Concentration (EC_{50}) of *Agelenopsis naevia* venom



Appendix III

Assembled Sequence of *A. naevia* venom gland

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