BIO-PROSPECTING FOR INSECTICIDAL SCORPION VENOMS AND IDENTIFICATION OF BIOACTIVE TOXINS
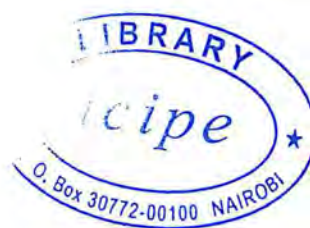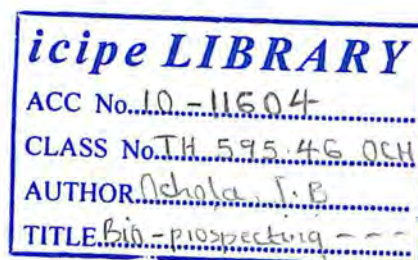
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A thesis submitted in partial fulfillment for the Degree of
Master of Science in Chemistry in the Jomo Kenyatta University of
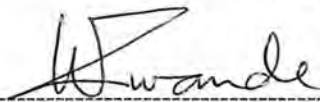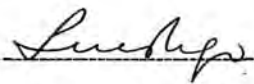Agriculture and Technology

2005

DECLARATIONS

The work described in this thesis was carried out at International Centre of Insect Physiology and Ecology (ICIPE). Information derived from published work is specifically acknowledged in the text and references appended. The experimental work described is entirely my own. This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my wife, Jackline and our son; Nathan
&
my parents, Sabastian and Celementina; my sisters; my cousin Hilary

ACKNOWLEDGEMENT

ACNONYMS AND ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
cDNA	Complimentary DNA
Da	Dalton
DDD	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichloroethane
DNA	Dioxyribonucleic acid
DPTU	Diphenylthiourea
DPU	Diphenylurea
EPA	Environmental Protection Agency
ESI	Electron Spray Ionisation
FDA	Food and Drug Administration
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatoraphy
ICIPE	International Centre of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KWS	Kenya Wildlife Services
LC	Liquid Chromatography
LC_{50}	Concentration that would kill 50% of test organism
LD_{50}	Dose that would kill 50% of test organism
MALDI	Matrix Assisted Laser Desorption Ionisation
MS	Mass Spectrometry
AMNH	American Museum of Natural History
NMR	Nuclear Magnetic Resonance
Plit	*Parabuthus leiosoma* insect toxin
Plmt	*Parabuthus leiosoma* mammalian toxin
Plt	*Parabuthus leiosoma* toxin
PMTC	N-Phenyl,o-Methyl-thiocarbonate
TCEP	Tris (2-carboxyethyl) phosphine
TDE	1,1-Dichloro-2, 2-bis (p-chlorophenyl) ethane
TOF	Time of Flight

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ABSTRACT

Scorpion venom contains insect and mammal selective toxins. However, despite their significance and potential application in insect-pest control, bioactivity of Kenyan scorpions is still unknown. As such this study initiated investigations on the inherent insecticidal activity of the venoms of scorpions collected in Kenya. Venom from four scorpion species namely *Parabuthus leiosoma, Parabuthus pallidus, Hottentotta trilineatus* and *Hottentotta eminii* was collected upon stinging a parafilm membrane. Crude venom was extracted by homogenization and centrifugation then the supernatant was lyophilized. Bioassay guided purification was carried out by cation exchange chromatography and reverses-phase chromatography. Amino acid sequence of the active fractions was determined by N-terminal automated Edman degradation. The structure elucidation of pure fractions was confirmed using LC-MS and UV. Subcutaneous injection and oral toxicity tests on crude and pure fractions were conducted in *Mus musculus* (Mice), *C. partellus* and *Busseola fusca*. The lowest LC_{50} value (i.e highest lethal potency) for *C. Partellus* was shown by venom from *P. leiosoma* at 0.689 µg/50mg weight of *C. partellus*. From this study, one lepidopteran-selective toxin, *Parabuthus leiosoma* insect toxin (Plit) was isolated, the partial N-terminal sequence was determined as – KDGYPVDNANCKYE- and the molecular weight was (6688.5, 7198 Da). It shows high homology to other discovered insect toxins. The second peptide isolated showed significant insect toxicity coupled with mild effect on mice, the partial N-terminal sequence was established as –LCEKFKVQRLVELNCVD- and the molecular weight was 6742.5 Da. It has no amino acid sequence similarity with any already discovered toxins. The study also led to purification and characterization of a third peptide that is selectively toxic to mice, with -ADVPGNYPlDKNGNRYY- as the partial N-terminal amino acid sequence and the molecular weight was (7145, 6688.50) Da. This is the first report of an insect selective peptide toxin from the Kenyan scorpion species- *Parabuthus leiosoma*. The scientific data obtained may be useful in the design and the development of environmentally safe target-specific and biodegradable pest control products.

CHAPTER ONE

INTRODUCTION

1.1 Background

Insects and other arthropods such as scorpions, spiders, ticks and mites constitute more than half of animal species, and are by far the most diverse group of organisms inhabiting earth (Bulet *et al.*, 1999). The problems associated with arthropod pests and disease vectors on one hand, and the opportunities for their conservation and utilization on the other (the rich beneficial arthropod resources of Africa) are among the most under utilized and threatened, this cuts across the key sectors of health, agriculture and the environment (ICIPE, 2003)

Research has revealed that many folk medicinal uses of insects and related arthropods have scientific base with potentials in pharmacological use and other applications of derived chemicals. Besides, their potential as sources or leads for important drugs and pest control products cannot be underestimated (Costa-Neto, 2005).

Although comprising a relatively small component of terrestrial arthropod diversity, scorpions are of considerable interest to scientist and layman alike. The envenomation represents a significant cause of morbidity and mortality in many parts of the world (Rajarajesward *et al.*, 1979). Apart from the medical importance, their relatively large sizes compared to other arthropods and the fearsome appearance notwithstanding the great age of their lineage, scorpions contribute to a fascination that has always surrounded them and continues to. They are especially well adapted to survive in extreme thermal environments sometimes constituting a major portion of the total animal biomass in these environments (Polis, 1990). They are also of considerable importance in ecological food webs, particularly with respect to controlling insect populations by feeding on other species, which are pests, vectors and nuisance species such as biting flies, as well as other spiders and scorpions (http://www.nature.nps.gov)

The vast majority of chemical and pharmacological studies of scorpion venoms were performed in the past primarily for medical importance as poisons (Lazarovici *et al.*, 1982). Currently, the study is motivated by their ability to serve as pharmacological tools for the study of excitation of biological systems (Catterall, 1980). It is now certain that the venoms of some scorpion species

contain several toxins that are selectively toxic to insects only. This offers alternative, biodegradable and environmentally safe tools for pest control (Minton, 1974; Lazarovici *et al.*, 1982).

Scorpions are unusual among arthropods in that all species are viviparous and several are parthenogenic. Unlike terrestrial arthropods, they resemble large vertebrates in their very slow reproductive rates and remarkable longevity. Furthermore, many scorpions are equilibrium species and valuable bioindicators, also considered as among the successful inhabitants of earth (Brownell *et al.*, 2001). As such, their disappearance is a signal of habitat degradation while their status as 'charismatic micro-fauna' predisposes them for use as flagship species in programs aimed at conserving terrestrial invertebrates. This group of arthropods is increasingly threatened by habitat destruction and harvesting for exotic pet trade, consequently reducing volume of the genetic resources and causing other valuable materials to disappear.

Environmental degradation is in alarming situation hence urgent call for value addition to biodiversity through bioprospecting; the systematic search for new applications of hitherto unstudied biological species, development and commercialization of useful chemical products (Weiss & Eisner, 1998). Through bioprospecting, there lies a last chance to rescue irreplaceable biological and chemical information evolved over billions of years before they are forever lost to humanity (Reid, 1993; Rouhi, 1997). This information could some day lead to cures for devastation diseases, natural ways to grow food crops, or food supplements for the improvement of health. In bioprospecting, we may also create economic incentive for conservation of biodiversity (Barret, 2000), serve as the foundation for modern biotechnology industry in the developing countries and provide the indigenous people with ways to benefit from their unique understanding of the environment in which they live.

1.2 Sustainable economic and environmental development

Sustainable economic and environmental developments are complimentary and mutually dependent. This concept is now widely accepted. In Africa there is need to address the twin challenges of rapid population growth and food deficit, while conserving the environment. Demand for food, which is predominantly plant based, will continue to increase as the world population increases. Since earth acreage remains constant, agriculture will become more intensive. This implies that the use of fertilizers, pesticides and soil disinfectants will continue in spite of their negative effect on the environment. This calls for a balance between

increased food production and sustenance of the ecological system (Briggs, 1998; Berdy, 1989).

Arthropod pests are a major threat to crop production, human and livestock health. Though there has been a significant advancement in technology towards production of cereals in Africa, the productivity is hampered by several abiotic and biotic constraints, which may cause losses up to 80% (Pinstrup-Andersen *et al.*, 1999). Abiotic constraints in this region are mostly due to seasonal unreliability of rain-fed agriculture, poverty and limited access to remedial inputs. Biotic stress, which includes diseases and pests, are ever present, and require effective management processes to support productivity and environmental protection.

In tropical Africa, several insect species are known to damage these crops (Ingram, 1958; Seshu Reddy, 1991). Lepidopteran stem borers are the most important insect pests for these crops because they are ubiquitous and far the most injurious (Ingram, 1958; Nye, 1960). These insects infest many graminaceous crops including maize, millet rice, sorghum and sugarcane, from germination to maturity. With the exception of sugarcane, the rest are the subsistence food crops in developing countries. Yield losses of maize and sorghum caused by stem borers vary widely in different regions depending on the pest population density and phenological stage of the crop at infestation. Recent estimates of losses due to stem borers alone in sub-Saharan Africa range from neighbourhood of 20-40% of the potential yield (Seshu Reddy & Walker, 1990). The magnitude of the losses indicates the importance of stem borers as a limiting factor affecting crop production in this region.

Twenty-one species of lepidopteran stem borers that infest cultivated grasses have been identified, and includes 7 noctuids, 2 pyralids and 12 crambids (Maes, 1998). Among them, two crambids *Chilo partellus* Swinhoe, *Chilo orichalcociliellus* Strand, one pyralid *Eldana saccharina* Walker, and two noctuids *Sesamia calamistis* Hampson and *Busseola fusca* (Fuller), are economically the most important pests of cereals in Eastern and Southern Africa (Seshu Reddy, 1998; Kfir, 1998). These species infest a large number of economically important plants such as maize, sorghum, sugarcane, rice and millet. As such, one way of contributing to sustainable food production and thus alleviating the recurrent food deficits is through the control of pests of food crops.

-3-

1.3 *Chilo partellus* (Swihoe)

The spotted stem borer, *Chilo partellus* (Swinhoe), from the family Pyralidae is one of the most important pests in tropical Africa and Indian sub-continent. It is essentially a pest in lowland areas and seldom-found above 1500 m (Hill, 1983). *C. partellus*, is native to Asia, and is suspected to have been introduced in Africa from the Indian sub-continent(Duerdon, 1953. It was first reported in Uganda (Ingram, 1958) and Tanzania (Duerdon, 1953). By 1977, it had spread to Ethiopia, Kenya, Malawi, Mozambique, Somalia, South Africa, Sudan, Tanzania and Uganda (Taneja & Nwanze, 1989).

Adults are nocturnal and live 2-3 days, during which each female lay 200–600 scale-like eggs in batches of 10-80 overlapping eggs on underside of leaves of the host plant, mostly near the midrid, with a preference for young plants (3-4 weeks after germination) (Taneja & Nwanze, 1989). Larvae hatch after 4-5 days. The damage caused by the larvae feeding on the young plants involves the attack of growing point and cause 'dead heart', characterized by dead central leaf that result in 100% crop loss. In older plants the larvae bore into the main stem, which is subsequently hollowed out over a considerable length. Stem tunneling reduces plant vitality, the grain filling process and promotes falling of plants as they mature. The affected plants have poor growth, reduced yield and are more susceptible to wind damage and secondary fungal and bacterial infections. Larval development is completed in 2-4 weeks and the larvae pupate in the damaged stem. The pupal period lasts for 5-12 days. The lifecycle is completed in 25-50 days (Taneja & Nwanze, 1989), and may be continuous in areas where suitable conditions for host plant growth are permanently present. However, the cycle is usually interrupted by cool or dry season during which plant growth is impossible.

1.4 Control of insect pests

Various control measures have been used in attempts to reduce the crop losses due to insect pests. These include cultural practices, biological control, chemical control and breeding of host resistant plants.

1.4.1 Cultural practices

Cultural practices have been attempted with different limited levels of success. These include the destruction of crop residue after harvest by farmers, destruction of wild host plants in the proximity of field (Ingram, 1958) and intercropping of host and non-host plants (Khan & Hassanali, 2003). Though many cultural practices are labour intensive, they have less adverse effects on the environment and are readily available without extra investment on equipment.

However, none of the cultural methods has been shown to be efficient and therefore reliable in the control *B. fusca* populations in the field (Nwanze & Mueller, 1989; Skovgård & Päts, 1996; Grisley, 1997). In addition, most African farmers lack management capabilities to adopt the cultural control methods (Harris, 1989).

1.4.2 Biological control

Biological control is a component of an integrated pest management strategy. It is defined as the reduction of pest populations by natural enemies and typically involves an active human role. Naturally occurring enemies and environmental factors also suppress all insect species with no human input. This is frequently referred to as natural control. Natural enemies of insect pests, also known as biological control agents, include predators, parasitoids, and pathogens. Predators, such as lady beetles and lacewings, are mainly free-living species that consume a large number of preys during their lifetime. Parasitoids are species whose immature stage develops on or within a single insect host, ultimately killing the host. Many species of wasps and some flies are parasitoids. Pathogens are disease-causing organisms including bacteria, fungi and viruses. They kill or debilitate their host and may be specific to certain insect groups.

1.4.2.1 Parasitoids

Classical biological control involving use of exotic parasitoids has been used as a strategy for controlling populations of moth pests in the tropics. The success of classical biological agents is generally attributed in part to the high searching efficiency of the natural enemy for its hosts (Waage, 1990). An important exotic parasitoid of stemborers, *Cotesia flavipes* (Cameron), introduced in Kenya in 1993 for the control of *Chilo partellus* (swinhoe) has shown varied degree of success in the control of insects (Overholt, 1998). However, *Busseola fusca* seems to be unsuitable host for the parasitoid. Laboratory studies indicate that although *C. flavipes* can locate and attack *B. fusca* (Ngi-Song *et al.*, 1996; Ngi-Song & Overholt, 1997), it is not able to complete its development cycle in the host since all the parasitoid eggs become encapsulated (Ngi-Song *et al.*,1998). *Cotesia sesamiae* (Cameron) has been reported as the predominant larval parasitoid of *B. fusca* in most parts of Africa (Polaszek & Walker, 1991; Kfir, 1992; Omwega *et al.*, 1995; Kfir, 1997). However, other studies reported that *C. sesamiae* cannot successfully develop in *B. fusca* populations originating from the coastal area of Kenya (Ngi-Song *et al.*, 1998; 2000). Although there appears to be synergistic parasitism of *B. fusca*

by *C. sesamiae* and *C. flavipes* these parasitoid species have not been shown to be efficient in the field (Ngi-Song *et al.*, 2001).

1.4.2.2   Predators

The arthropod predators of insects and mites include beetles, true bugs, lacewings, flies, midges, spiders, wasps, and predatory mites. Insect predators can be found on plants, including the parts below ground, as well as in nearby shrubs and trees. Some predators are specialized in their choice of prey, others are generalists. Some are extremely useful natural enemies of insect pests. Most beneficial predators will consume many insect pests during their developmental stages, but some are more effective at controlling pests than others. Some species may play an important role in the suppression of particular pests. Others may provide good late season control, but appear too late to suppress the early season pest population. Many beneficial species may have only a minor impact by themselves but contribute to overall pest mortality. Unfortunately, the role of many of the beneficial predators in pest control has not been adequately studied and some prey on other beneficial insects (Hoffmann & Frodsham, 1993).

Insect predators can be found in almost all agricultural and natural habitats. Each group may have a different life cycle and habits. Although the life history of some common predators is well studied, information on the biology and relative importance of many predatory species is lacking. Surveys of agricultural systems give an indication of the potential number and diversity of predators on a crop. For example, over 600 species of predators in 45 insect families and 23 families of spiders and mites have been recorded on Arkansas cotton. Eighteen species of predatory insects have been found in potatoes in the north eastern United States. There may be thousands of predators per acre, in addition, too many parasitoids. Although the impact of any one species of natural enemy may be minor, the combined impact of predators, parasitoids, and pathogens can be considerable (Hoffmann & Frodsham, 1993).

1.4.2.3   The "push-pull" strategy

This strategy involves the use of semiochemicals and other methods that manipulate insect behaviour in the management of stemborers. Semiochemicals are chemical compounds that act as signals to modify insect behaviour or development. They are reported to be widely used in the control of insect pests (Smart *et al.*, 1994; Pickett *et al.*, 1997; Khan *et al.*, 2000).

However, semiochemicals often give ineffective or insufficiently robust pest control strategy when employed alone. Use of semiochemicals should therefore be combined with other integrated pest management approaches that involve population reducing agents such as pesticides or biological control agents. The main components of such strategies are pest population monitoring (to allow accurate timing of treatments), combined use of semiochemicals, host plant resistance, trap crops (to manipulate pest behaviour) and selective insecticides or biological control agents (to reduce pest populations).

Based on the understanding of the volatile semiochemicals employed by stemborers in locating suitable hosts and avoiding non-host plants, a novel and highly promising integrated pest management strategy has been developed to control these pests in East Africa (Pickett *et al.*, 1997; Khan *et al.*, 2000).

This habitat management system often referred to as the "push-pull" or stimulo-deterrent diversionary tactics involves 'pushing' the insects away from the harvestable, agronomic crops, and pulling them onto trap crops where their populations are reduced by biological control agents or a highly specific but slow acting pesticide. Hence the strategy involves combining a harvestable agronomic crop trap crop, and intercrop. Host masking agents, repellents, anti-feedants, oviposition deterrents or compounds associated with plant defence protect the harvestable crop from attack by the insect (push). At the same time, aggregative semiochemicals, including host plant attractants and pheromones, stimulate colonisation of the 'pushed' pests on the nearby trap crops or entry into traps where pathogens can be deployed (pull). The individual components of the 'push-pull' strategy are not in themselves highly efficient, and therefore, do not select for resistance as strongly as conventional toxicant pesticides, making the approach intrinsically more sustainable (Pickett, 1998).

Therefore by combining this approach with others in the integrated management strategies, a robust and efficient control strategy for cereal stemborers can be developed. Using three graminaceous plant species, maize (*Zea mays*), sorghum (*Sorghum bicolor*) and napier grass (*Pennisetum purpureum*), it was shown that under laboratory conditions gravid females of *B. fusca* preferred to oviposit on maize and sorghum over napier grass (Le Rü *et al.*, 2004). In this study it was suspected that both host plant semiochemicals and plant physical characteristics were involved in the preferential choice of hosts and hence could be key factors involved in the "push-pull" strategy. Napier grass produces sap that traps and inhibits

further development of eggs laid by the stem bores hence disrupting the life cycle (Mohyuddin, 1990).

1.4.2.4 Biotechnology

The evolution of genetically modified crops took a major step in the mid-1990s with the approval and commercial release of insect-resistant maize hybrids with trans-genes derived from *Bacillus thuringiensis* (Bt maize). The release of Bt maize was met with great enthusiasm by many researchers and crop managers because of its ability to effectively control European corn borers and other lepidopteran insects without the use of foliar insecticides. Crop producers and the agricultural industry rapidly accepted the technology and began to incorporate it into their crop production practices (Munkvold *et al.*, 1997). But recently, controversy over production and use of genetically modified crop cultivars has focused a great deal of public attention on Bt maize. A number of organizations and individuals have raised questions about the safety and ethics of Bt maize production, despite EPA and FDA approvals that consider environmental impact, food safety, non-target effects, and pest resistance. The controversy has been fueled largely by the reluctance of European consumers to accept genetically modified crops. Although Bt technology has fairly obvious benefits for maize producers and biotechnology companies, some consumers have found it difficult to perceive the consumer benefits of Bt technology (Betz *et al.*, 2000)

One aspect of risk/benefit analysis is the influence that Bt technology may have on maize diseases and mycotoxin-producing fungi in maize. Because the fungi that produce mycotoxins in maize are frequently associated with insect damage to the plants, insect control has the potential to reduce mycotoxin concentrations in grain (Betz *et al.*, 2000)

Insects affected by currently available Bt maize hybrids are in the order lepidoptera, which includes moths and butterflies. The primary target species for Bt maize is a pest imported from Europe, *Ostrinia nubilalis*, or the European corn borer. Other lepidopteran pests of maize that can be controlled or partially controlled by current Bt technology are corn earworm *(Helicoverpa zeae)*, common stalk borer *(Papiapema nebris)*, armyworm *(Pseudaletia unipunctata)*, and southwestern corn borer *(Diatraea grandiosella)*. Currently available Bt hybrids are effective against European corn borer (Rice & Pilcher, 1997), stalk borer, and southwestern corn borer, and they can reduce damage by armyworm and corn earworm.

However, they have not shown much benefit for controlling damage by black cutworm (*Agrotis ipsilon*) or fall armyworm (*Spodoptera frugiperda*) (Lynch *et al.*, 1999; Pilcher *et al.*, 1997).

1.4.3 Insecticides

Control against insect pests has mainly been by the use of chemical insecticides. Insecticides are chemicals that are used to control damage or annoyance from insects. Control is achieved by poisoning the insects by fumigants (penetrate through the respiratory system) oral ingestion of stomach or contact poisons (penetrate through the cuticle) (Kirk & Othmer, 1981).They may be classified as inorganic, synthetic organic and natural organic compounds. Inorganic insecticides generally act as stomach poisons, while natural products act largely as contact poisons. Synthetic organic insecticides may have contact and stomachs poisons action and sometimes are used as fumigants.

The inorganic insecticides so far used include arsenicals and fluorides. Arsenicals like lead arsenate ($PbHAsO_4$), arsenic trioxide (As_2O_3), calcium arsenate (neutral) $\{Ca(AsO_4)_2$, calcium arsenate (basic) $\{Ca_3(AsO_4)_2\}_3.Ca(OH)_2$ copper acetoarsenate, $\{Cu(C_2H_3O_2)_2.3Cu(AsO_2)_2$, sodium arsenite $\{NaAsO_2\}$ among others have been used. Flourides include salts of hydrofluoric acid $\{HF\}$, fluorosilicic acid $\{H_2SiF_6\}$ and fluoroaluminic acid $\{H_3AlF_6\}$. Specific examples are sodium fluoride $\{NaF\}$, sodium fluorosilicate (Na_2SiF_6) and sodium fluoroaluminate (cryolite) (Na_3AlF_6) among many others (Kirk & Othmer, 1981).

Many synthetic organic insecticides have also been used to control insects. DDT (**1**) was synthesized in 1874 and its insecticidal properties discovered in 1939 (Kirk & Othmer, 1981). In the mid 1940s to mid 1960s DDT (**1**) was the most successful pesticide with tremendous benefits. It has been employed for control of several insects and is still widely used for control of insect vectors of public health importance like mosquitoes. DDT (**1**) analogues synthesized and attained commercial importance as insecticides include 1,1-dichloro-2, 2-*bis*-(*p*-chlorophenyl) ethene DDD (**2**) and TDE (**3**). DDD (**2**) and TDE (**3**) have the same environmental and resistance problems. Methoxychlor, 1,1,1-trichloro-2, 2-*bis* (*p*-methoxyphenyl) ethane (**4**) is related to DDT (**1**) and has also been used as an insecticide (Kirk & Othmer, 1981). It gives a rapid knockdown of many insects than DDT (**1**).
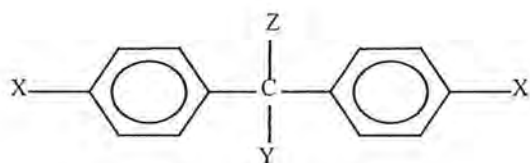
	X	Y	Z
1.	Cl	CCl_3	H
2.	Cl	$=Cl_2$	-
3.	Cl	$CHCl_2$	H
4.	OCH_3	CCl_3	H

The advent of synthetic pest control agents saw the birth of industries worth billion dollars. These synthetic wonders have facilitated astonishing gains in agricultural production. Moreover, the same compounds used in food crop protection have serious social and environmental repercussions. There are problems of rapidly increasing costs of modern synthetic organic pesticides, pest resurgence, detrimental effects on non-target organisms and environmental quality (Blagbrough *et al.*, 1992). Unfortunately, most insecticides like DDT **(1)** and its analogues are ineffective due to the resistance developed by insect pests. Moreover, DDT is non-biodegradable hence a major environmental problem. The methoxy groups in Methoxychlor are readily dealkylated *in vivo* by microsomal oxidases producing phenols that are easily eliminated. Unlike DDT **(1)**, methoxychlor **(4)** does not accumulate in nature and so it is favoured for general environmental use. Unfortunately, insects that are resistant to DDT **(1)** show cross-resistance to methoxychlor **(4)** (Kirk & Othmer, 1981).

Cyclodienes are polychlorinated cyclic hydrocarbons with endomethylene-bridged structures. Discovery of 2,3,4,5,6,7,8,8-octachloro-2, 3,3a, 4,7,7a-hexahydro-4, 7-methanoinoindene (Chlordane) **(5)** in 1945 resulted in the development of other chlorinated cyclodienes like indene **(6)** (Kirk & Othmer, 1981). Other chlorinated cyclodiene insecticides include 1,3,4,5,6,7,8,8-octachloro-3a, 4,7,7a-tetrahydro-4, 7-methanophthalon (telodrin) **(7)**, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-imethanonaphthalene (aldrin) **(8)**, lindane, hexachlorocyclohexane **(9)** and benzene hexachloride (BHC) **(10)**.

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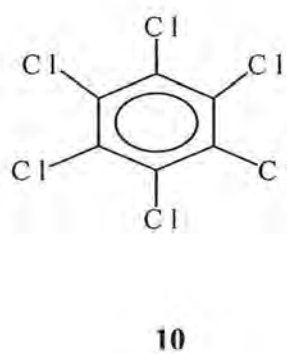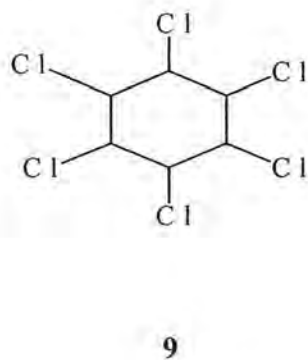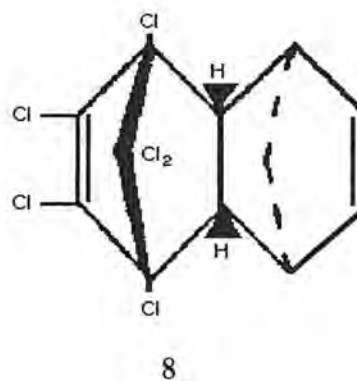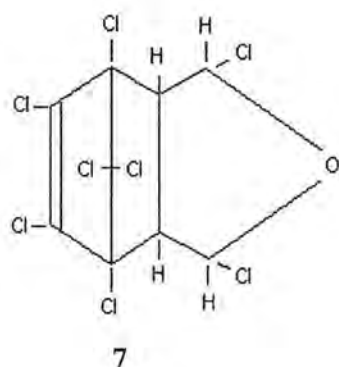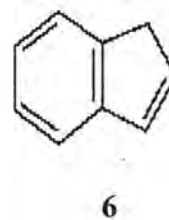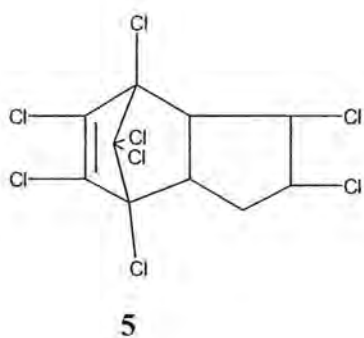
Organophosphorus insecticides include parathion (*o,o*-diethyl-*o,p*-nitrophenyl phosphorothio nate) **(11),** methylparathion (*o,o*-dimethyl-*o,p*-nitrophenylphosphorothionate) **(12),** chlorthion (*o*-3-chloro-4-nitrophenyl-*o,o*-dimethylphosphorothionate) **(13)** and dicapthion **(14)** among others.

	X	Y	Z
11.	H	H	OC_2H_5
12.	H	H	OCH_3
13.	Cl	H	OCH_3
14.	H	Cl	OCH_3

Compared to chlorinated hydrocarbons, organophosphate insecticides are non-persistent. Despite the advantage this group of insecticide has two major set backs: i) they are very toxic to mammals; and ii) they are rapidly broken down in moist conditions.

Examples of carbamate insecticides include 1-naphthyl-N-methyl carbamate (**15**) and 2-isopropyloxyphenyl-N-methyl carbamate (baygon) (**16**).
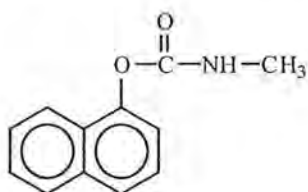
15
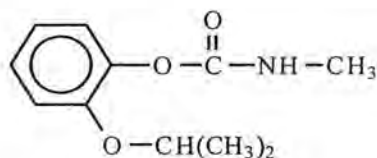
16

Cross-resistance to organochlorine, organophosphate and organocarbamate insecticides has been reported (Kirk &Othmer, 1981).

New brands of chemical pesticides are permeating the market routinely. Furthermore, there will be a continued increase in the quantity of chemical pesticides to control the increasing number of insect pests occasioned by their resurgence due to resistance development to chemicals insecticides. The indiscriminate use of chemical pesticides is known to increase cost of agricultural production and lower profit margins, has deleterious and pollutant effects on the environment, pose health hazards and induce ecological imbalances (Borrecco *et al.*, 1979; Ware, 1980).

It is imperative to investigate other environmentally friendly alternative pest control techniques that would minimize the cost of crop protection (Saxena, 1989; Gunn& Steren,

1976). The use of insect and disease resistant cultivars is one such approach. However, the development of such requires a thorough understanding of natural crop-resistance mechanisms. Which is a very expensive venture. Thus, the discovery of novel chemical compounds with agrochemical activity would have a dramatic impact on agriculture over the next decades. Because of the obvious need for novel non-cross resistant, biodegradable, and less toxic pest control products, natural products are investigated in this work.

1.5 Natural products for human welfare

Chemical substances derived from animals, plants and microbes have been used to treat human disease since the dawn of medicine. The investigation of natural products as source of novel human therapeutics reached its peak in the Western pharmaceutical industry in the period 1970-1980, which resulted in a pharmaceutical landscape heavily influenced by non-synthetic molecules. Of the 877 small-molecule New Chemical Entities (NCEs) introduced between 1981 and 2002, roughly half (49%) were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural-product pharmacophores (Newman et al., 2003). This is because natural products are readily biodegradable, selective and generally have low toxicity.

Scientists have shown special interest in biologically active compounds (from arthropods), such as vitamins, toxins, amino acids, hormones and other substances, which influence the life process of any organism. More attention has been turned to natural products in a bid to address the problem of resistance and indiscriminate use of pest control products. To this end a number of candidate compounds are under development.

1.5.1 Arthropod defense secretions

Arthropods as a group are endowed with efficient chemical defense systems. The weaponry serves both offensively (for incapacitation of prey) and defensively (against predators). Over half of all terrestrial arthropod orders contain species that use chemical deterrents (Whitman et al., 1990). Defense secretions by arthropods are a source of a large number of novel natural products that have fascinated man for along time because of their dramatic excitatory and depressant pharmacological effects (Harvey, 1995). Arthropods that produce insecticidal secretions include; insects, spiders and scorpions (Jacobson & Crosby, 1971). Insects that produce insecticidal secretions are predators belonging to Hemiptera (ruduviids, pentatomids, notonectids, belostomids), Diptera (tabanids, asilids, empids) and parasitic

Hymenoptera. Some Coleoptera and Neuroptera also producee insecticidal secretions (Jacobson & Crosby, 1971).

Arthropods secret various compounds during biting and stinging, leading to subcutaneous introduction of substances with dramatic physiological or pathological effects on plants and animals (Jacobson & Crosby *et al.*, 1971). From an ecological point of view, the use of chemicals for defense or obtaining food may be considered as an efficient and energy-saving method. Secretion of chemicals has a high requirement on the organism, and they are emitted in low but effective doses (Eisner *et al.*, 1978). The scorpion venom organ is a perfect example of this principle. Scorpions feed on freshly killed prey, consisting of arthropods and soft-bodied insects. However, as predators, scorpions appear to lack certain essential anatomical and physiological qualities. They move relatively slowly, are practically blind and have a rather undeveloped sense of smell. They actually do not seek their food actively but wait for their prey to approach their lairs (Stahnke, 1966). Thus a device for paralyzing the prey at the earliest moment of contact is essential. This is achieved by the venom, which is employed against arthropods and insects. This specific function is believed to be associated with highly specified chemical adaptations.

The specific action of scorpion toxins against insects provides possibilities for using them as neuropharmacological tools to study insect neuromuscular processes, candidate recombinant baculovirus 'warheads' and models for designing selective insecticides (Kozlov *et al.*, 1999).

Studies on arthropod defense secretions that can be used to develop recombinant bio-pesticide as a safer alternative to broad-spectrum chemical insecticides in Kenya are being conducted by the International Center of Insect Physiology and Ecology (ICIPE) as part of the bio-prospecting strategy to generate new effective pest control products that are safe to the environment. This will reduce over dependence on synthetic chemical pesticides and minimize their deleterious and pollutant effects.

CHAPTER TWO
LITERATURE REVIEW

2.1 The scorpions

Scorpions are venomous arthropods of the class Arachnida, and among the oldest (400 million years) living groups of animals (Lourenco, 1994). They are represented by 1500 distinct species (Lourenco, 1994) worldwide characterized by an elongated body and a segmented tail that is tipped with a venomous stinger. All of the potentially lethal scorpions belong to the family Buthidae, with the exceptions of one genus, Hemiscorpion, which belongs to the family Scorpionidae (Ischnuridae). Buthoid venom has been reported to strongly affect a wide variety of vertebrate and invertebrate organisms and its toxicity is attributed to the presence of a large variety of polypeptides cross-linked by 3-4 disulfide bridges (Zlotkin *et al.*, 1978; Rochat *et al.*, 1979).

2.2 Biosystematics and distribution of scorpion species in the world

According to Vachon (1952), numerous morphological characteristics serve to divide the order of scorpions into two groups the buthoids and the chactoids, without designating them as sub-orders. The buthoids consist of a single family, Buthidae, while the Chactoids include five others (Chactidae, Scorpionidae, Diplocentridae, Buthriudae and Vijodae). In addition, a characteristic biochemical feature is one of the classical differences between these groups. This follows the establishment of taxonomic characteristics of hymolyph protein by establishment of polyacrylamide gel electrophoregram (PAGE) (Goyffon and Kovoor, 1978). In this analysis, the hymolyph proteins of about 50 species, all showed one band, the slowest of the migrating fractions to be characteristic of the Buthidae. Buthidae comprises about 60% of the species described and the largest family of scorpions with more than 500 species distributed throughout the world (Shulov & Levy, 1978).

Although all known species of scorpions posses venom, only 25-50 species are considered medically important (Keegan, 1980). They are mainly found in the tropics (Fig. 1).

Figure 1: Distribution of scorpions of medical importance (Bucherl, 1971)
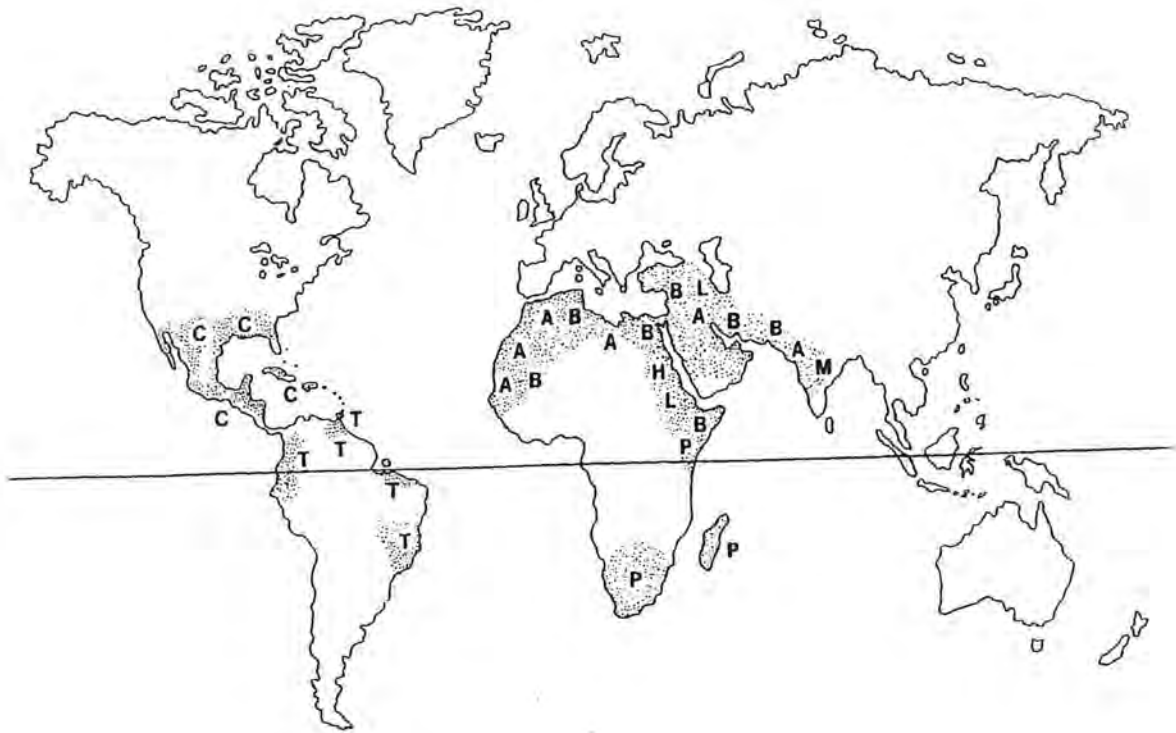
Androctonus (A), *Buthus* (B), *Centruroides* (C), *Hottentotta* (H), *Leiurus* (L), *Mesobuthus* (M), *Parabuthus* (P), *Tityus* (T)

Centruroides are found in the southern states of USA, Mexico, Central America, and the Caribbean. *Centruroids exilicauda* is found in Mexico and the southwestern parts of USA primarily in Arizona and parts of Texas, New Mexico, Nevada, and California. *Tityus* are found in Central and South America and the Caribbean while *Buthus* are dominant across the Mediterranean, Middle East and eastern Africa. *Mesobuthus* have been found in southern and central Asia. *Buthotus (Hottentotta)* are found in northern Africa. In this work we report it in East Africa (Kenya). *Leiurus* is found in eastern Africa and the Middle East while *Androctonus* has been reported in northern Africa to South East Asia.

The genus *Parabuthus* is composed of approximately 30 known species and all but a few are found in South Africa. In this work we report *Parabuthus leiosoma* and *Parabuthus pallidus* in

East Africa (Kenya). Several of species in this genus (*P. leiosoma, P. granulatus, P. pallidus* and *P. fulvipes*) have potent venoms (Inceoglu *et al.*, 2001).

Parabuthus leiosoma (Plate 1.) are found in several countries in East Africa (Probst, 1973). Adults are of medium size for scorpions and have yellow to yellowish-red body colour, except for part of metasoma and telson, which are red/brown. They have small, slender pedipalps and a thick, powerful metasoma. It was previously named *P. liosoma*, but later corrected to. *P. leiosoma* (Rein, 2003).

Plate 1: *Parabuthus leiosoma*

2.3   Habits and habitat of scorpions

Scorpions are commonly thought of as desert animals, but in fact they occur in many other habitats as well, including grasslands and savannahs, deciduous forests, mountainous pine forest, caves in the sea (littoral scorpions) and just about anywhere except perhaps the artic and Antarctica. Many species can and do dig burrows in the soil to create a more conducive micro-habitat, which may partially explain their vast distribution (Francke & Boos, 1986). Most scorpions would not survive if exposed to extreme environmental conditions. Extreme heat or cold kills them but the microhabitat within their burrows reduces such effects allowing some to survive in such conditions. Scorpions are nocturnal; a behavior that helps them manage temperature and water balance, important functions for survival in dry habitats (Rudd, 1996)

Scorpions detect and capture prey by the sense of touch. They also have an extremely well developed sense of hearing. However they do not see well and have poor sense of smell. Scorpions hide under stones, bark, wood or other objects on the ground where they wait or search for prey. A few species exhibit social behaviors beyond the mother-child association, such as over-wintering aggregations, colony burrowing and sometimes even living in extended family groups that share burrows and food (Ward, 1996).

2.4   Biology of scorpions

2.4.1 Morphology

All scorpions have a long, slender body with a five-segmented tail that can be arched over the back. The tail ends in a bulb-like poison gland or stinger (telson). Scorpions have four pairs of legs and two large pincer-bearing arms (pedipalps) in front. They are well equipped to defend themselves or attack prey with their pincers and stinger. Between the last pair of legs is a comb like structure (pectines) that is used to identify surface textures and to detect prey (McCormick & Polis, 1990) (Plate 2&3)

Although scorpions have two eyes on the top of the head, and usually two to five pairs of eyes along the front corners of the head, they do not see well. They must rely on the sense of touch, using their pectines and other organs for navigation and hunting. The flat body allows them to hide in small cracks, under rocks and barks. Scorpions range in size from 0.5-7.5" in length (including the tail) depending on the species (McCormick & Polis, 1990).

Plate 2: Dorsal view of a scorpion (http://wrbu.si.edu/www/stockwell/photos/photos.html)

CARAPACE
CHELICERA
PEDIPALP — PROSOMA
LEG I — MESOSOMAL TERGITE I
MESOSOMAL TERGITE II
MESOSOMAL TERGITE III
MESOSOMAL TERGITE IV
LEG II
MESOSOMAL TERGITE V
LEG III
MESOSOMAL TERGITE VI
LEG IV — MESOSOMAL TERGITE VII
OPISTHOSOMA
METASOMAL SEGMENT I
METASOMAL SEGMENT II
TELSON
METASOMAL SEGMENT III
METASOMAL SEGMENT IV — METASOMAL SEGMENT V

Plate 3: Ventral view of a scorpion (http://wrbu.si.edu/www/stockwell/photos/photos.html)

Labels on figure:

CHELICERA

PEDIPALP — PROSOMA

STERNUM

GENITAL OPERCULUM

LEG I

PECTEN

LEG II

MESOSOMAL STERNITE III

MESOSOMAL STERNITE VI

MESOSOMAL STERNITE V

LEG III

MESOSOMAL STERNITE VI

MESOSOMAL STERNITE VII

LEG IV

OPISTHOSOMA

TELSON

METASOMAL SEGMENT I

METASOMAL SEGMENT II

METASOMAL SEGMENT V

METASOMAL SEGMENT III

METASOMAL SEGMENT IV

2.4.2 Life cycle of a scorpion

Following an elaborate mating process, which lasts from 24 to 36 hours, the female undergoes a gestation period ranging from 5 months to greater than 1 year (depending on species) in which the young develop as embryos in the female ovariuterus. Adult scorpions may have several broods of young ones. The young are born alive in semi-transparent sacs. A female gives birth to about 25-35 young ones. They remain on her back until they molt for the first time, usually within a 1-2 weeks (McCormick & Polis, 1990). As soon as the young scorpions free themselves from the thin wrappers, they climb onto their mother's back. The young scorpions leave the mother after several days and begin to fend for themselves and are capable of stinging. Once they climb down, they assume an independent existence, and periodically molt to reach adulthood. Scorpions reach maturity in a year or more, depending on the availability of food. Five or six molts over two to six years are required for the scorpion to reach maturity. Most scorpions live for three to five years, but some species live for 10-15 years (Polis, 1990).

2.4.3 Feeding habits of scorpions

All scorpions are predators. Depending on locality, availability of food, species and size, the prey includes spiders, isopods, insects, myriapods (centipedes), diploid (millipedes), crabs, mice, birds, lizards and snakes (Polis, 1979; McCormick & Polis, 1990). The list is long and the general rule is that a scorpion may prey on any creature smaller or equal to itself but occasionally on larger ones. This is made possible by the venomous sting. Preys are located primarily by sensing vibrations (Pocock, 1893).

Scorpions grasp with pincers, arch their tails over their bodies and deliver venom with stingers (telson). They inject venom from glands located lateral to the tip of the stinger. Once the prey is captured, they use the large pincers to crush, draw it toward the mouth and suck the body juices (Casper, 1985).

Despite its thick armour, powerful pincers and venomous stings, scorpions have predators. Mammals, frogs, salamander, other predating insects, spiders and birds prey upon scorpions. Some animals seem to have partial immunity against that of scorpion venoms while others may have thick hides or fur to prevent envenomation, and yet others device numerous tactics to tackle this nutritious 'food item' (Casper, 1985). Significant predators of scorpions are other scorpions. Large scorpions prey upon smaller ones. Female scorpions sometimes eat males (except for some *Tityus spp* where the reverse is true). Predation may be within the

same species (intraspecific) or between species (interspecific). Polis (1990) in his studies of sand scorpion, *Smeringerus (Paruroctonus) mesaensis*, observed that almost half of the scorpions prey on others (Polis, 1979).

2.5 Scorpion venom

Scorpions have developed the ability to produce and deliver small quantities of very potent toxins to their prey (DeBin *et al.*, 1993). Venoms of certain scorpions are known to contain several toxins, which are selectively toxic to insects (Zlotkin *et al.*, 1971a; 1972a; Minton, 1974; Lazarovici *et al.*, 1982). Consequently, the venom is one of the arthropod defense secretions that has received much attention and has played an important role in folklore and medicine since ancient times (Costa-Neto, 2005). Indeed scorpion envenomation (tarantism) is among the subjects about which there has been a lot of speculation. In the last decade or so, a large number of scientific papers have been devoted to arthropod venoms. Of late, the chemistry and mode of action of several arthropod venoms have been thoroughly studied and some of these substances will probably be used as pharmacological tools and therapeutic agents.

The venom is secreted by a pair of ampullated glands located in the last caudal segment (telson), each of the glands has its own efferent duct, extending to the curved sting, and opening separately on each side near the tip of aculeus (Bucherl, 1971). It is a complex mixture of water, salts, small molecules, peptides and proteins (Inceoglu *et al.*, 2003). In its natural condition, venom is somewhat opalescent, milky translucent substance with a pH of 7.12 (Stahnke, 1966). When the lyophilized venom is reconstituted in water it contains some insoluble material, most of which is cellular debris. The water filtrate of this solution contains the toxic factor. The toxicity of scorpion venom is due to the presence of numerous polypeptides containing 30-70 amino acid residues bridged by three or four disulfide bridges (Lala & Narayanan, 1994; Possani *et al.*, 1999). The chains constitute toxins that exert physiological effect by specifically interacting with the voltage-dependent ion-channels (Lazarovici *et al.*, 1982).

The venom can be characterized by defining the individual components of the system (identification of peptide toxins), analysis of the structure of the components (primary, secondary and tertiary structure determination), analysis of the function of each component (determination of the mode of action), analysis of the relationships between these components (synergism) and the target sites or environment (binding sites and kinetics). The

knowledge of a phenomemon depends on the way in which it is observed (Inceoglu *et al.*, 2003).

2.6 Toxicity acquisition and toxin diversity in scorpions

Generally animal secreting or applying toxins either use their own genetic and metabolic machinery or that of other organisms for the production of these compounds. Two methods for intrinsic synthesis of toxin are available. These include gene expression leading directly to peptide or protein toxin through a complex metabolic pathway, that includes numerous chemical reactions (catalyzed by specific enzymes) leading to secondary metabolites exhibiting allelochemical activity (toxicity). Alternatively, toxicity can be by uptake, accumulation and storage of toxic compounds produced by another organism (microbe, plants or other animals) through feeding or association. In animals, both strategies have been equally adopted (Mebs, 2001)

In complex metabolic pathways, like in scorpions and other terrestrial and marine animals, a large number of toxic compounds (secondary metabolites) are synthesized denovo, and used mainly as allelochemicals for defense against predators and for immobilizing prey during food acquisition. During evolution a lot of "combinatorial chemistry" where compounds with similar structural properties are synthesized goes on. In such cases selection pressure is considered to trigger the biosynthetic process and shape the optimal structure. When the product ideally serves the function it may have been designed for, variability of a particular compound in the particular organism appears to be low (Mebs, 2001).

Toxin diversity increases dramatically by enlarging the gene pool encoding toxic peptides or proteins. Point mutations, gene duplications and recombination coupled with post-translation modifications of the gene products lead to a wide array of peptide and protein toxins (Mebs, 2001). Natural selection is assumed to sort out inappropriate and inefficient genes.Variability in venom composition occurs practically at all levels; interfamilial, intergeneric, inter- and intraspecies (Chippaux *et al.*, 1991). In addition, scientists have reported cases where scorpions of the same species but different locality (geographic race) have different venom potency. This is represented in *Buthus occitanus* venom that is lethal in North Africa (Algeria) but does not have the same properties in South France (Balozet, 1971). This emphasises the need to test venoms from different scorpion species in various geographical locations to discover the existing diversity. Indeed data is accumulating on the

high degree of venom variation under genetic control. However, the genetic mechanisms that underlie the high variability of toxic venomous peptides and protein remain unknown (Mebs, 2001)

2.7   Chemistry of scorpion venoms

The history of the study of scorpion venom composition parallels the new concepts and techniques developed in protein chemistry. The low mode of preparations as used by Wilson (1904), based on saline (0.15 M) extraction of telsons and preparation by ethanol, later adopted with certain modifications by Mohammed (1942) and Adam and Weiss (1959), did not result in pure products. This process involved grinding dried telsons in a mortar and extracting the 'venom' with distilled water, physiological saline or glycerine. This method is still used by some producers of antivenin. Obviously the technique extracts other soluble products present in the whole telson.

Contemporary methods obtain venom by electric and manual stimulation (Gopalakrishnakone & Gwee, 1995; Lazarovici et al., 1982). Electric stimulation causes muscles to contract and expel the venom, which is collected in special pipettes. A pair of tweezers, each arm of which is connected to, provides + and - poles of a square wave generator, and can be placed around the area containing the venom glands: telson for scorpions and chelicerare for mygalomorph spiders. Stimulation is done at appropriate voltage, whereby a field builds up around the muscle cells and depolarizes them, causing a brief contraction. In order to prevent breaking the aculeus, the orifice of the pipette must be great enough to accommodate for this movement. The voltage frequency should be high enough to elicit a continuous contraction and applied for only 4-5 seconds. Very high voltage causes the muscles to die from overwork while too little do not set up enough fields. The selection of optimum voltage therefore requires experimentation. Some workers have used up to 150 V for Heterometrus scorpions (Gopalakrishnakone & Gwee, 1995).

Pure venoms have been achieved through the introduction of modern column chromatographic techniques based on gel filtration and ion exchange (Wudayagiri et al., 2001). Detailed chemical analysis of scorpion venom has been possible following recent advances in electrical and manual stimulation milking techniques of venom from scorpion (Gopalakrishnakone & Gwee, 1995; Lazarovici et al., 1982) and sensitive analytical techniques like Nuclear Magnetic Resonance (NMR) spectroscopy, Mass Spectrometry (MS), Reverse

Phase High Performance Liquid Chromatography (RPHPLC) and automated peptide sequencing.

Scorpion venoms are complex mixtures of neurotoxins (toxins which affect the nervous system). The lethal and paralytic activity of scorpion venom is due to presence of polypeptide neurotoxins of low molecular weight (Zlotkin, 1973). A typical scorpion venom contains numerous toxins, biogenic amines, enzymes, salts, water and other unidentified substances. Each species has a unique mixture. The scorpion venom has been found to be a rich source of various polypeptides with diverse physiological and pharmacological activities, and generally act via target specific modulation of ion-channel function (Wudayagiri et al., 2001; Possani, 1984; Froy et al., 1999a). The polypeptide toxins are single chain composed of 60-70 amino acids and cross-linked by four disulfide bridges, the other type contain peptides with 30-40 amino acid residues and three disulfide bridges (Gong et al., 1998).

2.7.1 Amino acid sequencing

Protein sequencing, the determination of the amino acid sequence of a peptide or protein, has come a long way since the structure of the protein insulin was determined (Finelli, 1990). It provides information about the amino acid residues, which make up the protein, and specifically how they are arranged. Studies seeking to characterize both natural and recombinant proteins utilize this technique (Findlay & Geison, 1989). The fields of protein sequence and structure determination are ever expanding, as science demands more information about these macromolecules that hold the key to life. The technique has evolved from a manual process through automation up to pairing with DNA sequencing for the increasingly faster elucidation of amino acid sequences for peptides and proteins. With its many uses and the array of questions still left unanswered, protein sequencing is proving to be a bio-technique that will be required more frequently in the future.

The chemical processes employed in the protein sequencer to determine the amino acid arrangement based on Edman degradation (Edman, 1970) as summarized in scheme 1. Briefly, phenylisothiocyanate (PITC) (18) reacts with the amino acid residue at the amino terminus of a protein (17) under basic conditions (provided by N-methyl piperidine/methanol/water) to form a phenylthiocarbamyl derivative (PTC-protein) (19). Trifluoroacetic acid is then used to cleave off the first amino acid as its anilinothialinone derivative (ATZ-amino acid) (20) and leaves the new amino terminus (21) for the next

degradation cycle. The ATZ amino acid (20) is then removed by extraction with n-butyl chloride and converted to a phenylthiohydantoin derivative (PTH-amino acid) (22) with 25% TFA/water. Other by-products like DPTU (24), DPU (25) and PMTC (26) are also formed during the Edman degradation as shown in scheme 2. The PTH-amino acid is transferred to a reverse-phase C_{18} column for detection at 270 nm. A standard mixture of 19 PTH-amino acids is also injected onto the column for separation (usually as the first cycle of the sequencing run). This chromatogram provides standard retention times of the amino acids for comparison with each Edman degradation cycle chromatogram. The HPLC chromatograms are analysed using a computer data analysis system. To determine the amino acid present at a particular position, the chromatogram from the residues of interest is compared with the chromatogram from the previous residue by overlaying one on top of the other. From this, the amino acid for the particular residue can be determined. This process is repeated sequentially to provide the N-terminal sequence of the protein/peptide.

Scheme 1: Edman degradation on the N-terminal amino acid sequence
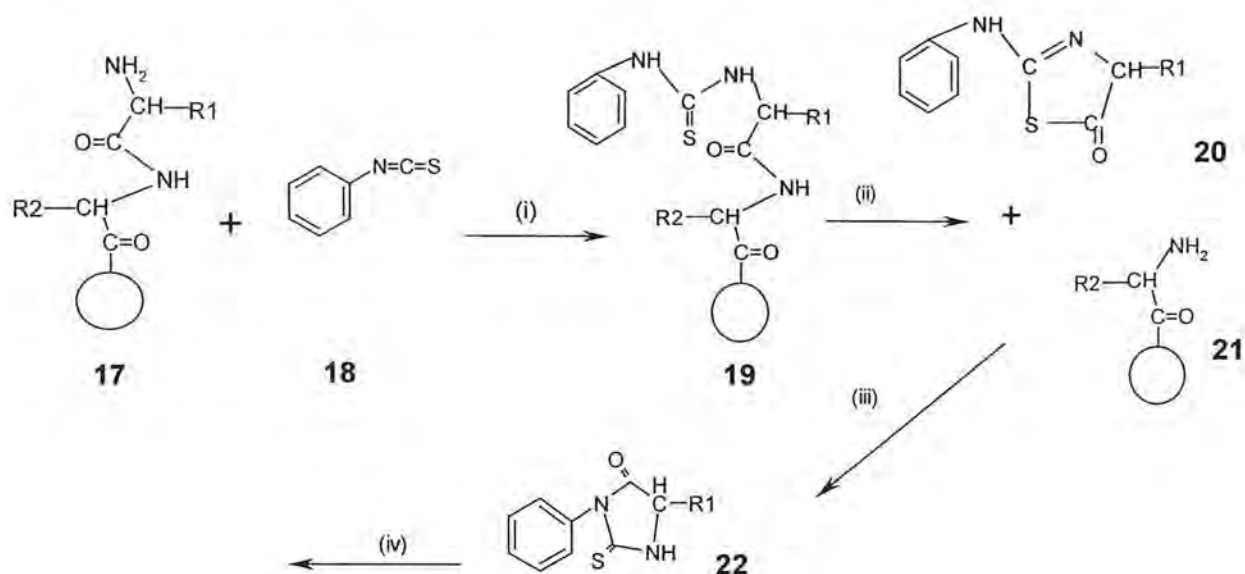
i)   5% PITC in n heptane, N-methyl piperidine/H_2O/MeOH
ii)   n –BuCl/EtOAc, TFA
iii)   n- BuCl/EtOAc, 25% TFA/H_2O
iv)   Evaporate, 20% Acetonitrile/H_2O

Scheme 2: By products of Edman degradation of the *N*-terminal sequence of peptides
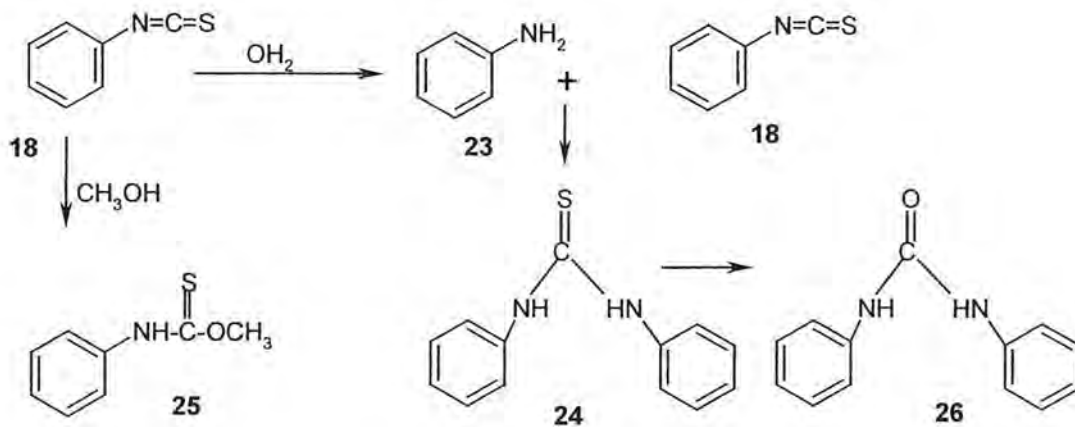
All amino acids possess the same generalized structure (**27**) and the only difference between them is the nature of the R- group. The 20 possible side-chain for DNA encoded amino acids are summarized in Appendix I.

27
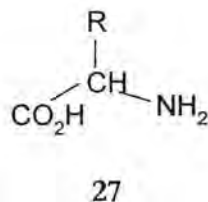
2.7.2 Composition and structure of scorpion toxins

Due to target specificity of toxins, studies on selective anti-insect polypeptides toxin has been a subject of great interest in a bid to come up with insect pest control tools that are environmentally friendly (Pelhate *et al.*, 1998). This has resulted in isolation and characterization of a large number of peptide toxins for the past two decades. Possani (1984) reported 80 toxins in the venom of several scorpion species. With rapid scientific advances, many more are presumed to have been discovered to date. Most of these toxins have low histidine and phenylalanine, no methionine and are highly hydrophobic and basic. There are numerous cysteine residues giving rise to stable disulphide bonds in tertiary structure. The neurotoxins isolated so far can be classified as α and β - toxins based on their physiological kinetics. A few examples are presented in figure 2.

Figure 2. The alignment of selected amino acid sequence of isolated scorpion toxins showing disulfide bridges (http://www.ebi.ac.uk/emboss/align/).


```
                10        20        30        40        50        60        70          %S   %I
                 |         |         |         |         |         |         |
PhTx    ----KFIK-HKDESFIECQQLIGTQQY-CVDACQAHGSK-EKGICKGHAPFGLPGGCYCPKLPDNRVRHCFGALESK-CA--   100  100


LqqV    -LKDGYIV-DDKNCTFFC--G--RMAY-CNDECKKKG--GESGYCQWASPYGN--ACWCYKLPDRVSIKEKG-----RCN--    49   30
BoII    -GRDAYIA-QPENCVYEC--A--XNSY-CHDLCTKNG--AKSGYCQWLGRWGN--ACYCIDLPDRVPIRIEG-----KCKF-    46   30
AmmV    -LKDGYII-DDLNCTFFC--G--RMAY-CDDECKKKG--GESGYCQWASPYGN--ACWCYKLPDRVSIKEKG-----RCN--    44   31
MeM9    -ARDAYIA-KPKNCVYECYNP--KGSY-CNDLCTENG--AESGYCQILGKYGN--ACWCIQLPDNVPIRIPG-----KCK--    44   31
BMK1    -VRDAYIA-KPKNCVYEC--A--RNBY-CNDLCTKNG--AKSGYCQWVGKYGN--GCWCIELPDNVPIRVPG-----KCH--    44   31
EmKM8   -GRDAYIA-DSENCTYFC--G--SNPY-CNDVCTENG--AKSGYCQWAGRYGN--ACYCIDLPASERIKEPG-----KCG--    41   32
BotIII  -VXDGYIV-DDRNCTYFC--G--RMAY-CHEECTKLX--GESGYCQWASPYGN--ACYCYKVPDHVRTKGPG-----RCN--    39   28
AaHII   -VKDGYIV-DDVNCTYFC--G--RMAY-CNKECTKLK--GESGYCQWASPYGN--ACYCYKLPDHVRTKGPG-----RCH--    38   29
Bo XI   -LXDGYIV-DDRNCTYFC--G--TMAY-CNEECVKLK--GESGYCQWVGRYGN--ACWCYKLPDHVRTVQAG-----RCRS-    38   26
AaH3    -VRDGYIV-DSKNCVYHC-VP--P----CDGLCKKNG--AKSGSCGFLIPSGL--ACWCVALPDNVPIKDPSY----KCHSR   33   24
LqhVII  -VRDGYIA-KPENCAHHC-FP--GSSG-CDTLCRENG--GTGGHCGFKVGHGT--ACWCNALPDKVGIIVDGV----RCH--    30   22


Ta7     --KEGYLMD-HEGCKLSCF--IRPSGY-CGRECGI--KKGSSGYCAW-------PACYCYGLPNWVKVWDRA---THKC---    35   28
Cn2     --KEGYLVDRNTGCKYECLK-LGDNDY-CLRECKQQYGKGAGGYCYA-------FACWCTHLVEQAIVWPLPN---KRCS--    30   23
Birtoxin ADVPGNYPLDKDGNTYKCFLLGGNEE--CLNVCKL--HGVQYGYCYASK-------CWCEYLEDDKDSV-----------    38   21


Depressant
AaITS   ---DGYIK-RHDGCKVTCLIN---DNY-CDTECKREG-GSYGY-CYSV---GF--ACWCEGLPDDK-AWKSET---MTCD--    32   22


Excitatory
Bj'xtrIT -KKNGYPLDRNGKTTE-CSGVNAIAPHYCNSECTKVYYAESGY-CCW-------GACYCFGLEDDKPIGPMKDITKKYCDVQIIPS  22   17
AaHIt1  -KKNGYAVDSSGKAPE-CLL----SNY-CNNQCTKVHYADKGY-CCL-------LSCYCFGLNDDKKVLEISDTRKSYCDTTIIN   21   15
```

AaH, *Androctonus australis* Hector; Amm, *Androctonus mauretanicus mauretanicus*; Bj, *Buthotus judaicus*; Bot, *Buthus occitanus tunetanus*; Cn, *Centruroides noxius*; Lqh, *Leiurus quinquestriatus hebraeus*; Lqq, *L. q. quinquestriatus*; Me, Mesobuthus eupeus; Bo, Buthus occitanus; Bm, Buthus martensi Karsch; Ts, Tityus serrulatus. The S-percentage similarity, I-identity to Phaiodotoxin. The brackets indicate how the disulfide patterns are arranged. - indicate the disulfide bridges common to all of them, ----- lines are special disulfide pairing specific to the toxin. - are introduced to increase similarities. The alignments were obtained with the program CLUSTAL-X. Similarities and identities were calculated using the pair wise alignment algorithms by EMBOSS

Phaiodotoxin is shown in the first line (PhTx) and two additional groups of sequences are shown thereafter. The first group (11 sequences) consists of α scorpion toxins (α-ScTXs), while the second (6 sequences) β-Scorpion toxins (β-ScTXs). Birtoxin is the shortest of the selected scorpion toxins for the above alignment. The depressant and the long-chain excitatory toxins are in the last two lines. All the toxins have a conserved core of three disulfide bridges as shown in the figure 2. However, the fourth disulfide pair of the excitatory toxins has a distinct pattern. Exceptions include birtoxin, which has only three disulfide bridges and is the shortest one.

2.8 Pharmacology and toxicology of scorpion venoms

Bioassay methods are frequently used in toxicological studies to evaluate the effect of chemicals and pathogens on test organism. These provide data on acute oral and dermal toxicity, and on reproductive and developmental impairment (Wilkinson, 1987). Rosen & Greth (1987) quoted Frawly et al., (1957) who used oral administration of 3 μg of parathion, 4 μg of ethyl p-nitrophenylthionobenzenephosphonate (EPN), 5 μg of lindane and 15 μg of DDT in houseflies in their bioassay. Heinrichs et al., (1981) suggested simple bracketing in non-replicated experiments and ending with at least three replications in the final tests for bioassays to determine LD_{50} values of insecticides in the laboratory. They also suggest that quantities be administered as 0.2 μl for plant hopper and leafhoppers and 1 μl for spider and larger insects. However, dosages used by different researchers may vary considerably depending on type of bioassay and whether administered per open space, sub-cutaneously or topically. Probit analysis (Finney, 1971) is a useful tool for estimation of quantals and dose responses, and has been used by many researchers to estimate lethal concentration (LC) as well as effective dose (ED) or effective concentrations (EC) of elements, compounds or pathogens and their products against test organisms. LD_{50} gives the dose of the test substance that would kill 50% of test organism in a given period of time (usually 24 h, 48 h, 72 h or 96 h).

Based on their effects on specific ion Na^+, K^+, Ca^{2+} and Cl^- channels, scorpion venoms are classified into four groups namely; Sodium, Potassium, Calcium and Chloride groups (Garcia et al., 1997b; Gordon et al., 1998; Tytgat et al., 1999; Valdivia & Possani, 1998; Possani et al., 1999). They are also classified according to the peptide length (the number of amino acid residues), as long and short chain toxins (Garcia et al., 1997b; Possani, 1984). Long chain toxins contain 60-70 amino acid residues with four disulfide bridges (Possani, 1984). Their targets are the voltage sensitive Na^+ channels of excitable muscle and nerve cells. The Na^+ channel toxins have been grouped into α-and β-toxins, based on their physiological kinetics in Na^+ channels. The α-toxins prolongs the action potential by blocking Na^+ channel inactivation (Dauplai et al., 1995). The β-toxin on the other hand, shift the voltage of activation towards more negative potential thereby affecting Na^+ channels activation and promoting spontaneous and repetitive firing (Pintar et al., 1999).

The difference in the effects of the α- and β-toxins is attributed to their ability to bind to two distinct sites on the Na$^+$ channel receptor. The α –toxin binds to site 3 while β-toxins bind to site 4 of the Na$^+$ channel (Jover et al., 1980). Alternatively, Na$^+$ channel toxins are classified based on their target organisms as insect, mammal and crustacean specific toxins (Garcia et al., 1997). Some toxins act across species. An example is AaH IT4, which affects both insects and mammals (Loret et al., 1991).

It has also been reported that a group of short-chain scorpion toxins that interacts with the voltage-dependent or Ca$^+$ activated K$^+$ channels or with Cl$^-$ channels exists (Wudayagiri et al., 2001). Voltage-gated K$^+$ toxins typically contain 31-38 amino acids with three or four disulfide bridges (Possani et al., 1999). Recently, a 23-residue toxin purified from *Tityus cambridge* was reported to block K$^+$ channels (Batista et al., 2000).

2.9   Potentials of scorpion toxins in agriculture

Scorpion toxins have been put to different uses, and demonstrated that their folding pattern could be used as templates for creating stable molecules with different biological properties (Harvey, 1995). In particular, a metal binding site typical of carbonic anhydrate was engineered onto the molecular scaffold of charybdotoxin, opening the possibility of producing small, stable proteins with wide variety of biological and therapeutic activities (Harvey, 1995).

Following the discovery of scorpion neurotoxins with total specificity for insects there are deliberate attempts by scientists to develop insect-selective biopesticides. Efforts have been directed to enhancing efficacy of baculoviruses by expressing genes for selective toxins (Chejanovsky et al., 1995). Similar efforts have gone into the discovery of more of such insect-selective toxins from biological sources. These studies exploit the lepidopteran cell system and aims at introducing the toxins into target insect pests using baculoviruses, which are natural invertebrate-specific entomo-pathogens that are currently being explored as biopesticides for the control of insect pests, particularly Lepidoptera (Black et al., 1997). Expressing the genes in baculoviruses would increase their insecticidal efficacy (Chejanovsky et al., 1995; Gershburg et al., 1998). Baculoviruses have a limited host range and are harmless to non-target organisms (Harrison et al., 2000). As such they provide alternatives to chemical insecticides for controlling insect pests. They can be applied, with reduced environmental and occupational risks by spraying (Cunningham 1982; Huber, 1986).

Baculoviruses act relatively slowly once dissolved in the midgut of insect larvae to release infectious virions, which enter gut epithelial cells and begin to replicate. Replications in other organs cause extensive tissue damage and eventual death. This process can take 4-5 days in the laboratory, but in the field may last for more than a week, allowing the insect larvae to feed longer and thereby damaging the host plant (Harrison et al, 2000). Baculovirus vectors expressing foreign genes such as those for insect-specific toxins can increase the rate of action (Stewart et al., 1991). They have been genetically modified by deletion of viral genes or insertion of insecticidal toxin genes (Van Beek & Hughes, 1998). Many of these modifications can reduce the time it takes for the virus to incapacitate its host, which in turn enhances crop protection by baculovirus application (Gershburg et al., 1998). Recombinant viruses engineered to express insect-selective peptide neurotoxins from scorpion venom are considered to have high potential for pest control (Harrison et al., 2000).

Biotechnology has already proved useful in generating novel biopesticide aiming at reducing recurrent overuse of harzardous insecticidal chemicals (Gershburg et al., 1998). The restricted host range of baculoviruses and their ability to penetrate the internal tissues of lepidopterous pests has made them ideal vectors for mobilizing selective toxins into target insects with minor predicted effects on the environment (Gershburg et a.l, 1998). The approach has been recently been demonstrated in ACMVPS bearing scorpion toxin gene (Stewart et al., 1991; McCuthen et al., 1991; Chejanovsky et al., 1995).

Baculoviruses expressing the toxin AaIT (from the North African scorpion, *Androcotonus australis* (Hector)(Zlotin et al; 1971a) or LqhIT2 (from yellow Israel scorpion, *Leirus quin-questriantus hebraeus* Hemprich & Ehrenberg) (Zlotkin et al., 1971b) have been tested in field for control of lepidopterous pests (Dupont, 1996; Black et al., 1997; Gard, 1997). A baculovirus engineered with the AaIT gene encoding scorpion toxin provided a ten-fold improved efficacy (ISB, 1997). While crude scorpion venom affected spiders, crustaceans and mice, the purified protein toxin (AaIT) does not and has no affinity to mammalian neurons (Stewart et al., 1991). The toxin or the recombinant virus has no clinical effects on non-target insects, rodents and other beneficial species (Harrison et al., 2000). The excitatory toxin (AaIT) of a West African scorpion (*Androcotonus autralis*) has been the focus of this experiment. To date, the toxin is considered to be the most potent insect specific scorpion neurotoxin due to its immediate contraction effects on fly larvae upon injection. This has

made it a primary target for genetic engineering of baculoviruses (Stewart *et al.*, 1991; McCuthen *et al.*, 1991).

Although many toxins are too large or too poorly absorbed to be used as insecticide, advances in analytical techniques for 3D structures of proteins and peptides and computer aided drug design may facilitate the use of toxins as lead compounds in drug and pesticide discovery (Bowman & Harvey, 1995). Since all scorpions have venom glands, continued research on the venom should provide diverse sources of natural target selective biopesticide.

2.10   Statement of the problem

Local scorpion toxins with potential as environmentally friendly biodegradable, insect-selective pesticides have not been investigated as alternatives to synthetic insecticides.

2.11   Research hypothesis

There are local scorpion venoms and derived toxins with highly selective insecticidal potency that vary with species and geographical locations.

2.12   Objectives

2.12.1 General objective

The general objective was to screen, isolate, purify, perform bioassay and characterize insect selective toxins from venoms of several scorpion species in the family Buthidae collected in Kenya.

2.12.2 Specific objectives

The following were the specific objectives:

   (i)   To extract scorpion venom from scorpion species collected in Kenya
   (ii)   To screen for insecticidal activity of crude scorpion venoms
   (iii) To fractionate and bioassay active scorpion venoms
   (iv)   To purify bioactive principles of local scorpion venoms
   (v)   To characterize the bioactive principles of local scorpion venoms

2.13   Scope of the study

In this study, we restricted our variables to taxonomic identification of local scorpions and neurotoxic peptides with activity against mice or insects. It dealt with venom extracted from, *Parabuthus leisosma,* a scorpion species of the Buthidae family.   The genus *Parabuthus* is composed of 30 documented species, and all of which except for a few are found in South Africa. In this thesis we report on *P. leiosoma* found in Kenya, East Africa and the specific peptides isolated from it for the first time. The isolation, purification and partial amino acid sequence determination of an insect selective toxin and a mammalian toxin from the venom of *P. leiosoma* is described.

2.14   Justification of the study

The ongoing over-use of non-specific chemical insecticides poses risks to the environment and to human health resulting from toxicity to non-target organisms and increased insect resistance to these agents. Many efforts have been invested in trying to minimize the use of these compounds by employing novel insecticides offered by nature. Scorpions produce insect selective polypeptide toxins that bind and modulate voltage-sensitive Na^+ channels in neurons and muscles, thus offering alternative, biodegradable and environmentally safe insecticides. Owing to their high target specificity increasing efforts has been directed at discovering insect-selective toxins that can be used to develop recombinant biopesticide as safe alternatives to broad-spectrum chemical insecticides (Stewart *et al.,* 1991; McCuthen, 1994).

Selectivity and specificity in the action of different components of crude venom strongly encourages investigation of composition and structure, leading to the knowledge of structure function relationships. Since the venom of a particular scorpion species can be potent in one region and harmless in the other chemical investigations of toxins of local scorpions should be encouraged to confirm potency (Harvey *et al.,* 1995).

Furthermore, out of the estimated 1,500 species of scorpion worldwide, the scorpion fauna in Kenya and Tanzania provide 25 different 'forms' whose venoms have not been evaluated for their insect and mammalian toxicity. These 'forms' belong to two families: Buthidae and Scorpionidae represented by 11 genera and 23 species (Probst, 1973). The biological diversity reflects underlying molecular diversity of venom, which may eventually assist in the discovery and design of new selective and environmentally friendly biopesticides.

Scorpion toxins have been employed in research aiming at direct utilization of the toxin genes to engineer vectors known to infect insect pests to increase their efficacy. This requires systematic analysis of potent toxins and establishing their selectivity. It was against this background that this work was undertaken.

CHAPTER THREE

MATERIAL AND METHODS

3.1   Scorpion collection, rearing and identification

The scorpions were collected at Nguruman, Nanyuki and Mbololo areas in Kenya (Fig. 3.).
However, due to limited number of scorpions in Nanyuki and Mbololo, most collections
were from Nguruman, which provided a high species diversity and enough numbers for
venom extraction

Figure 3: Map of Kenya showing scorpion collection sites

Nguruman is located near Lake Magadi on the escarpment of Narok hills, in Rift Valley Province of Kenya. The area lies within latitude $01^0 45.830$ S and longitude $36^0 03.991$ E, at an altitude of 781 m above the sea level. The area has a hot humid climate, an average annual rainfall of 597 mm and average temperatures minimum of 18.96 ^{0}C, an average temperature maximum of 31.83 ^{0}C and relative humidity ranging from 40 to 45%. It is rocky with fallen rotting logs that provide suitable habitats for scorpion breeding. Deserted Manyattas (Masai houses) are also a common feature and habitat for scorpions.

Scorpions were collected during the day by turning stones, logs, tree bark, and inspecting other potential diurnal retreats in addition few other specimens were collected at night with the use of a portable ultraviolet light, comprising two mercury-vapor tubes attached to a chromium parabolic reflector and powered by a rechargeable 7 A/h, 12 V battery. UV detection is known to greatly increase collecting yields and has led to the discovery of numerous un-described species, even in previously well-collected areas (Lamoral, 1979; Williams 1980; Sissom *et al.*, 1990).

Scorpions were kept individually in plastic containers under 12:12 h photoperiod at 27 ^{0}C constant temperature. Experts from International Centre of Insect Physiology and Ecology and Zoology Department, Cape Town University, South Africa jointly identified the scorpions.

3.2   General procedures
All the glassware used in this work was cleaned thoroughly with water, rinsed in distilled water, and acetone and dried in oven at 110 ^{0}C. Plastic tubes and vials were used only once and disposed.

All solvents were supplied by Sigma-Aldrich or Merck at 97.5 -99.9% purity and were used without any further purification.

3.3 Biological materials
3.3.1 Scorpion venom
The venom was collected by manual stimulation, which is considered as the natural method for collecting scorpion venoms (Froy *et al.*, 1999b). The venom collected presents a high degree of purity, as it is free from contamination with cellular materials from neighboring

gland tissues. Briefly, a live scorpion was released in an arena (tray) and made to sting by tapping on its tail (Plate 7 & 8). A parafilm wrapped open tube (50 ml centrifuge tubes) was immediately provided for the scorpion to attack using its stinger, and deposited small quantities of the venom on the surface of the parafilm. The deposited venom was reconstituted in double distilled water and put into a vial placed on ice bath using a micropipette, and the crude venom frozen at -20 ^{0}C.

3.3.2. Crude venom extraction

Crude venom collected (milked) from each of the scorpion species and lyophilized separately, was weighed out in a 1.5 ml eppendorf tube, re-suspended in 2000 µl of de-ionized water and homogenized using a Potter-Elvehjam homogenizer. The insoluble material was removed by centrifugation at 26,000 x g (15000 rpm) for 20 min. at 4 ^{0}C. Additional 1000 µl of de-ionized water was added to the pellet, homogenization repeated, the contents centrifuged and supernatant collected. The procedure was repeated 2-3 times to maximize the yield of peptide toxins from the crude venom. The supernatant (soluble venom) extracted from the venom was pooled, lyophilized and stored at -20 ^{0}C for purification. Crude venom from the same scorpion species collected from the same locality was pooled together and the yield recorded (Table 2).

3.3.3. Insect larvae

Third instar *Chilo partellus* and *Busseola fusca* larvae, reared at 12:12 h photoperiod at 45% relative humidity at the Animal Rearing and Quarantine unit (ARQU), ICIPE, were used in insecticidal assays.

3.3.4. Mice

Albino laboratory mice were reared at the Animal Rearing and Quarantine unit (ARQU), ICIPE were used for mammalian toxicity assays. They were fed on 5% fat Agway Prolab 3000 rat and mouse chow. Colonies were maintained at 20-24 °C on a 14:10 h light:dark cycle photo period.

Plate 5: Scorpion milking apparatus
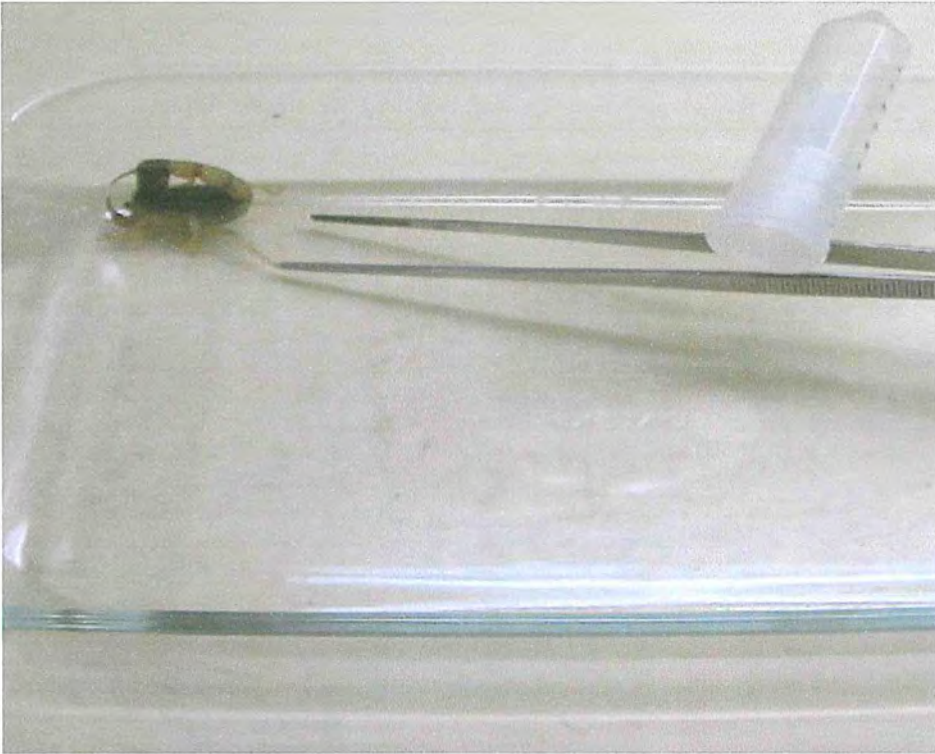
Plate 6: Scorpion milking process

3.4 Bioassay of crude venom

3.4.1. Oral administration of crude venom

Feeding starved larvae on maize leaves dipped into toxic venom or toxin rich solution was used to test oral toxicity (Quistad, 1992). The experiment was set up by applying the crude venom on leaf disks measuring 9cm i.d cut with a cork borer from 2 weeks old maize leaf (variety Katumani) planted in a greenhouse at ICIPE. The leaf was placed in a 9 cm i.d petri dish and 2 starved third instar *C. partellus* or *B. fusca* larvae added. Serial dilutions of 0.75, 1, 1.25, 1.5 and 2.0 µg/1µ of the crude venom were prepared in $NH_4CH_3CO_2$ buffer and used to soak the disc shaped maize leaves. Control experiments were similarly set with bovine serum albumin (BSA) in $NH_4CH_3CO_2$ buffer. The treatment and control experiments were kept closed in the petri dish to prevent test insect escape. The different concentrations were classified as lethal, toxic or non-toxic depending on the reactions evoked by orally administered venom and the area eaten determined. The lethal concentration means that the component at the ingested dose was enough to kill the test insects within 24 hours. Toxic ones are those components that when orally administered induced symptoms like temporary paralysis but recovered within 24 h. Non-toxic components are those that evoked no reaction like the control. The LD_{50} was estimated by the method of 50% end point according to Reed & Muench (1938).

3.4.2. Subcutaneous administration of crude venoms

Pooled crude venom for each of the collected species was tested for toxicity by subcutaneous injection into 3rd instar larvae of *C. partellus.*. The design of the bioassay experiment was 5x5 Latin square design that was run for 48 hours. The larvae were subcutaneously injected with 0.75, 1, 1.25, 1.5 and 2.0 µg of the venom per 50 mg larval weigh in 2µl of BSA using a micro syringe. Percentage mortality against time was scored at 24 and 48 h after injection, whereas contractile and flaccid paralysis symptoms were noted for evidence of mild toxicity. The venom solution was injected (2 µl in BSA) through the dorsal surface of the 3rd instar *C. partellus* larvae in a small petri (9 cm i.d) dish with a filter paper (9 cm i.d) at the bottom to soak up any hymolyph. Petri dishes with larvae that experienced contractile paralysis were characterized by more hymolyph being squeezed from their body during contraction. In most cases, at high concentrations, the larvae were paralyzed and shriveled within the first 10 to 15 min. Albino Swiss mice (18-20 g) was injected intracerebroventricularly with 0.75, 1, 1.25, 1.5 and 2.0 µg of crude venom in 0.2 ml of BSA in 10 mM $NH_4CH_3CO_2$buffer

(Inceoglu, 2003). Control experiments were set with BSA in 10 mM $NH_4CH_3CO_2$ buffe instead of venom or toxin. Percentage mortality was calculated using the formula.

$\%M = [y/x] \times 100$, where y is the mean deaths defined by the difference between mean control deaths and the mean test deaths and x is the initial larvae population (WHO, 1996).

Probit analysis was done to establish the regression equations. Briefly, this analysis was done using log dose and probit transformation of corrected percentage mortality employing probit plane model by Busvine (1971). Probit transformations were plotted against log (+1) dose using excel programme. A regression equation was obtained and lethal concentrations (LC) calculated.

The scorpion venoms with low LC_{50} values were considered for further investigation and the toxins purified and characterized.

3.4.3. Subcutaneous administration of semi and purified toxins

Lethality of the various protein fractions was tested by subcutaneous injection with 0.75, 1, 1.25, 1.5 and 2.0 µg of proteins in 0.2 ml of BSA in $NH_4CH_3CO_2$ buffer for Swiss Albino mice (18-20g) (Inceoglu, 2003) and 2 µl of BSA in $NH_4CH_3CO_2$ buffer for *C. partellus* or *B. fusca* larvae (50 mg). The larvae were injected with 2 µl of test solution by sticking the micro-syringe into the lateral side about three segments up from the posterior end and allowed to move on the needle for 5 seconds to ensure that it was in good condition for conducting contractile paralysis test. The needle was then removed gently and the larvae observed for toxicity symptoms for 5 min. After 10 minutes of injection of the toxic components in mice, induced symptoms like excitation, salivation and urination.

Lethality defined, as the complete immobility of the test animal, was determined after an interval of 24 hours. Paralysis, defined as the inability of the test animal to change its location, was employed as an addition criterion of toxicity for insect larvae, determined after 5 min. following the injection. Toxicity was determined on sampling and calculations according to Reed and Muench (1938). At least five animals were employed in each experimental group.

3.5. Toxin purification

Lyophilized venom of the scorpion species that gave the highest LD_{50} value was subjected to several chromatographic steps. Starting with cation-exchange (Wudayagiri *et al.*, 2001) and finally reverse phase high performance liquid chromatography (RPHPLC) (Wudayagiri *et al.*, 2001) were carried out for toxin purification.

3.5.1. Cation exchange chromatography

Separation was carried out on cation-exchange chromatography using column type 16/20 (Pharmacia, Uppsala, Sweden) with a bed volume of 3 ml CM-sepharose-6B-C (Sigma). The column (1.6 x 25 cm) was pre-equilibrated with 0.1 M $NH_4CO_2CH_3$ buffer (pH 6.4). Lyophilized venom (6 mg) was dissolved in 10 mM $NH_4CO_2CH_3$ buffer (pH 6.4) and passed through a CM-sepharose-6B-Cl (Sigma) column. Elution of unbound fractions was achieved by the salt gradient (0.01 - 0.5 M $NH_4CO_2CH_3$) at a flow rate of 0.6 ml/min using a peristaltic pump (MP-3, Rikakikai Co. Ltd, Tokyo). Fractions (1.2 ml) were collected using a BioRad 2128 fraction collector. All the chromatographic separations were carried out at 4 ^{0}C and fractions monitored at λ 280 nm by a Beckman DU 640-B spectrophotometer (Fullerton, California). The fractions in the same absorbance peak were pooled and concentrated to 50 µl in polyethylene glycol (PEG) 6000 (Serva). After overnight dialysis (dialysis membrane MWCO 6,000 - 14,000) with two changes against 10 mM $NH_4CO_2CH_3$ (pH 6.4), the protein concentrations determined and the sample was frozen at -20 ^{0}C.

3.5.2. Reverse Phase High Performance liquid chromatography purification

Reverse Phase High Performance liquid chromatography (HPLC) was performed on Beckman System Gold model 126 linked to Waters 991 photodiode array detector. The freeze dried CM-sepharose fractions were re-suspended in water and aliquots (10 µg) loaded onto a Vydac C_4 reverse-phase analytical HPLC column (25 x 4.6 (i.d) cm) equilibrated in solvent A (A=5% acetonitrile, 95% water, 0.1 TFA. The individual proteins were eluted from the column with a linear gradient reaching 70% solvent B (B=95% acetonitrile, 5% water and 0.1% TFA) in 80 min. at a flow rate of 0.6 ml/min. All the fractions were collected, the protein content determined, freeze dried and assayed for biological activity.

Biologically active fractions were further purified using a Michrome Magic 2002 micro-bore HPLC machine equipped with C_{18} column and an online 5 µl peptide trap (Michrome Bioresource Inc., Auburn, USA) with a linear gradient of 5-70% solvent B in 30 min. and

from 70-90% of solvent B in 12 min. at a flow rate of 50 µl min⁻¹. Fractions were collected manually into BSA tubes by following the UV traces and pooled.

3.5.3. Estimation of protein concentration

Protein estimation was carried out using the standard bicinchoninic acid (BCA) method (Smith *et al.*, 1985) as per the manufactures instructions (Pierce, Rockford III, USA). Bovine serum albumin (BSA) was used as the protein standard. Working reagents were prepared by mixing 50 parts of reagent A (1 ml of base reagent containing; Na_2CO_3, Na_2HCO_3, bicinchonin detection reagent and sodium tartrate in 0.2 M NaOH) with 1 part of reagent B (25 ml of 4% CuSO4 solution. Working reagent (2 ml) was added to each test tube and protein samples pipetted to appropriately labeled tubes. A control consisting of 0.1 ml of buffer (0.1 M $NH_4CH_3CO_2$) in 2 ml working reagent was also included. All tubes were incubated for 30 min at 37 °C in a water bath. After incubation, the tubes were cooled to room temperature and the absorbance of each tube measured at λ 562 nm in Beckman Du 680 series spectrophotometer. The absorbance obtained was compared to those for a standard curve of BSA (appendix II) and the corresponding protein in each sample determined.

3.3. Chemical characterization of the peptide

3.6.1 High Performance Liquid Chromatography - Mass spectroscopy

The molecular mass of collected HPLC fractions was determined using electrospray ionization mass spectrometry on a Mass platform LCZ single quadrupole mass spectrometer. The delivery of mobile phase (50% acetonitrile/0.05% formic acid; v/v) was performed with ISCO µLC-500 syringe pump at a flow rate of 5 µl/min. The purified toxin collected from micro-bore HPLC, using Magic 2002 C_{18} column, 1.0 x 150 mm, 5 um bead 200 Å column particle size from (Michrom Bioresources, Inc. Auburn California) and diode array detector at: λ 214 and 280 nm was analyzed by direct flow injection of 10 µl. The spectral analysis was done in a positive ion mode at a capillary voltage of +3.5 kV, a cone voltage of 60 V and at a source temperature of 790 °C. Spectra were scanned over the range of 200-6600 m/z at a rate of 20 sec/scan; 20 scans were combined using the VGMCA acquisition mode. The molecular weight of the toxin was determined using the maximum entropy deconvolution algorithm (MaxEnt) to transform the range of 650/1500 m/z to give a true mass scale spectrum. The mass calibration was performed using horse heart myoglobin (Sigma) and purity following HPLC was evaluated by MALDI-TOF

3.6.2. Amino acid sequence determination

For amino acid sequence determination, the cysteine residues of the peptide were reduced by incubating in TCEP (100-fold excess: 1/100, w/w) at pH 8.0 and 37 ^{0}C overnight, TCEP in 0.1 M $NH_4CH_3CO_2$ (pH: 7.5) and 0.1M $NH_4CH_3CO_2$ (pH: 7.5). Maleimide (10-fold excess) at pH 8.0 and 50 ^{0}C was then added for an additional 2 h. The reduced and alkylated peptide was further purified on reverse phase liquid chromatography. The *N*-terminal sequence of the toxin was determined using HP GS1000 sequence analyzer by automated Edman degradation at the Molecular Structure Laboratory, Stine-Haskell Research Centre, Newark, and USA.

3.6.3 Structural analysis

The amino-acid sequence of Plit and Plmt were subjected to peptide tools from the Expasy web site for mass calculation of sequenced peptide. Sequence homologies were determined using the CLUSTALW program at the EMBL server (http://www2.ebi.ac.uk/clustalw). Sequence alignments were visualized using the ESPRIPT tool at the Expasy server.

3.7. Data analysis

Probit analysis (Finney, 1971) was employed. This is a useful tool for estimation of quantal and dose responses, and has been used by many workers to estimate LC, LD as well as effective doses (ED) or concentrations (EC) of elements, compounds or pathogens and their products against test organism. LD_{50} is the dose of the substance to be tested that would kill 50% of the organism in a given period of time. LC_{50} is the concentration of the substance to be tested that would kill 50% of the organism in a given period of time. For cases where mortality was not scored, LD_{50} was estimated using the method used by Reed and Muench (1938) with 95% confidence level using simple calculations.

CHAPTER FOUR

RESULTS

4.0 BIO-PROSPECTING FOR INSECTICIDAL SCORPION TOXINS

4.1 Scorpion collection, rearing and identification

During the bio-prospecting exercise, a total of 190 scorpions were collected from Nguruman area, Mbolo and Nanyuki (Sabuk-Retreat) in Rift Valley province, Kenya. They were reared under ambient conditions at the International Centre of Insect Physiology and Ecology (ICIPE), Animal Rearing and Quarantine Unit (ARQU).

Identification of scorpions was based entirely on male and female characteristics and traits (color, size etc). The total collection was categorized into 2 families, 2 genera and 5 species. Nevertheless this was a rather small portion of the approximately 1,500 scorpion species found world wide (Lourenco, 1994) to conclude on the trend. Some were identified only to family level and are coded pending identification to genera and species level. Identification was done at ICIPE in collaboration with the curator at Biosystematics Unit, Cape Town University Museum, South Africa. Voucher specimens have been deposited at the National Museums of Kenya.

Table 1. Collected and identified scorpions

Family	Genera	Species	Region Nguruman	Nanyuki	Mbololo	Total
Buthidae	*Parabuthus*	*leiosoma*	35	18	15	68
Buthidae	*Parabuthus*	*pallidus*	25	4	2	31
Buthidae	*Hottentotta*	*eminii*	40	2	-	42
Buthidae	*Hottentotta*	*trilineatus*	30	2	-	32
Buthidae	*M0001102**	*	1	3	6	10
Buthidae	*G0000103**	*	-	-	5	5
Ishchrunidae	*Opisthacanthus*	*rugiceps*	1	1	-	2

* Pending identification

The collection was done all year round and all the species were found to be seasonal. It was more common to collect large numbers of all the species during rainy season than when it was very dry and hot. This can be explained by the fact that under extreme weather

conditions (high temperature) scorpions burrow underground or below tree barks. When it rains the burrows are filled with water forcing the scorpions to venture out due to lack of space and extremely low temperature. Each scorpion species had different preference for particular areas as it was discovered in their varied abundance in different collection sites.

4.2 Scorpion venom yields

The yields of venoms from three different scorpion species are summarized in table 3.

Table 2: Venom yield from scorpion species

Species	No. Scorpions	Total venom weight (mg)	Mean venom weight (mg)
Parabuthus leiosoma	35	10.83	0.32 ± 0.03
Parabuthus pallidus	25	04.20	0.18 ± 0.08
Hottentota eminii	40	10.10	0.25 ± 0.17
Hottentotta trilineatus	7	0.90	0.15 ± 0.02

Production of venom varied considerably (Table 2), and the amount released was directly proportional to the body size of the scorpion. *P. leisosoma*, the most aggressive and relatively bigger species than the rest gave high quantity of venom. Out of the 30 *H. trilineatus* sp scorpions collected, 23 were very young and only 7 were subjected to milking.

3.3 Bioassay of crude venom

4.3.1 Oral toxicity

Oral toxicity was assessed by considering the percentage damage caused by 3rd instar *C. partellus* and *B. fusca* larvae feeding on the young (2 weeks) leaves of maize dipped into venom solution of different concentrations. The test recorded no significant activity. The starved larvae fed on the leaves soaked in venom solution but showed no sign of envenomation.

These results suggest that the two insect (*C. partellus* and *B. fusca*) midgut contains enzymes responsible for small peptides degradation rendering the neurotoxin non-toxic. This also helped rule out oral toxicity as a means of administering venom or toxin for further work.

4.3.1 Subcutaneous injection toxicity

The crude venom from the three scorpion species was also screened for insecticidal activity against third-instar *Chilo partellus* larvae (Table 4). Venoms from the rest of the species were not analysed because of low quantities obtained.

Table 3.   Percentage mortality of 3rd instar *C. partellus* larvae injected with *H. eminii* crude venom after 24 hours

Conc. (µg/50mg larvae)	Mean of alive larvae	Mean of dead larvae	%Mortality	Corr % mortality
0.00 (control)	5	0	0	0
0.75	5	0	0	0
1.00	5	0	0	0
1.25	4	1	20	20
1.50	3	2	40	40
2.00	1	4	80	80

Table 4:   Percentage mortality of 3rd instar *C. partellus* larvae injected with crude *P. pallidus* venom after 24 hours

Conc. (µg/50mg larvae)	Mean of alive larvae	Mean of dead larvae	%Mortality	Corr % mortality
0.00 (control)	5	0	0	0
0.75	5	0	0	0
1.00	4	1	20	20
1.25	1	3	60	60
1.50	2	3	60	60
2.00	2	4	80	80

Table 5.   Percentage mortality of 3rd instar *C. partellus* larvae injected with crude *P. leiosoma* venom after 24 hours

Conc. (µg/50mg larvae)	Mean of alive larvae	Mean of dead larvae	%Mortality	Corr % mortality
0.00 (control)	5	1	0	0
0.75	2	3	60	60
1.00	1	4	80	80
1.25	1	4	80	80
1.50	0	5	100	100
2.00	0	5	100	100

The bioassay data was subjected to probit analysis (Busvine, 1971; Finney, 1971). The results are summarized in tables 6 – 8

Table 6: Probit analysis of insecticidal activity data for *H. eminii* venom

Conc. (μg/50mg larvae weight)	LogDose + 1	Corr% Mortality	Empirical probit	Expected probit	Working probit	Weighing coefficient	Weight		
	x			Y	y		w	wx	wy
Control	-	0	-	-	-	-	-	-	-
0.75	0.875	0	-	2.30	1.97	0.031	0.155	0.137	0.305
1.00	1.000	0	-	3.33	2.83	0.208	1.040	1.040	2.943
1.25	1.097	20	4.16	4.14	4.15	0.503	2.515	2.759	10.44
1.50	1.176	40	4.75	4.79	4.73	0.627	3.135	3.687	14.83
2.00	1.301	80	5.84	5.82	5.84	0.503	2.515	3.272	14.68

The regression equation obtained was $y = 8.2792x - 4.9466$

$Sw = 9.36$; $Swx = 10.895$; $Swx^2 = 12.78$; $\bar{x} = \dfrac{Swx}{Sw} = 1.164$; when $y = 5$, $x = m = 1.201$

$LC_{50} = 1.588$ μg/50mg larvae weight;

$$V = \frac{1}{b^2}\left\{\frac{1}{Sw} + \frac{(m-\bar{x})^2}{Swx^2 - \frac{(Swx)^2}{Sw}} - \right\} = \frac{1}{8.2792^2}\left\{\frac{1}{9.36} + \frac{(1.589-1.164)^2}{12.777 - \frac{(10.895)^2}{9.36}} - \right\} = 0.0292$$

Table 7: Probit analysis of insecticidal activity data for *P. pallidus* venom

Conc. (μg/50mg larvae weight)	LogDose + 1	Corr% Mortality	Empirical probit	Expected probit	Working probit	Weighing coefficient	Weight		
	x			Y	y		w	wx	wy
Control	-	0	-	-	-	-	-	-	-
0.75	0.875	0	-	3.75	3.21	0.370	1.85	1.619	5.939
1.00	1.000	20	4.16	4.39	4.18	0.558	2.79	2.790	11.66
1.25	1.097	60	5.25	4.87	5.25	0.634	3.17	3.477	16.64
1.50	1.176	60	5.25	5.29	5.25	0.026	0.13	0.153	0.683
2.00	1.301	80	5.84	5.93	5.84	0.471	2.36	3.070	13.78

The regression equation obtained was $y = 5.1287x - 0.7396$

$Sw = 10.3$; $Swx = 11.109$; $Swx^2 = 12.196$; $\bar{x} = \dfrac{Swx}{Sw} = 1.0785$; when $y = 5$, $x = m = 1.119114$

$LC_{50} = 1.31$ μg/50mg larvae weight;

$$V = \frac{1}{b^2}\left\{\frac{1}{Sw} + \frac{(m-\bar{x})^2}{Swx^2 - \frac{(Swx)^2}{Sw}} - \right\} = \frac{1}{5.1287^2}\left\{\frac{1}{10.3} + \frac{(1.119114-1.0785)^2}{12.196 - \frac{11.109^2}{10.3}} - \right\} = 0.0040$$

Table 8: Probit analysis of inseciticidal activitydata for *P. leiosoma* venom

Conc. (µg/50mg larvae weight)	LogDose + 1	Corr% Mortality	Empirical probit	Expected probit	Working probit	Weighing coefficient	Weight		
	x			Y	y		w	wx	wy
Control	-	0	-	-	-	-	-	-	-
0.75	0.875	60	5.25	5.17	5.26	0.627	3.14	2.748	16.52
1.00	1.000	80	5.84	5.84	5.84	0.503	2.52	2.520	14.72
1.25	1.097	80	5.84	6.36	5.60	0.302	1.51	1.656	8.456
1.50	1.176	100	7.33	6.78	7.26	0.076	0.38	0.447	2.759
2.00	1.301	100	7.33	7.45	7.85	0.050	0.25	0.325	1.963

The regression equation obtained was **y = 5.3613x + 0.4752**

$Sw = 7.8;$ $Swx = 7.696;$ $Swx^2 = 7.6899;$ $\bar{x} = \dfrac{Swx}{Sw} = 0.9867;$ when $y = 5$, $x = m = 0.844$

$LC_{50} = 0.6981 \mu g/50mg$ larvae weight;

$$V = \frac{1}{b^2}\left\{ \frac{1}{Sw} + \frac{\left(m - \bar{x}\right)^2}{Swx^2 - \dfrac{(Swx)^2}{Sw}} - \right\} = \frac{1}{5.3613^2}\left\{ \frac{1}{7.8} + \frac{(0.844 - 0.9867)^2}{7.6899 - \dfrac{(7.696)^2}{7.8}} - \right\} = 0.0118$$

From the regression lines obtained, the LC_{90}, LC_{50} and LC_{25} of the crude scorpion venom were calculated (Table 9).

Table 9: Lethal concentration (LC) of venom from three scorpion species on *C. partellus* after 24 hours

	LC_{25} (µg/50mg larvae weight l)	LC_{50} (µg/50mg larvae weight)	LC_{90} (µg/50mg larvae weight)
Hottentotta eminii	0.79b	1.59b	4.84a
Parabuthus pallidus	0.43a	1.31b	7.93b
Parabuthus leiosoma	0.36a	0.70a	5.85c

LC values with same letter are not significantly different

From the LC_{50} values *P. leiosoma* venom exhibited the highest insecticidal activity after 24 hours while activities of *P. pallidus* and *H. eminii* were slightly lower. The venom from *P. leiosoma* was therefore considered for further investigation and identification of the insect-selective toxin. The crude venoms from two *Parabuthus* species (*leiosoma* & *pallidus*) were analysed by reverse phase HPLC (Fig. 3) for quantitative and qualitative comparisons.

The chromatographic comparison of the revealed that the venom from *P. leiosoma* and *P. pallidus* are different both quantitatively and qualitatively (Fig. 3). *P. leiosoma* venom contained

more peaks that were absent from *P. pallidus* venom. *P. leiosoma* venom had a large proportion of protein in the retention time range known to include neurotoxins. Such differences may reflect a response to local ecological conditions

Absorbance 214nm

0.0000

0.3000

0.0000

0.00

2.65; 3.06; 3.57

6.01; 6.44; 6.

16.06
16.65

18.77; 19.56

22.98

25.17

12.01; 12.71
13.56
14.47

15.32; 16.21
17.30
17.74; 18.47
19.00; 19.53
20.37
21.41; 21.86
23.18
23.44
25.41
25.68; 25.92
27.26; 27.73
28.29; 28.78

20.00

30.63;
31.75; 32.35
33.17
33.70
35.06; 35.79
37.05
37.55; 37.98
38.77
39.84;

41.89

45.94 L@h172

46.98
47.53; 47.98; 48.44
48.90; 49.28; 49.82

50.92

51.42

54.67
55.45; 55.87

58.50

40.00

42.44
43.22; 43.68
44.25
45.65
46.47; 47.22
48.34

51.22; 51.87

60.00

59.49
60.56
61.64

66.83

B

C

A

Figure 4. C₄ RP-HPLC analysis of crude venom of: [A] *P. pallidus*, [B] *P. leiosoma* and [c] active fraction (viii) from ion-exchange chromatography of *P. leiosoma* venom.
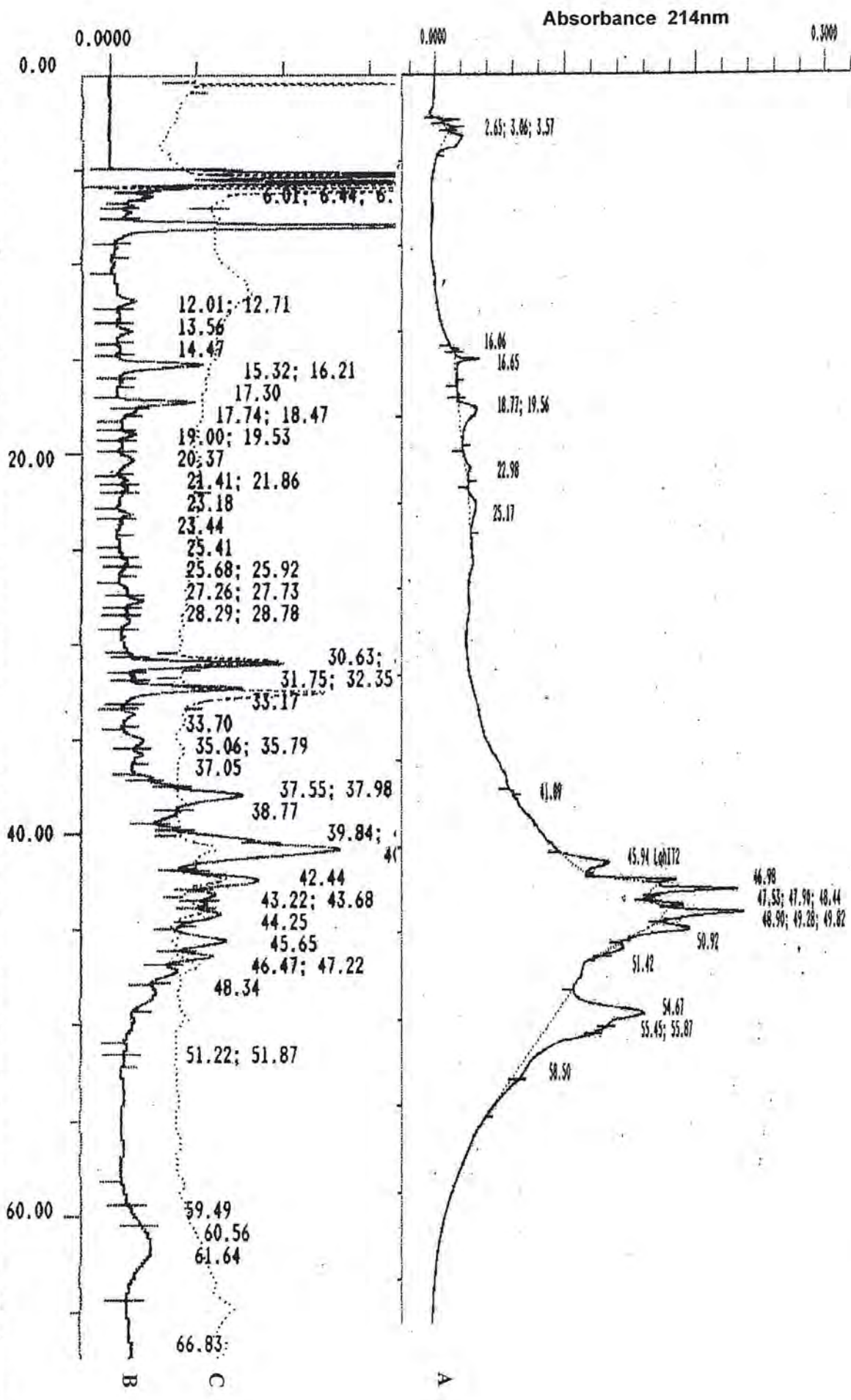
4.4 Bioassay guided purification of toxins from *Parabuthus leiosoma* venom

Soluble venom was initially pooled into 15 fractions from ion-exchange chromatography (Fig. 5).

Figure 5: Ion exchange chromatogram of crude *P. leiosoma* venom
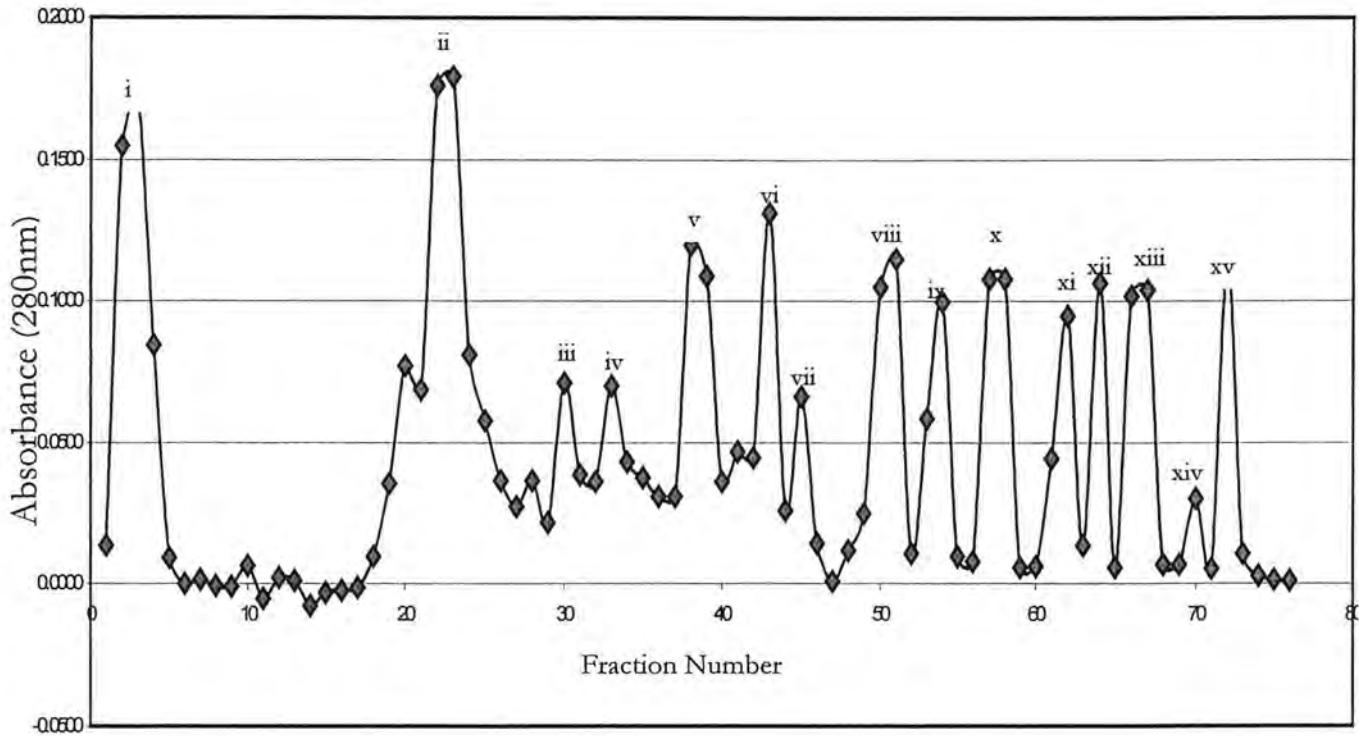
The fractions were tested on *C. partellus* and *B.fusca* larvae for lethality by subcutaneous injection. Fraction viii was found to be the most toxic (lethal) at 2 µg /50 mg of *C. partellus* larvae (Table 11). It also recorded significant activity on mice (Table 11). In a bid to identify an insect selective toxin, fraction viii was used for further chemical analysis.

Table 10: Bioassay of fractions of *P. leiosoma* venom from ion exchange chromatography

Pooled fractions	Time range (Minutes.)	Lethality		
		C. partellus larvae	*B. fusca* larvae	Mice
i	2 -10	+	-	+
ii	12 -17	+	+	+
iii	18 -26	+	-	+
iv	27 -29	-	-	-
v	34 -38	+	-	++
vi	39 -44	+	-	+
vii	46 - 51	+	-	+
viii	76 - 87	+++	++	++
ix	93 - 97	+	-	+
x	98 -102	+	-	+
xi	108 - 113	+	-	+
xii	114 - 116	-	-	-
xiii	121 - 131	-	-	-
xiv	138 - 143	-	-	-

Relative toxicity denoted as: - non-toxic; + mildly toxic; ++ moderately toxic; and +++, highly toxic

Further purification of the excitatory fraction viii was achieved by ion paired reverse phase high performance liquid chromatography (IPRPHPLC) yielding 5 major component sub-fractions (Figure 6). They were collected, pooled according to their retention time, lyophilized and their protein concentrations established by BCA protein assay (Smith *et al.*, 1985)
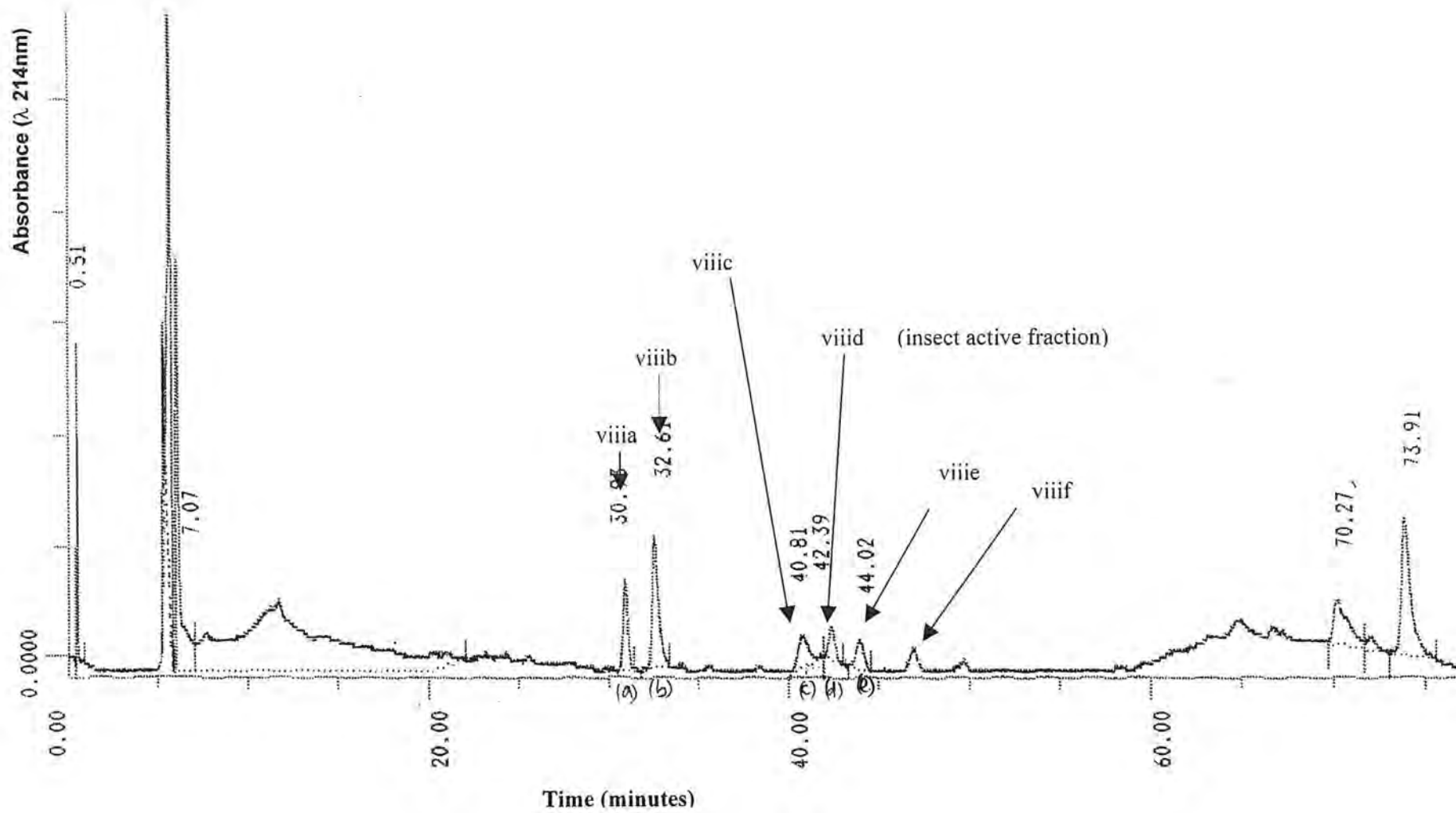
Figure 6: C₄ RPHPLC profile of active fraction (viii) of *P. leiosoma* venom from ion exchange chromatography

Bioassay of each of the five fractions on *C. partellus* larvae led to location of insect selective toxin in sub-fraction viiid with the highest toxicity (Table 11). The sub-fraction showed prolonged contractile paralysis of *Chilo partellus* larvae by sub-cutaneous injection with 1 µg/50 mg (Plate 6). The same sub-fraction had significant effect on *B. fusca* larvae but showed no effect on mice. Sub-fraction viiid was finally purified to homogeneity on a C_4 RPHPLC column. The yield of the purified fraction was estimated to account for 3.3 % of the total protein content of the dry venom.

Plate 6. Visual comparison of toxicity stages of active fraction of *P. leiosoma insect* selective toxin (Plit)
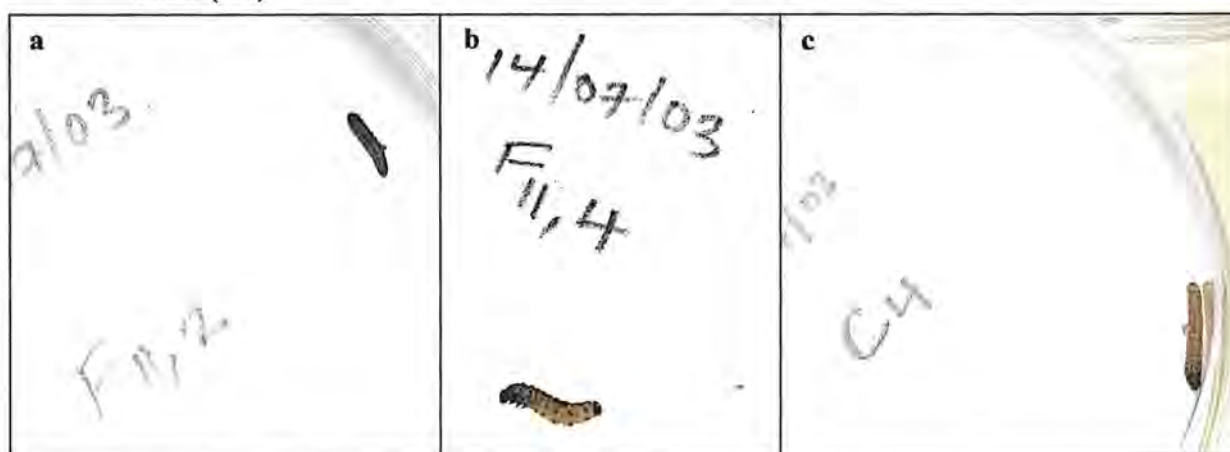
(a) Contractile paralysis of *C. partellus* after 24 hrs (b) Contractile paralysis of *C. partellus* after 12 hrs (c) *C. partellus* larvae injected with buffer as control

Sub-fraction viiie had high toxic effect on mice but no effect on *B. fusca* and *C. partellus* (Table 11). It was potent against mice. When injected, the mice were first stunned then started to tremble, the severity of the tremors increased with time and lasted up to 24 h. The paws were contracted and the body took the shape of hunchback with frequent whole body jerks. The toxicity symptoms did not start immediately after the injection with fraction viiie was completed but increased in intensity for 30 min after a 10 min. lag. No biological activity against *C. partellus* and *B. fusca* larvae was detected for fraction viiie. Control animals injected with BSA in buffer rapidly recovered within three minutes after injection. From the UV absorbance of the C_4 RPHPLC column profile, sub-fraction viiid was estimated to constitute 3.3% of the crude venom while viiie constituted 6-7%.

Sub-fraction viiib exhibited relatively moderate effect on *C. partellus*, *B. fusca* larvae and mice while sub-fraction viiif showed significant insect toxicity coupled with mild effect on mice. Sub-

fraction viiia was isolated in small quantities that could not be assayed. Sub-fractions viiic was found to have mild effect on *C. partellus* only.

Table 11: Biological activity of C_4 RP-HPLC sub-fractions of fraction viii of *P. leiosoma* venom against, *C. partellus, B. fusca* larvae and mice

Sub-fraction number	*C. partellus*[a]	*B. fusca*	Mice[b]	Molecular mass $(M + H)^+$
viiia	*nd*	Nd	*nd*	**6742.5**, 7591.5,
viiib	+	+	+	Not detected
viiic	+	-	-	4094, 3508, **4078**, 2818
viiid	++++	++	-	7145, **6688.5**, 7198, 6715, 6781, 6688
viiie	-	-	++++	**7145.5**, 6688.5
viiif	++	+	+	**6742.5**, 7591, 5057

Relative toxicity denoted as: nd, not done –, nontoxic; +, mild toxic; ++, moderately toxic; and ++++, highly toxic;
$(M+H)^+$ detected molecular masses: * Bold indicates the most abundant mass

Sub-fraction viiid and viiie were further purified to homogeneity on a C_{18} micro-bore column after reduction and carboxymethylation resulting in >97% purity as determined by MALDI-TOF. The fractions were further assayed on insects and mice, respectively by sub-cutaneous injection and their biological activities confirmed Toxicity was only observed in the high intensity UV fraction collected from the microbore-run. The purified toxins were used for amino acid sequencing as detailed in the next chapter.

5.0 ELUCIDATION OF *PARABUTHUS LEIOSOMA* TOXINS

The sub-fractions with insecticidal activity or mammalian toxicity were considered for molecular characterization and structural analysis.

5.1. Structure elucidation of fraction viiid

BCA assay (Smith *et al.*, 1985) indicated that the protein concentration of fraction viiid (KN-1876) was 0.22 μgμ^{-1}.

LC-MS analysis of sub-fraction viiid (Fig. 7-8) revealed 4 peaks (1- 4) at 17.23, 17.59, 18.02 and 18.68 min (Fig. 8). The MS spectrum (Fig. 9) shows the m/z for a specific time that was matched to the UV and MS data and the peak. The MS region where the largest ions occur corresponding to the proposed molecular mass of peptide/protein is also shown (Fig. 9b). Computer assisted correlation of the time of flight (TOF) of a particular ion (m/z) facilitated the construction of the MS data for a peak and therefore the spectrum.

Figure 7: LC-UV profile for sub-fraction viiid (KN-01876)
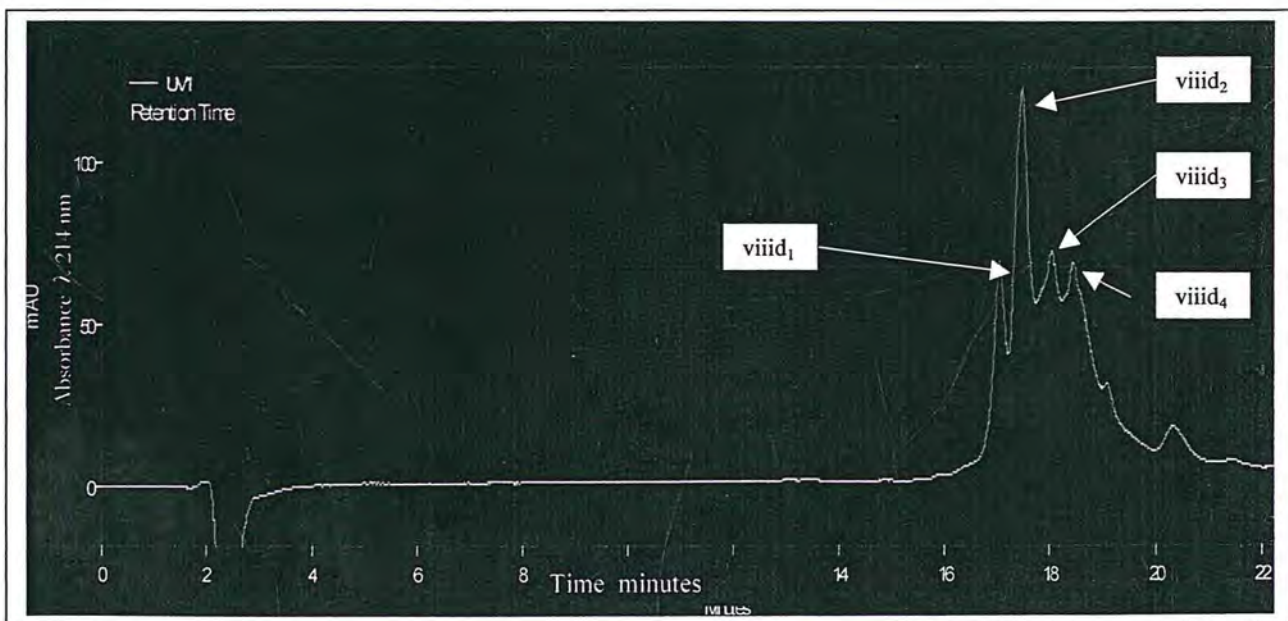
Figure 8: LC-MS profile of sub-fraction viii d (KN-1876)
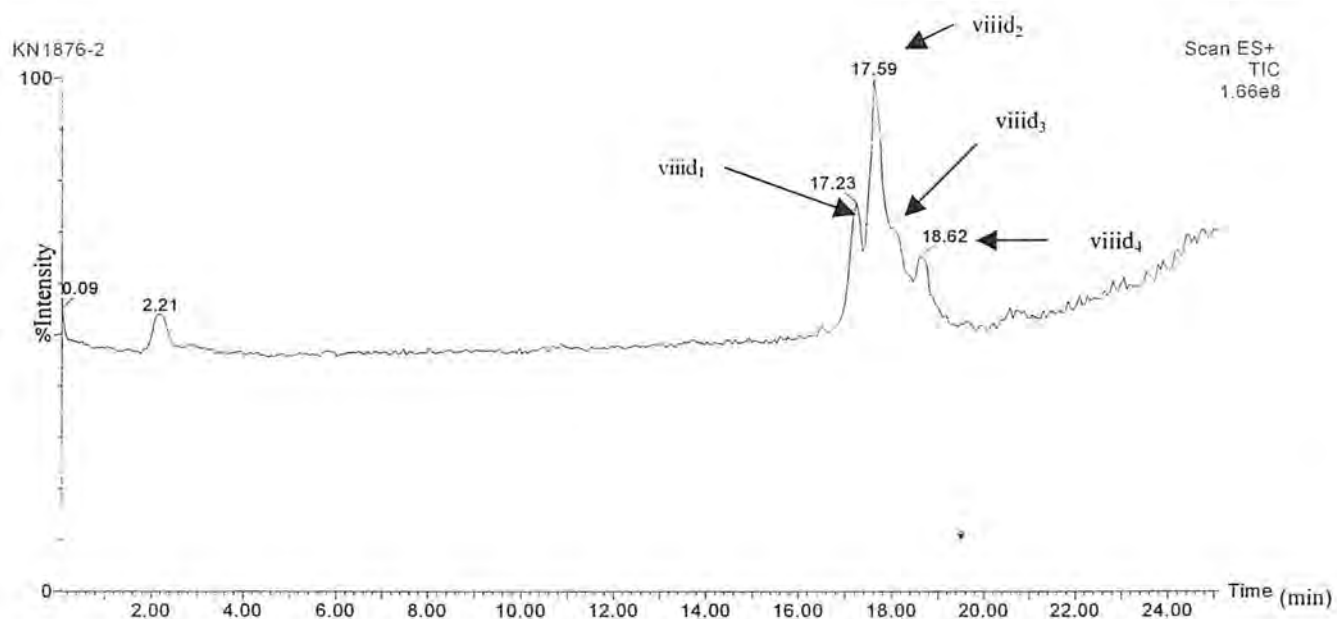
Figure 9a: Positive ESI-MS of low mass region for sub-fraction viiid₁
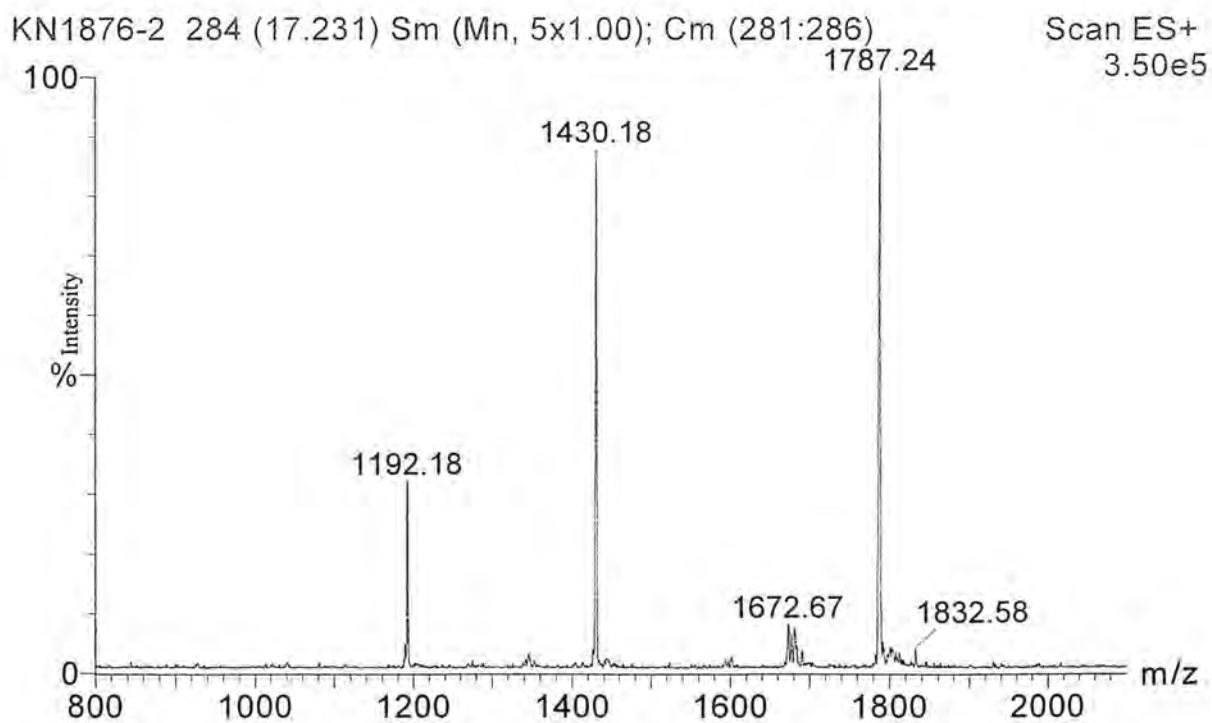
Figure 9b: Positive ESI-MS of high mass region for sub-fraction viiid₁

KN1876-2 284 (17.231) M1 [Ev-77827,It13] (Gs,2.500,800:2100,0.50,L20,R20
100
7145.50
3.53e6

% Intensity

5358.50 5716.50
8338.50 8575.00
0
5000 5500 6000 6500 7000 7500 8000 8500 mass
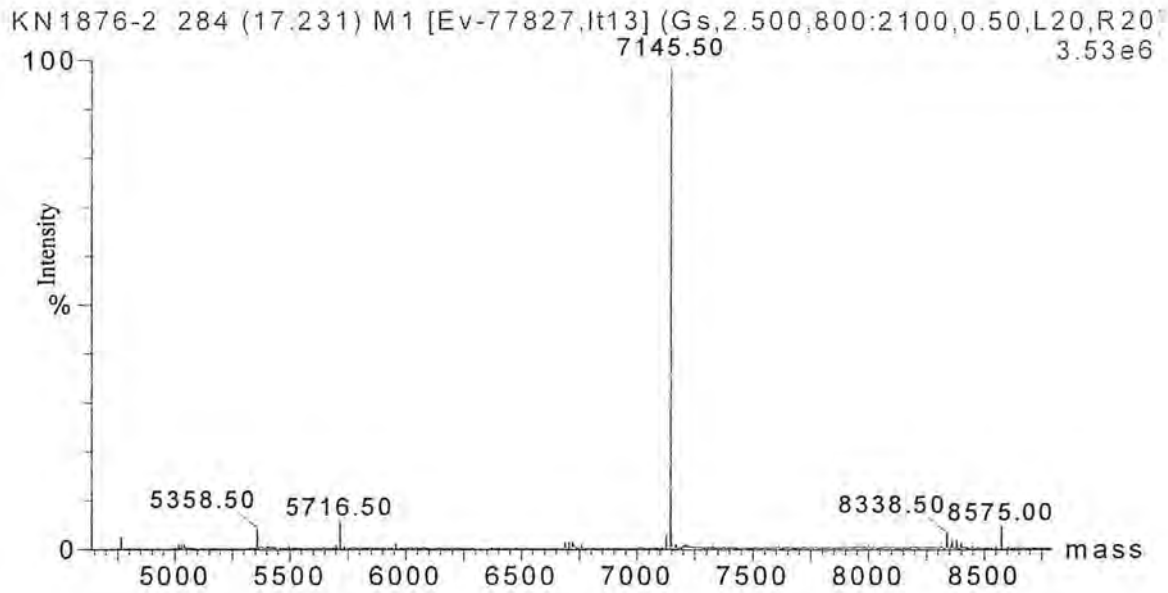
Figure 10a: Positive ESI-MS of low mass region for viiid₂

KN1876-2 290 (17.594) Sm (Mn, 5x1.00); Sm (Mn, 5x2.00); Cm (289:293)
100
1673.12
4.92e5

% Intensity

1800.21

1440.58

1338.86

1593.36

1200.72

0
800 1000 1200 1400 1600 1800 2000 m/z
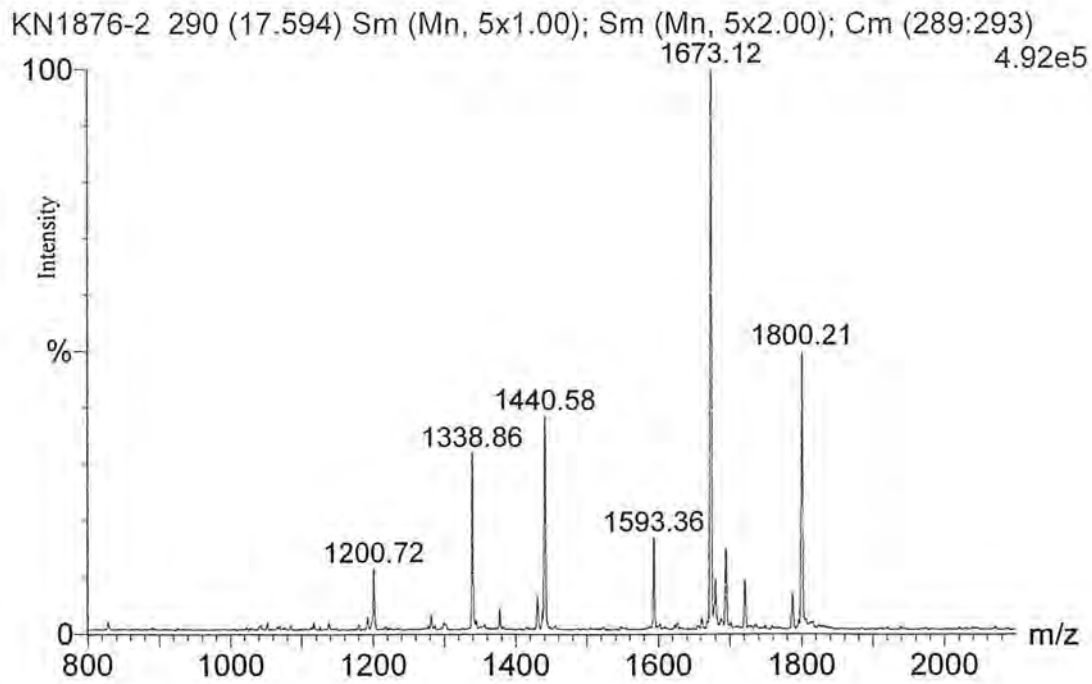
Figure10b: Positive ESI-MS of high mass region for sub-fraction viii d$_2$

KN1876-2 290 (17.594) M1 [Ev0,It21] (Gs,2.500,800:2100,0.50,L20,R20); Sm
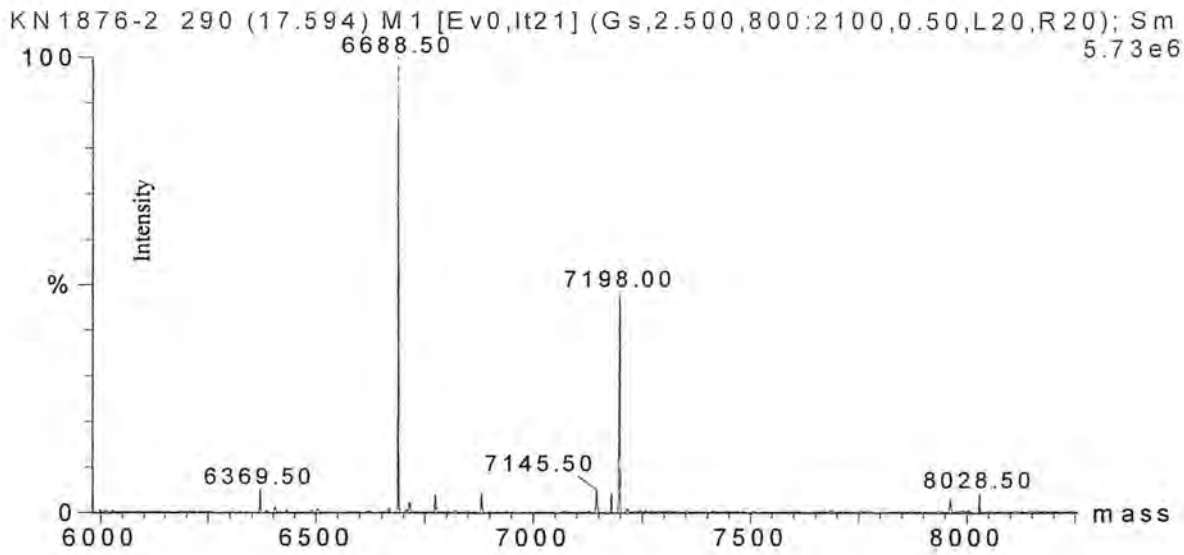
Figure 11a: Positive ESI-MS spectrum of low mass region for sub-fraction viiid$_3$

KN1876-2 297 (18.019) Sm (Mn, 5x1.00); Cm (296:301)   Scan ES+
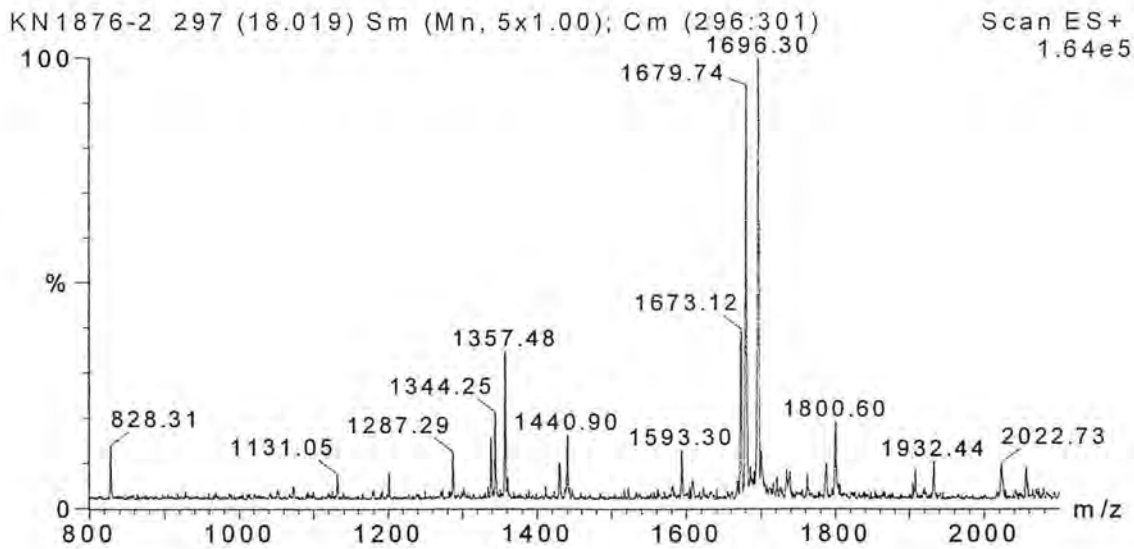
Figure 11b: Positive ESI-MS of high mass region for sub-fraction viiid$_3$

KN1876-2 297 (18.019) M1 [Ev-73482,It16] (Gs,2.500,800:2100,0.50,L20,R20
100 ― 6715.50 6781.50   1.24e6
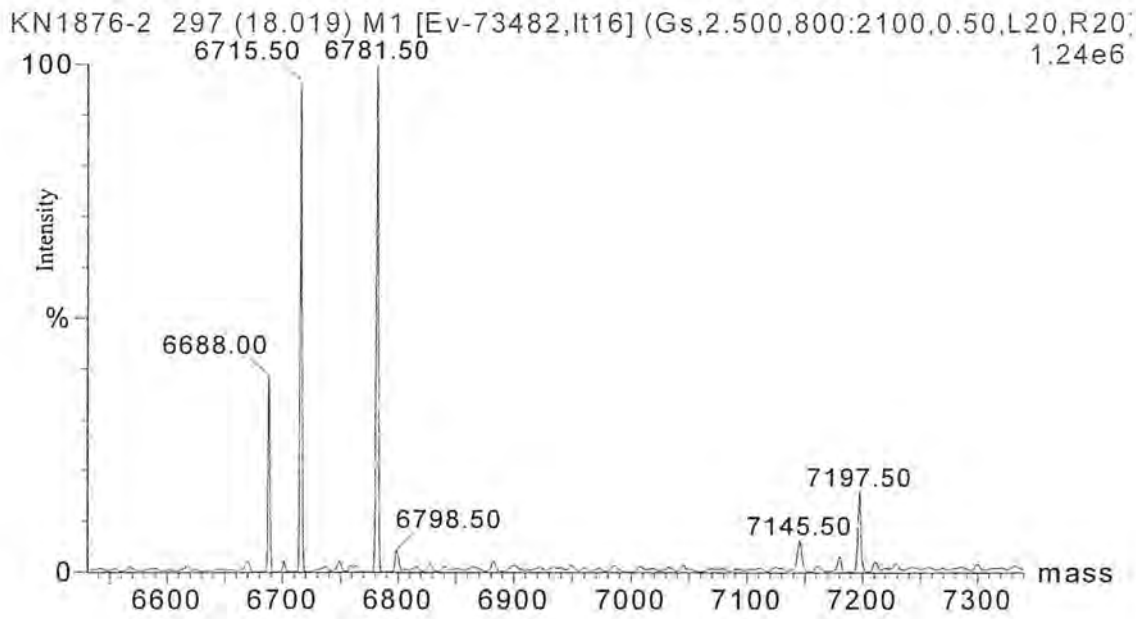
Figure 12: Positive ESI-MS spectrum of low mass region for sub-fraction viiid$_4$
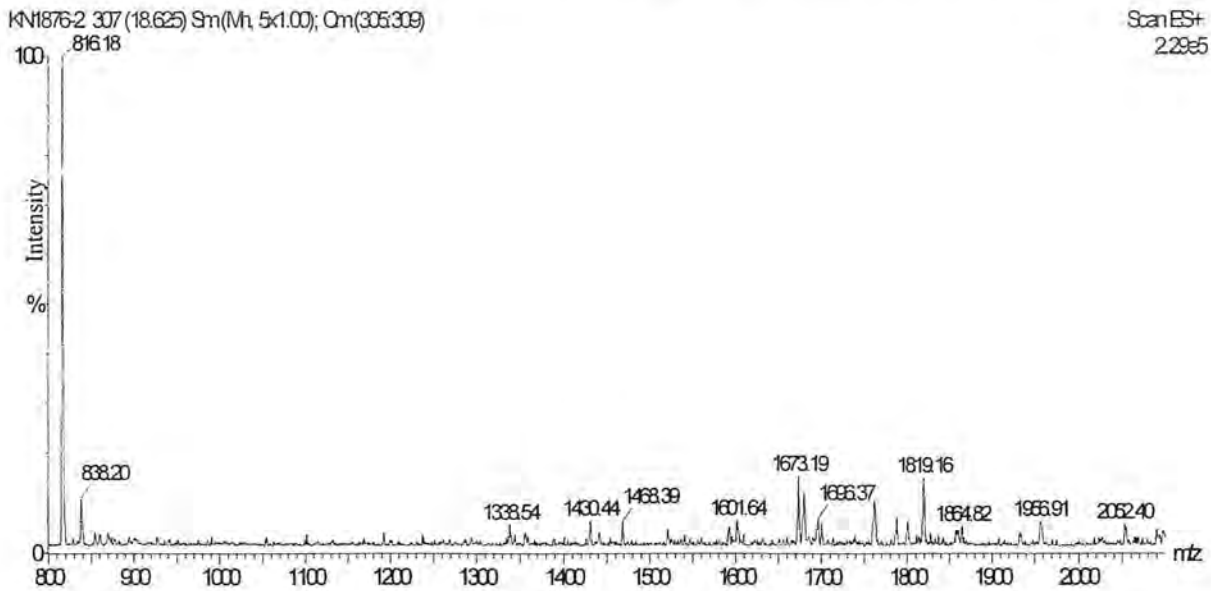
From the MS spectrum it would appear that either there is little to no protein or that it is a small peptide hence leading to low resolution to for maximum entropy process to determine the high mass region spectrum for sub-fraction viiid$_4$

-60-

Sub-fraction viii d (KN1876) was reduced, alkylated and subjected to LC-UV analysis (Figure 13) as described in the methodology and further purified by micro-bore LC, the peaks manually collected according to retention times. Peaks 9,12 and 16 were sequenced

Figure 13: LC-UV profile of alkylated and reduced sub-fraction viiid of *P.leiosoma* venom
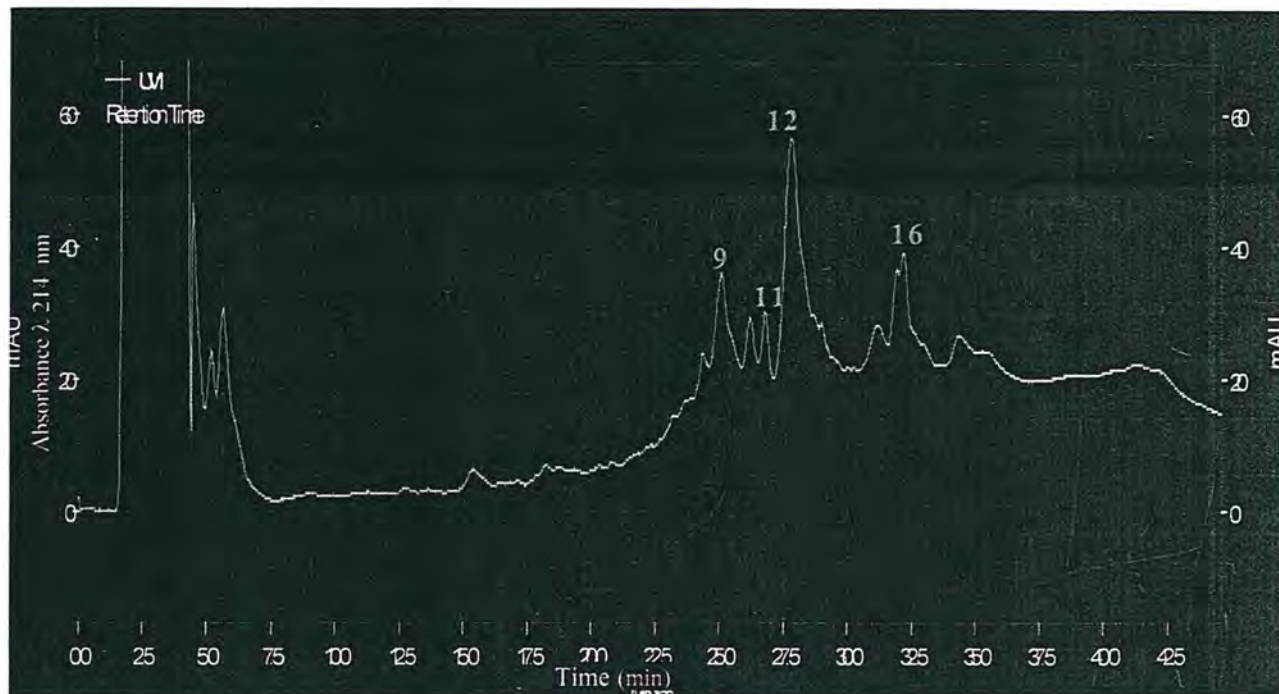
The partial *N*-terminal amino acid sequence of alkylated and reduced components of sub-fraction viiid is summarized in table 12.

Table 12: N-terminal amino acid partial sequences for components of alkylated and reduced sub-fraction viiid of *P. leiosoma* venom

Peak No.	File	Sequence
9	KN1876-9	KDGYPVDNAN C KYE
12	KN1876-12	KDGNY GP NYP LN ASK G
16	KN1876-16	ADVPGNYPL DDNCGN KRY

5.1.1 Structural analysis of Parabuthus *leiosoma* insect toxin (Plit)

The average molecular mass $(M+H)^+$ for the most abundant peaks; viiid$_1$ was 7145.5 Da viiid$_2$ (6688.5, 7198 Da) and viiid$_3$ was (6715.5, 6781.5 Da), determined by MALDI-TOF. The partial *N*-terminal sequence for the most active peak viii d$_9$ (Plit) after reduction and alkylation consisted of 15 amino-acid residues – KDGYPVDNANCKYE Multiple sequence alignment of partial N-terminal sequence of Plit with other related scorpion derived insect toxins using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) to access other databases revealed the 5 to some known toxins (Fig. 14).

Figure 14. The *N*-terminal amino acid partial sequence of *P. leiosoma* insect toxin aligned with known scorpion toxins using CLUSTALW program

```
Plit              -KDGYPVDNANCKYE----------------------------------------------- 14
Cse-V             KKDGYPVDSGNCKYECLKD--DYCNDLCLERKADKGYCYWGK---VSCYCYGLPDNSPT--KTSGKCNPA 63
Lqh II (Lqh2)     IKDGYIVDDVNCTYFCGRN--AYCNEECTKLKGESGYCQWASPYGNACYCYKLPDHVRT--KGPGRCR-- 64
Neurotoxin TbIT-I -KDGYPVDSKGCKLSCVAN--NYCDNQCKMKKASGGHCYAMS-----CYCEGLPENAKVSDSATNIC--- 59
CsE V5            -KDGYPVDSKGCKLSCVAN--NYCDNQCKMKKASGGHCYAMS-----CYCEGLPENAKVSDSATNICG-- 60
Insect Toxin      GKEGYPVDSRGCKVTCFFTGAGYCDKECKLKKASSGYCAWPA-----CYCYGLPDSVPVYDNASNKCB-- 63
                   *:**:**.  .*.
```

Entry names/abbreviations are given to the left of each sequence. Consensus or regions of identity are marked by symbol *. Discordantor regions are marked by : or .. All amino acid sequences are full length except Plit, which represents partial N-terminal sequence. Gaps (-) introduced to maximize homology (sequence identity). Toxin abbreviations used are as follows: Cse-V neurotoxin from *Centruroides exilicauder* (bark scorpion), Lqh II (Lqh2) neurotoxin from *Leirus quinquestriatus hebraeus*, , TbIT–I insect toxin from *Tityus bahiensis* and V-5 (CsEV5) neurotoxin from *Centruroides exilicauder* (bark scorpion).

Full sequence shall be determined from the cDNA library, which is currently under construction.

5.2 Structure elucidation of sub-fraction viiie (KN-1877)

BCA assays (Smith *et al.*, 1985) indicated that the protein concentration of sub-fraction viii e (KN-1876) was 0.34 $\mu g\ \mu l^{-1}$.

LC-MS analysis on sub-fraction viiie was done (Fig. 15-16). Two peaks (5 and 6) with retention time of 1.6 and 2.8 min., respectively, were observed. The m/z of various ions were matched to time of flight (TOF) and assigned to a particular peak thus giving rise to the mass spectra of corresponding compounds (Fig. 17-18). The mass spectral data for the regions with the low and high ion masses, corresponding to the mass of peptides/proteins, are also shown.

Figure 15: LC-UV profile for sub-fraction viiie of *P. leiosoma* venom (KN-01877)
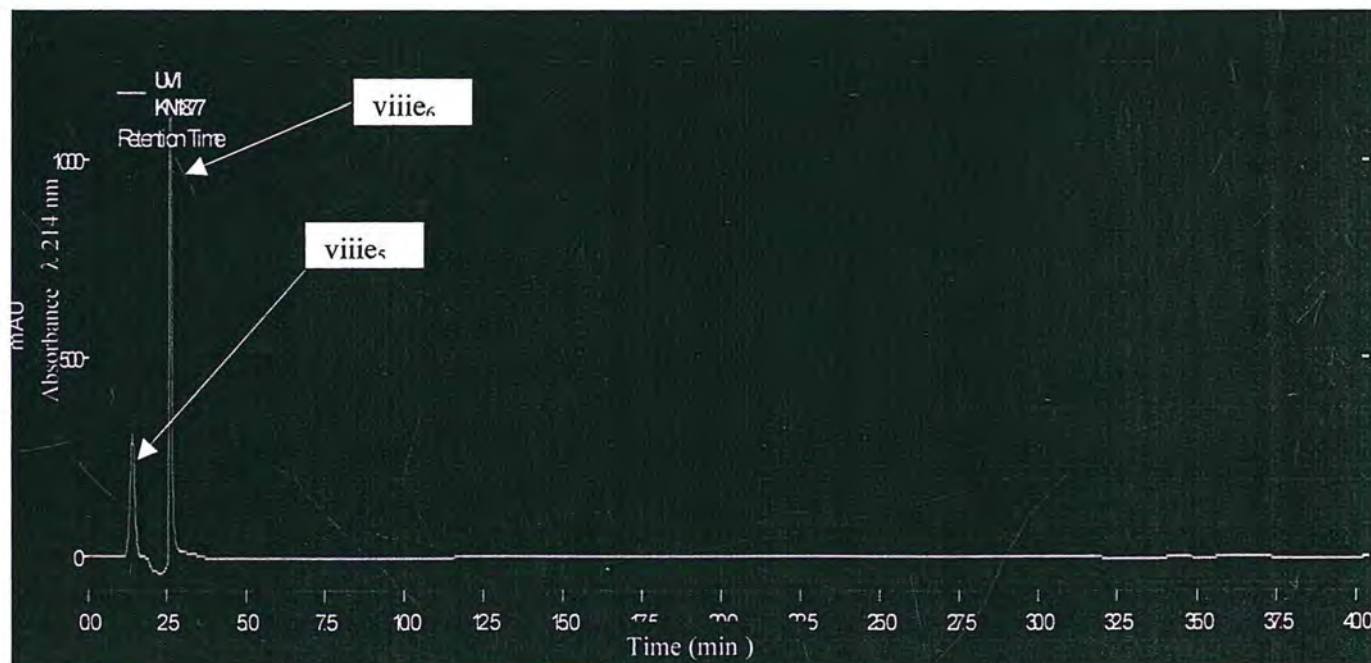
Figure 16: LC-MS profile of sub-fraction viiie of *P. leiosoma* vonom
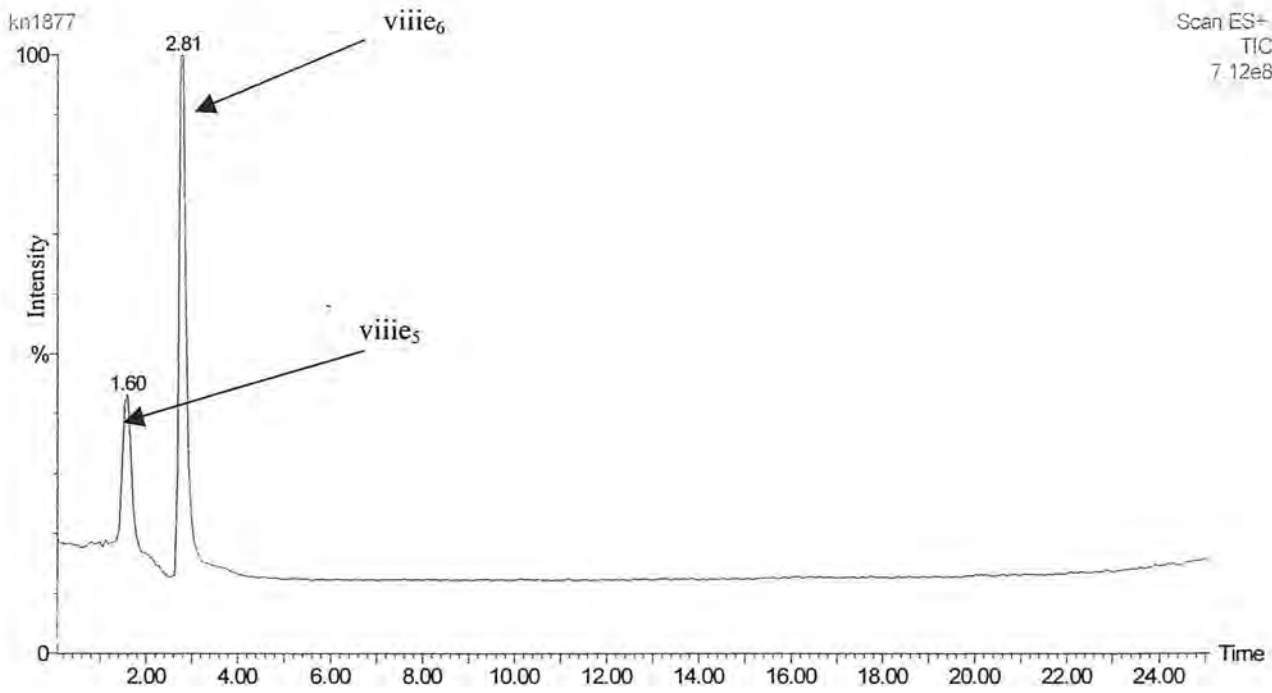
Figure 17a: Positive ESI-MS of low mass region for sub-fraction viiie$_5$
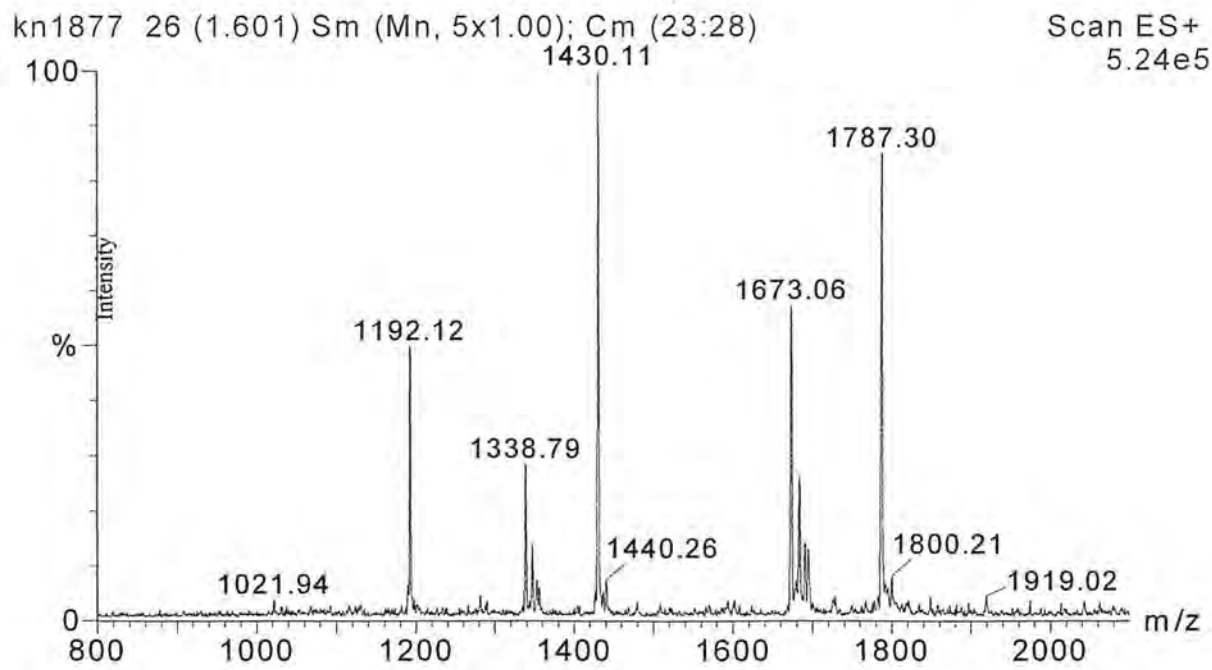
Figure: 17b Positive ESI-MS for high mass region for sub-fraction viiie$_5$

kn1877 26 (1.601) M1 [Ev0,It22] (Gs,2.500.800:2100,0.50,L20,R20); Sm (Mn,
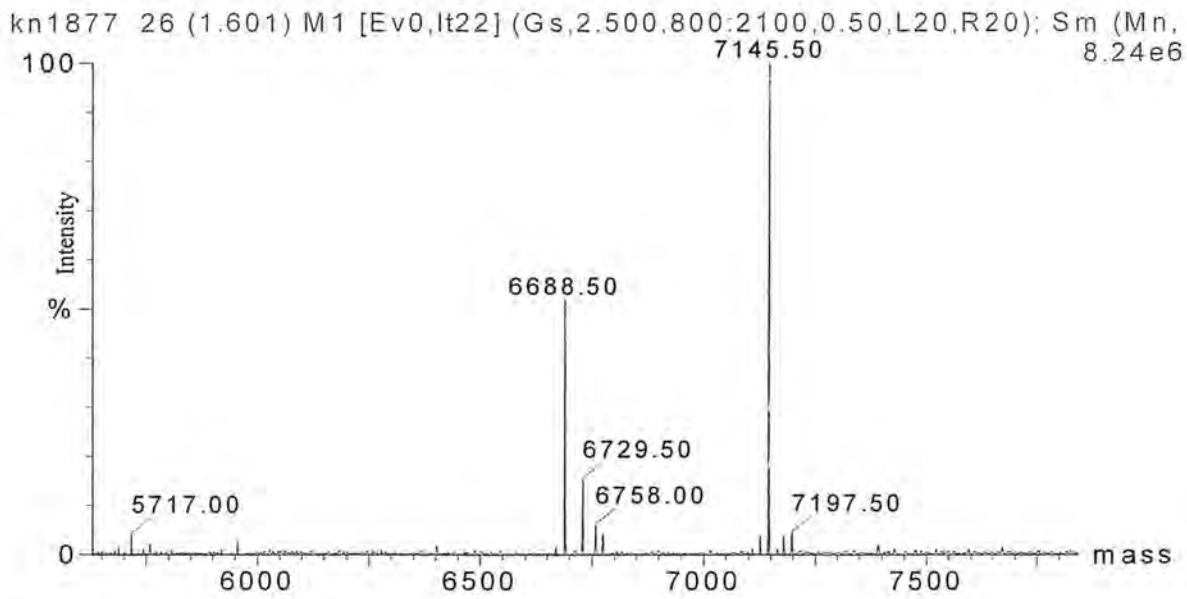
Figure 18a: Positive ESI-MS of low mass region for sub-fraction viiie$_6$

kn1877 46 (2.813) Sm (Mn, 5x1.00); Cm (44:47)   Scan ES+
                                                    1.42e6
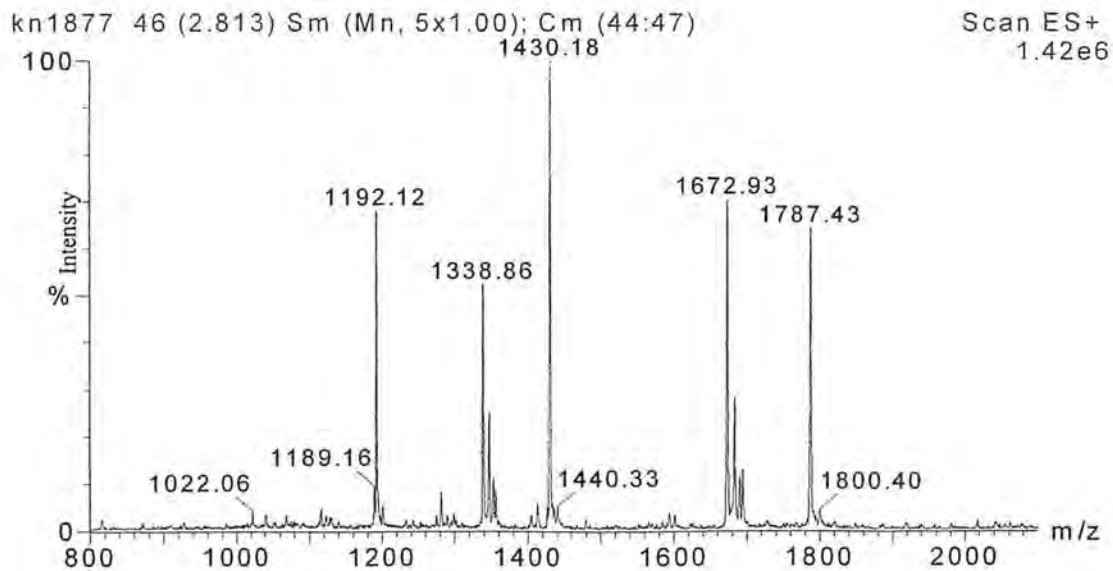
Figure 18b: Positive ESI-MS of the high mass region for sub-fraction viiie[6]

kn1877 46 (2.813) M1 [Ev-90188,It14] (Gs,2.500,800:2100,0.50,L20,R20); Sm
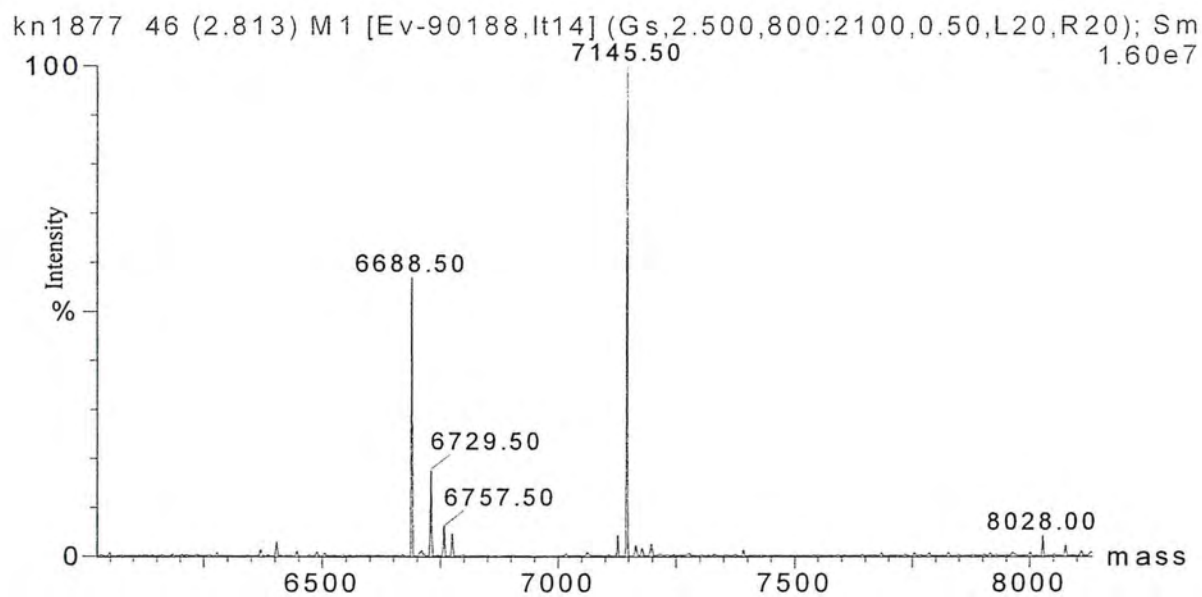
Sub-fraction viiie was alkylated, reduced and subjected to LC-UV analysis (Fig.19).

Figure 19: LC-UV profile of alkylated and reduced sub-fraction viiie of *P. leiosoma* venom
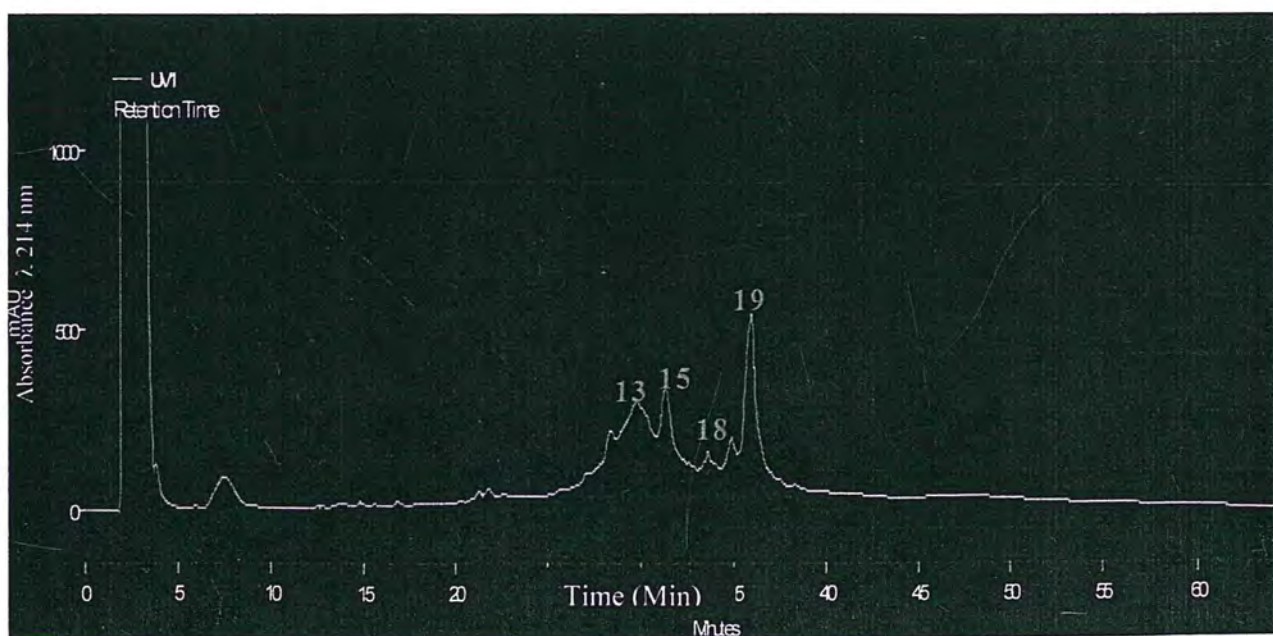
The LC peaks (14-20) from the alkylation and reduction of sub-fraction viiie were further purified by micro-bore LC and each peak collected manually. Sub-fractions viii e_{13}, viii e_{15}, viii e_{18}, and viii e_{19} were sequenced. The partial N-terminal amino acid sequences for peaks 13, 15, 18 and 19 are summarized in table 14.

Table 13: N-terminal amino acid partial sequences for components of reduced and alkylated sub-fraction viiie of *P. leiosoma* venom

Peak No.	File	Sequence
13	KN1877_13	KDGYPVDNAN CKYEAWY
15	KN1877_15	KDGYGNYNANGKYE
18	KN1877_18	ADVPGNYPLDKNGNRYY
19	KN1877_19	ADVPGNYPLDKNGNRYY

5.2.1 Structural analysis of *Parabuthus leiosoma* mammalian toxin (Plmt)

The average molecular mass $(M+H)^+$ for the most abundant peak viiie$_5$ and viiie$_6$ (R$_t$ 2.8 min.) was determined to be (7145.5, 6688.5 Da) by MALDI-TOF. This indicates that the peaks were similar. The partial N-terminal sequence for the most abundant peak (19) after alkylation and reduction had 17 amino-acid residues (-ADVPGNYPLDKNGNRYY-).

Multiple sequence alignment of N-terminal amino acid partial sequence of Plmt with other related scorpion derived toxins using Basic Local Alignment Search Tool (BLAST) software to access other databases revealed the 3 closest homologs (Fig. 20).

Figure 20: Alignment of the *N*-terminal amino acid partial sequence of *P. leiosoma* mammalian toxin (Plmt) with known scorpion toxins using CLUSTALW program

```
Plmt toxin         ----------------------ADVPGNYPLDKNGNRY------------------------Y------------------------------------- 17
Birtoxin           ----------------------ADVPGNYPLDKDGNTYKCFLLGGNE--ECLNVC-KLHGVQYGYCYASKCWCEYLEDD--------------KDSV--- 58
Toxin-KBT          MMKFVLFGMIVILFSLMGSIRGDDDPGNYPTNAYGNKYYCTILGENE--YCRKIC-KLHGVTYGYCYNSRCWCEKLEDK--------------DVTI--- 80
Neurotoxin-variant ---MKFFLMCLIIFPIMG---VLGKKNGYPLDRNGKTTECSGVNAIAPHYCNSECTKVYYAESGYCCWGACYCFGLEDDKPIGPMKDITKKYCDVQIIPS 94
                                       .  ..**  :    *:
```

Entry names/abbreviations are given to the left of each sequence. Consensus or regions of identity are marked by symbol *. Discordantor regions are marked by : or . All amino acid sequences are full length except Plmt, which represents partial *N*-terminal sequence. Gaps (-) introduced to maximize homology (sequence identity). Toxin abbreviations used are as follows: Plmt toxin a neurotoxin from *P. leiosoma*, Birtoxin a neurotoxin from *Parabuthus transvaalicus* (South African fat tail scorpion). Toxin-KBT a neurotoxin from *Mesobuthus martensii* and Neurotoxin-variant from *Hottentotta judaica*.

Complete sequence shall be determined from the cDNA library, which is currently under construction.

5.3 Structure elucidation of sub-fraction viii f (KN-1878)

BCA assays (Smith *et al.*, 1985) indicated that the protein concentration of sub-fraction viii f (KN-1878) was 0.0895 $\mu g \, \mu l^{-1}$.

LC-MS analysis on sub-fraction viiif revealed one major peak (7) with retention time 20.6 min. (Fig. 22-23). The m/z of various ions were matched to time of flight (TOF) and assigned to a particular peak thus giving rise to the mass spectra of corresponding compounds (Fig. 24-25). The mass spectral data for the regions with the highest ion masses corresponding to the mass of peptide/protein is also shown (Fig. 24b).

Figure 21: LC-UV profile for sub-fraction viiif, of *P. leiosoma* venom (KN-01878)
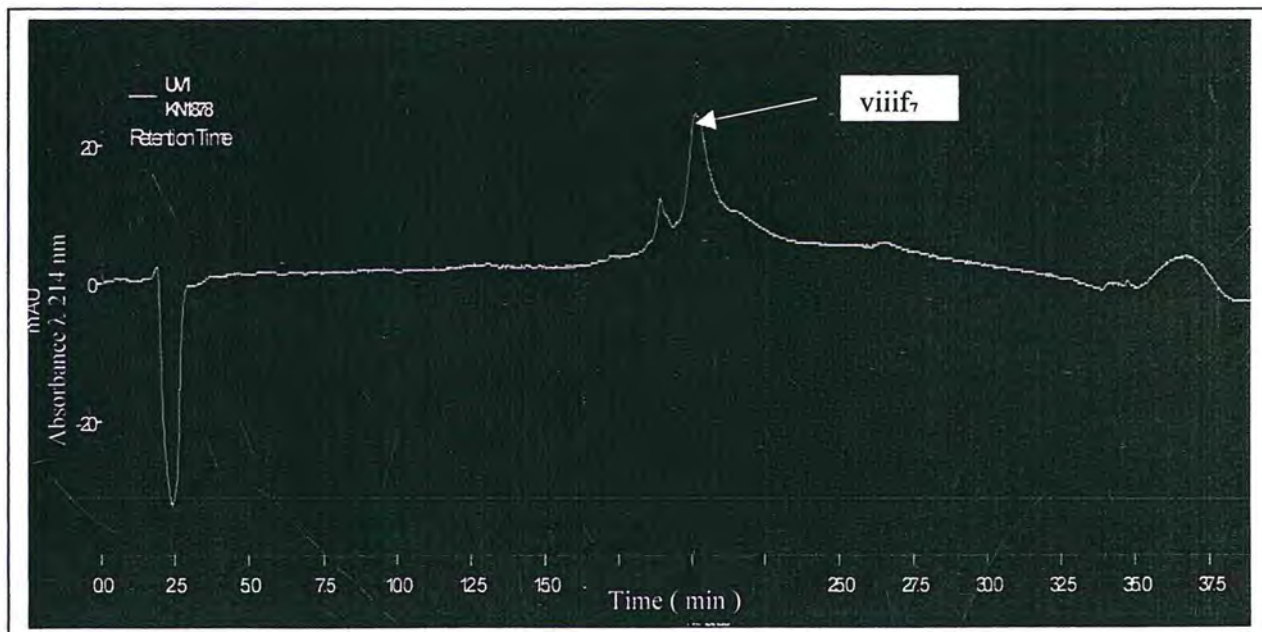
Figure 22: Positive ESI-MS sub-fraction viii f of *P. leiosoma* toxin
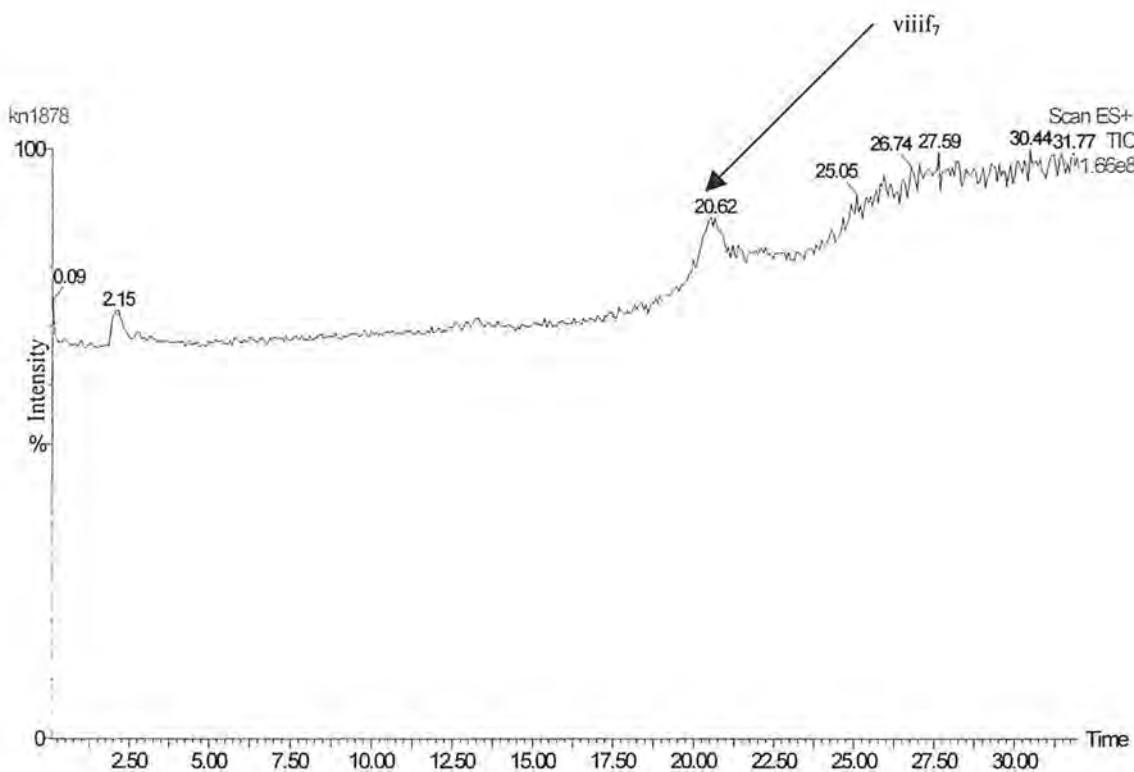
Figure 23a: Positive ESI-MS of low mass region for sub-fraction $viiif_7$.
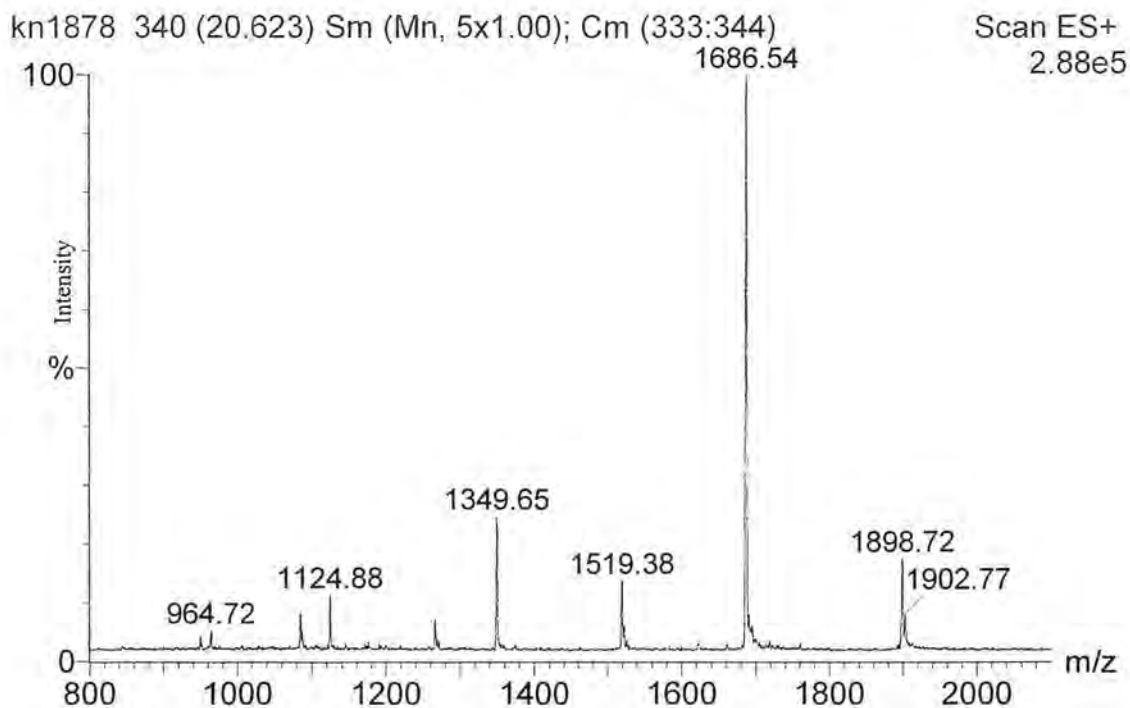
Figure 23b: Positive ESI-MS of high mass region of sub-fraction viiif₇

kn1878 340 (20.623) M1 [Ev-81824,It9] (Gs,2.500,800:2100,0.50,L20,R20); Sr
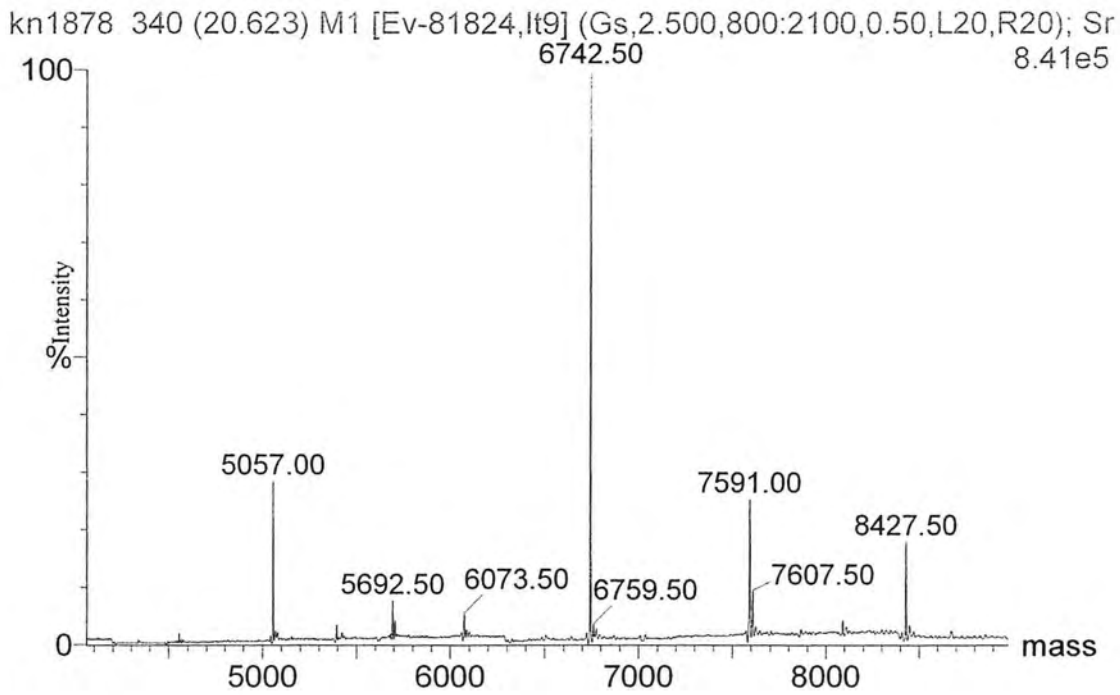
Sub-fraction viiif was alkylated, reduced and subjected to LC-UV analysis (Fig. 24).

Figure 24: LC-UV profile of alkylated and reduced sub-fraction viiif of *P. leiosoma* venom
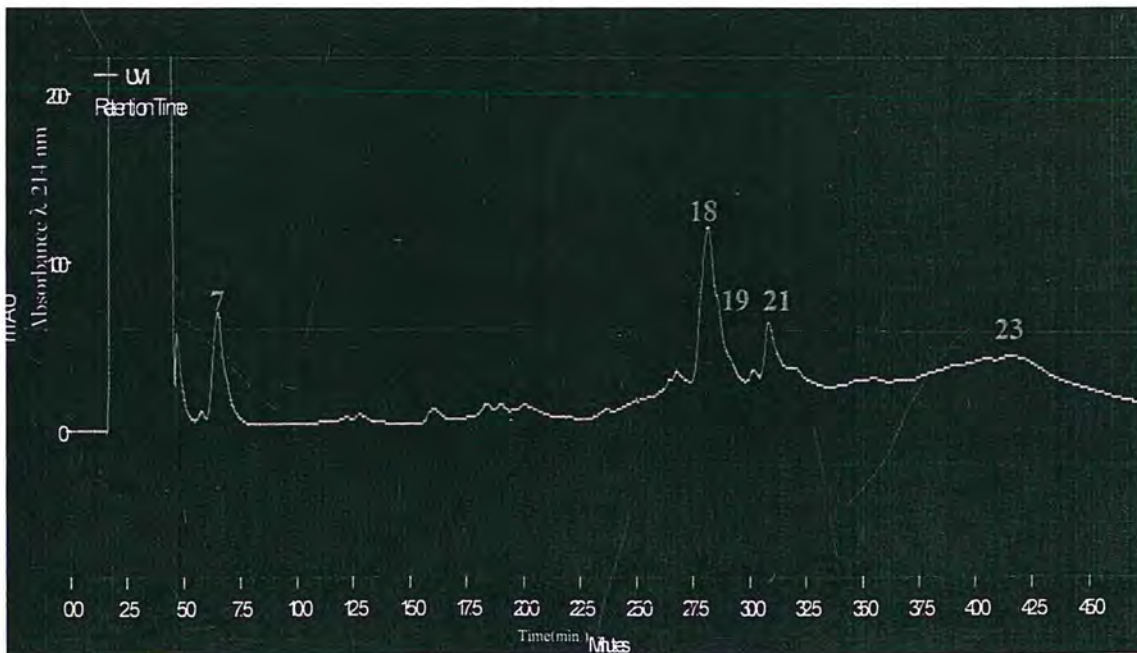
The profile revealed alkylation and reduction and at least 2 proteins. The MS data of the reduced and alkylated sub-fraction viiif revealed masses of ions corresponding to a protein

consistent with the presence of 1, 2, 3, 4 and 5 cysteine moieties in the same LC-MS peak at 20.62 minutes (Fig. 22).

The LC peaks (7-23) from the alkylation and reduction of sub-fraction viiif were further purified by micro-bore LC and each peak collected manually. Sub-fractions viii f_7, viii f_{18}, viii f_{19}, viii f_{21}, and viii f_{23} were sequenced. The partial N-terminal amino acid sequences for peaks 7, 18, 19, 21 and 23 are summarized in Table 14.

Table 14: Partial *N*-terminal amino acid partial sequences for components of reduced and alkylated sub-fraction viiif of *P. leiosoma* venom

Peak	Filename	Sequence
07	KN1878_07	Probably Chemical
18	KN1878_18	L QEKFKDKLTDAKN
19	KN1878_19	LCEKFKDKLTDAKNMVK
21	KN1878_21	GLREKHVQRLVELALPD
23	KN1878_23	GLR

5.3.1 Structural analysis of *Parabuthus leiosoma* toxin (Plt) from sub-fraction viiif

The average molecular mass $(M + H)^+$ for the most abundant sub-fraction viiif$_7$ (R_t 20.62 min.) was determined to be 6742.5 Da using MALDI-TOF. The partial *N*-terminal sequence for the most abundant and active peak (19) after alkylation and reduction had 17 amino acid residues (-LCEKFKDKLTDAKNMVK-). When matched with known peptides, the sequence gave no homolog to already discovered toxins. Complete sequence shall be determined from the cDNA library, which is currently under construction.

5.6 Discussion

In this study, we provide insight into the biological effect of principles isolated from scorpion venoms on insect and mice. Comparing previous and present data on the composition and action of *Parabthus leiosoma* venom with well established information on scorpion venoms the following conclusions could be made; in both toxins

  (i) The active principles are peptides that demonstrate animal group specificity with regard to their toxicity.

  (ii) Some peptides were toxic to insects only

  (iii) Others were effective on mice (mammal) only.

  (iv) Yet others were toxic to both insects and mice.

Interesting and essential differences were shown as demonstrated in insects subcutaneously injected with the isolated toxins. The insect-toxic components in the scorpion venom included a fast reversible paralytic factor and a slow lethal paralytic factor

Oral toxicity test showed no significant effect of the crude venom of the three scorpion species. The observation is supporting results that were obtained earlier by Quistad (1992) while testing venom from scorpions and spiders. The same was observed in other toxins tested through oral administration (Quistad *et al.*, 1988). Apparently venoms and toxins encounter different enzymes (peptidases) upon ingestion, affecting mostly peptides that may not survive proteolytic attenuation. In the digestive system, small proteins (7 kD) have been recorded to penetrate through the digestive track of certain insects such as flesh flies and *Sarcophagi* sp (Primor & Zlotkin, 1980).

The major insecticidal components of venoms are polycationic polyamines and proteins that cannot penetrate the cuticle restricting topical application as method of testing scorpion toxin potency (De Diamous *et al.*, 1988). Insect selective peptide toxin, AaIT, was found to be 500 times less effective through topical application than injection in *Musca domestica* L.

The partial *N*-terminal amino acid sequence of the lepidopteran-selective toxin; Plit (*Parabuthus leiosoma* insect toxin) was determined as – KDGYPVDNANCKYE-. It shows high homology of partial *N*-terminal sequence to other 5 known insect toxins (Fig. 13). A search of protein sequences using BLAST P (Basic Local Alignment Search Tool – Proteins) under short exact matches - revealed that Plit is homologoues to Cse-V neurotoxin from *Centruroides exilicauder* (bark scorpion), Lqh II (Lqh2) neurotoxin from *Leirus quinquestriatus*

hebraeus, TbIT–I insect toxin from *Tityus bahiensis* and V-5 (CsEV5) neurotoxin from *Centruroides exilicauder* (bark scorpion). The first set of cysteine residues at position 12 are perfectly aligned. Other degrees of conserved amino acids include K in position 2, G in position 4, Y in position 5, V in position 7 and D in position 8. The multiple alignments also showed conserved substitution at position 3 where D has been substituted for K. This indicates the high degree of homology of the purified toxin, Plit at partial *N*-terminal amino acid sequence level to other members of scorpion toxins, more so, in respect to their functionality as insect selective toxins. The mode of action may be similar to the known homologous toxins.

This is the first report of an insect selective peptide toxin (Plit) from a Kenyan scorpion species *P. leiosoma*. The isolation of Plit from scorpion venom gives new insights into the mode of action of toxins and opens new avenues for discovery of novel insecticidal molecules from arthropod venoms. Since the peptide was only partially sequenced, it would be interesting to establish the other amino acid residues for further structural analysis and comparison. From bioassay observations the novel neurotoxin Plit exhibits excitatory activity on the insect although the details of its mechanisms of action remain to be determined.

The study also led to purification and characterization of another peptide selectively toxic to mice, *Parabuthus leiosoma* mammalian toxin (Plmt) with partial *N*-terminal amino acid sequence -ADVPGNYPlDKNGNRYY-. The peptide shows high homology with 3 other known toxins; Birtoxin neurotoxin from *Parabuthus transvaalicus* (South African fat tail scorpion), Toxin-KBT neurotoxin from *Mesobuthus martensii* and Neurotoxin-variant from *Hottentotta judaica* (Fig. 19). The neurotoxin Plmt showed highest homology to Birtoxin, a neurotoxin from *Parabuthus transvaalicus*, this could be described as a chemotaxonomic neurotoxin since the two source scorpions fall in the same genus. The alignment clearly indicates a degree of conservatism at positions 29, 30 and 35 occupied by Tyrosine, Proline and Glycine, respectively. The toxin may be useful for understanding the salient characteristics for mammal selective scorpion toxin. The mode of action may be similar to that of the known homologous toxins. The full amino acid sequence is under investigation through the cDNA library construction.

The other peptide that showed significant insect toxicity coupled with mild effect on mice displayed partial *N*-terminal sequence –LCEKFKVQRLVELNCVD-. This peptide (Plt) has

no similarity with any known scorpion toxin.. It may be a new neurotoxin that may be used for further studies on understanding the characteristics of non-specificity. The full amino acid sequence is under analysis through cDNA library construction

The isolation of peptide toxins from scorpions leads to the determination of amino acid composition and sequence of new scorpion toxins. This is important for structure-activity relationship studies (Darbon *et al.*, 1983) and discovery of new types of scorpion toxins (Jover *et al.*, 1980).

CHAPTER SIX

CONCLUSION AND FUTURE PERSPECTIVES

6.1 Conclusion

The isolation of the insect selective toxin demonstrates that the defensive secretion of scorpions continues to be more promising source of insect selective toxins. All species in Buthidae family produce a series of polypeptides in their telson glands, which appear to be responsible for the potency of their venoms as animal poisons. The increases in the number of toxins that have been identified in scorpion venoms constitute a class of compounds that vividly illustrates the insecticidal potential of the venoms from arthropods. Although the chemistry of the venoms of only a few of the stinging scorpions has been examined in detail, it can be assumed that the poisonous venoms are predominantly proteins. From this study, two bioactive principles viiid and viiif were obtained from the venom of *P. leiosoma*. Fraction viiid was selectively active on insects (*C. Partellus* and *B. fusca*) and had no activity on Mice. Whereas, fraction viiif had mild activity on *C. partellus*, weak activity on *B. fusca* and mice. Fraction viiid is an excitatory insect selective peptide, here refereed to as Plit, isolated from the venoms of *Parabuthus leiosoma*, a scorpion species dominant in southern and eastern Africa that had not been examined before.

6.2   Future perspective

Scorpion venom is a promising tool for study of essential physiological activities controlled by the nervous system. Highly purified and potent toxins isolated from these   venoms and active upon excitable tissue could be used to investigate molecular changes responsible for important functions such as the synthesis, storage and release of neurotransmitters. In addition, the study of the interaction of scorpion venom with terrestrial invertebrate organism may constitute an interesting model of biological interaction. As such efforts in these directions are worth pursuing.

The scope of this work was wide but was limited by funding and listed objectives. Consequently, further work needs to be done to reveal more information on the discovered toxin. This study was limited to identification of the primary structure by amino acid sequencing. This was partially achieved by providing partial *N*-terminal sequence. To determine the complete primary structure of Plit, the corresponding cDNA need to be

cloned and total RNA isolated from telson glands of the scorpion with an aim of getting complete sequence. Once the full sequence of Plit is obtained, the gene expressing the toxin need to be cloned and introduced in a baculovirus to form a rocombinant virus. This can be tested in the field for its efficacy, effect to the environment and non-target toxicity before incorporating it into existing in integrated pest management strategies.

From the identified scorpion species, only a few have been analysed, hence more work need to be done to discover insect selective toxins therein.

REFERENCES

Adam K.R., Weiss C. (1959b). Action of scorpion venom on skeletal muscle. *Brit. J. Pharmacol.* **14**: 334-339.

Balozet L. (1971). Scorpionism in the old world. In: Bucherl W. & Buckley E.E. (eds), *Venomous animals Animals and their Venoms*, Vol. 3: *Venomous Invertebrates*. Academic Press, New York. p349-371.

Barret C.B. (2000). Is bioprospecting a viable strategy for conserving tropical ecosystem. *Ecol. Econ.* **34**: 293-300.

Batista C.V., Gomez L.F., Lucas S., Possani L.D. (2000). TCL from *Tityus cambridgei*, is the first member of a new sub-family scorpion toxin that blocks K^+ channels. *FEBS Lett.* **486**: 117-120.

Berdy J. (1989). The discovery of new bioactive microbial metabolites: Screening and identification. In: Bushell M.E., Graefe U. (eds), *Progress in Industrial Microbiology*, 27th Edn. Elsevier, Amsterdam, p398

Betz F.S., Hammond B.G., Fuchs R. (2000). Safety and advantages of *Bacillus thuringiesis* – protected plants to control insect pests. *Reg. Toxicol. Pharmacol.* **32**: 156 - 173.

Black B.C., Brennan L. A., Dierks P.M., Gard I.E. (1997). Commercialization of baculoviral insecticides. In: Miller L.K. (ed), *The Baculoviruses*. Plenum, New York.

Blagbrough I.S., Brackley P.T.H., Bruce M., Bycroft B.W. Matter A.J., Millington S., Sudan H.L Usherwod P.N.R. (1992). Arthropod toxins as leads for novel insecticides: An assessment of polyamine amides as glutamate an antigenenists. *Toxicon* **30**: 303-322.

Borrecco J.E., Black H.C., Hooven E.F. (1979). Response of small mammals to herbicide induced vegetation changes. *Northwest Sci.* **53**:97-106.

Briggs G.G. (1998).*Advances in the Chemistry of Insect Control, III*. Royal Society of Chemistry, London, pp315.

Brownell P.H. (2001). Sensory ecology and orientational behaviors. In: Brownell P.H. & Polis A.(eds), *Scorpion Biology and Research*. New York,Oxford University Press, p159-183.

Bowman W.C., Harvey A.L. (1995). The discovery of drugs. *Proc. Royal Coll. Physic. Edinburgh*. **25**: 5-24.

Bucherl W. Buckley E.E. (eds) (1971). *Venomous animals and their venoms*, Vol. 3: *Venomous Invertebrates*. Academic press, New York & London.

Bulet P., Hetru C., Dimareq J.L., Hoffman D. (1999). Antimicrobial peptides in sinsects; structure and function. *Dev. Comp. Immun.* **23**: 329-344.

Busvine R.J. (1971). *A critical Review of the Techniques for testing Insecticides*, Commonwealth Agricultural Bureau, London, p263-288.

Catterall W.A. (1980). Neurotoxins that act on voltage-sentive sodium channels in excitable membranes. *A. Rev. Pharm.* **20**: 15-45.

Casper C.S. (1985). Prey capture and stinging behavior in the emperor scorpion, *Pandinus imperator* (Koch) (Scorpiones, Scorpionidae). *J. Arachnol.* **13**: 277-283.

Chejanovsky N., Zilberberg N., Rivkin H., Zlotkin E., Gurevitz M. (1995). Functional expression of an alpha anti-insect scorpion neurotoxin in insect cells and lepidopterous larvae. *FEBS Lett.* **376**: 181-184.

Chippaux J.P., Williams V., White J. (1991). Snake venom variability: Methods of study, results and interpreations. *Toxicon* **29**: 1279 - 1303.

Costa-Neto E.M. (2005). Animal based medicines: biological prospection and the sustainable use of zootherapeutic resources. *Ann. Brazil Acad. Sci.* **77**: 33-43.

Cunningham J.C. (1982). In: Kurstak E. (ed), *Microbial and Viral Insecticides.* Dekker, New York, p335-386.

Darbon H., Jover E., Coraud F. Rochat H. (1983). Scorpion neurotoxin derivatives as potential markers of sodium channels. *Int. J. Peptide Protein Res.* **22**:179-199.

Dauplais M., Giquin B., Possani L.D., Gurrola-Briones G., Roumestand C., Menez A. (1995). Determination of three dimensional solution structure of noxiustoxin: analysis of structural differences with related short-chain scorpion toxins. *Biochemistry* **34**: 16563-16573.

De Dianous S., Hoarau F., Rochat H. (1988). The effect of the mode of application on toxicity of *Androctonus australis* (Hector) insect toxin. *Pestic. Sci.* **23**: 35-40.

DeBin J.A., Maggio J.E., Strichartz G.R. (1993). Purification of chlorotoxin, a choloride channel ligand from venom of scorpion. *Am. J. Physiol. (Cell Physiol)* **264**: 361-369.

Duerdon J.C. (1953). Stem borers of cereal crops at Kogwa, Tanganyika 1950-52. *E. Afr. Agric. J.* **19**: 105-118.

DuPont (1996). Notification to conduct small-scale field-testing of a genetically altered baculovirus. *EPA.* No. 352-NMP-4.

Edman P. (1970). Sequence determination determination. In: Needleman S.B. (ed), *Protein Sequence Determination.* Springer Verlag, Berlin, p224.

Eisner T., Alsop D., Meinwald J. (1978). Secretions of opilionids, whip scorpions, and pseudoscorpions. In: Bettini S. (ed.), *Handbuch der Experimentellen Pharmakologie.* Springer-Verlag, Berlin, p87-99.

Findlay J.B.C., Geisow M.J. (1989). *Protein Sequencing: A Practical Approach.* Oxford Univeristy Press, New York.

Finelli V.N., Carlo F., Ardesio F., Brigitte W. (1990). *Laboratory Methodology in Biochemistry: Amino Acid Analysis and Protein Sequencing.* CRC Press Inc., Boca Raton.

Finney D. (1971). *Probit Analysis*, 3rd Edn. Cambridge University Press, Cambridge.

Francke O.F., Boos J. (1986). Chactidae (Scorpiones) from Trinidad and Tobago. *J. Arachnol.* **14**: 15-28.

Frawley J.P., Fuyat H.N., Hagan E., Blake J.R. Fithugh O.G. (1957). Marked potential in mammalian toxicity from simultatneous administration of two anticholineterase compounds. *J. Pharmacol. Expt. Ther.* **212**: 96-106.

Froy O., Sagiv T., Poreh M., Urbach D., Zilberberg N., Gurevitz M. (1999a). Dynamic diverfication from a putative common ancestor of scorpion toxins affecting sodium, potassium and choloride channels. *J. Mol. Evol.* **48**: 187-196.

Froy O., Zilberberg N., Gordons D., Turkov M., Gilles N., Stankiewicz M., Pelhate M., Loret E., Oren D.A., Shaanan B., Gurevitz M. (1999b). The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J. Biol Chem.* **274**: 5769-5776.

Garcia C., Becerril B., Selisko B., Delepierce M., Possan L.D. (1997a). Isolation, characterization and comparison of a novel crustacean toxin with mammalian toxin from the venom of the scorpion *Centruroides noxious* (Hoffmann). *Comp. Biochem. Physiol.* **116:** 315-322.

Garcia M.L., Hanner M., Knaus H.G., Koch R., Schmalhofer W., Slaughter R.S., Kaczorowski G.J. (1997b). Pharmacology of potassium channels. *Adv. Pharmacol.* **17**: 4125-4171.

Gard I.E. (1997b). Field testingField-testing a genetically modified baculovirus. In: *Microbial insecticides: Novelty or necessity.* Proceeding of British Crop Protection Council Symposium, No. 68., British Crop protection Council, Surrey, p101-114.

Gershburg E., Stockholm D., Froy O., Rashi S., Gurevitz M., Chejanovsky N. (1998). Baculovirus-mediated expression of scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. *FEBS Lett.* **422**: 132-136.

Gong W., Dong-Sheng W., Fa-Hu H., Guo-Yuan H., Hou-Mitng W. (1998). A K$^+$ channel-blocking peptide from venom of Chinese scorpion *Buthus martensii* Karsch. *Acta Pharm. Sinica* **4**: 453-462.

Gopalakrishnakone C., Gwee T. (1995). Black scorpion (*Heterometrus longimanua*) as a laboratory animal: mainteinance of colony of scorpion for milking of venom for research, using a restraining device. *Lab. Anim.* **29**: 456-458.

Gordon D., Savarin P., Gurevitz M., Zimm-Justin S. (1998). Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol. Toxin. Rev.* **17**: 131-159.

Goyffon M., Kovoor J. (1978). In: Bettini S. (ed.), *Arthropod Venoms, Vol. 48.* Springer-Verlag, Berlin, p317-369.

Grisley W. (1997). Crop-pest yield loss: a diagnostic study in the Kenya highlands. *Int. J. Pest Manag.* **43**: 137-142.

Gunn D.L., Steren J.C.R. (1976). *Resistance and Human Welfare.* Oxford University Press, Oxford, p181-192.

Harris K.M. (1989). Recent advances in sorghum and pearl millet stem borer research. In: Nwanze K.F. (ed). *Proceedings of International Workshop Sorghum Stem Borers,* Patancheru, 17-20 ICRISAT, November 1987, Patancheru, India, p9-16.

Harrison R.L., Bonning B.C. (2000). Use of scorpion neurotoxins to improve the insecticidal activity of *Rachiplusia* on multicapsid nucleopolyhedrovirus. *Biol. Control* **17**: 191-201.

Harvey A.L. (1995). From venom to toxins to drugs. *Chem. Ind.* Available at: http://ci.mond.org/952214.html.

Heinrichs, E.A., Chelliah S., Valencia S.L. Arceo M.B., Fabellar L.T., Aquino G.B. and Pickin S. (1981). Manual for testing insecticides on rice.

Hill S.D. (1983). *Agricultural Insect Pests of the Tropics and their Control,* 2nd Edn. Cambridge University Press, London, p50-54.

Huber J. (1986) In: Granados R.R. & Federici B.A (eds), *The Biology of Baculoviruses II. Practical Applications for Insect Control.* CRC Press, Boca Raton, p181-202.

Hoffmann M.P., Frodsham A.C. (1993). *Natural Enemies of Vegetable Insect Pests.* Cooperative Extension, Cornell University, Ithaca, NY, p63.

Inceoglu B., Lango J., Wu J., Hawkins P., Southern J., Hammock B.D. (2001). Isolation and characterization of a novel type of neurotoxic peptide from the venom of South African Scorpion *Parabuthus transvaalicus* (Buthidae). *Eur. J. Biochem.* **268**: 5407-5413.

Inceoglu B., Lango J., Jing J., Chen L., Doymaz F., Pessah N., Hammock D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechansism action. *Proc. Natl. Acad. Sci:*USA **100**: 922-927.

ICIPE (2003). *Meeting the Needs of a Changing World: ICIPE's Vision and Strategy 2003-2012.* ICIPE Science Press, Nairobi, Kenya, p1

Ingram W.R. (1958). The lepidopterous stalk borers associated with Gramineae in Uganda. *Bull. Entomol. Res.* **49**: 67-83.

Information Systems for Biotechnology (ISB)/NBIAP.News Report (January 1997), http://www.gene.ch/gentech/1997/8.96-5.97/msg00060.html

Jacobson M., Crosby D.G. (eds)(1971). *Naturally Occurring Insecticides.* Marcel Dekker New York.

Jover E., Couraud F., Rochat H. (1980). Two types of scorpion neurotoxin characterized by their binding to two separate receptor sites on rat brain synaptosomes. *Biochem. Biophys. Res. Commun.* **64**: 1607-1614.

Keegan H.L. (1980). *Scorpions of Medical Importance.* University of Mississippi Press, Jackson, Mississippi, p140.

Kfir R. (1992). Seasonal abundance of the stem borer *Chilo partellus* (Lepidoptera: Pyralidae) and its parasites in summer grain crops. *J. Econ. Entomol.* **85**: 518-529.

Kfir R. (1997). Natural control of the cereal stemborers *Busseola fusca* and *Chilo partellus* in South Africa. *Insect Sci. Appl.* **17**: 61-67.

Kfir R. (1998). Maize and grain sorghum: southern Africa. In: Polaszeck A. (ed.). *African Cereal Stem Borers: Economic Importance, Taxonomy, Natural Enemies and Control.* CABI, Wallingford, UK, p29-37.

Khan Z.R., Hassanali A. (2003). Habitat management strategies for control of stemborers and striga weed in cereal based farming systems in Eastern Africa. In: *ICIPE Annual Scientific Report*, ICIPE Science Press, Nairobi.

Khan Z.R., Pickett J.A., Van den Berg J., Wadhams L.J., Woodcock C.M. (2000). Exploiting chemical ecology and species diversity: stem borer and striga control for maize and sorghum in Africa. *Pest Manag. Sci.* **56**: 957-962.

Kirk R.E., Othmer D.F. (1981). *Encyclopedia of Chemical Technology*, 3rd Edn. Vol. **13**. John Wiley & sons, Toronto, p413-458, p483.

Kozlov S., Lipkin A., Nosyreva E., Blake A., Windass J.D., Grishin E. (1999). Purification and cDNA of an insecticidal potein from the venom of the scorpion *Orthochirus scrobiculosus*. *Toxicon*. **38**: 361-371.

Lala K., Narayanan P. (1994). Purification, *N*-terminal sequence and structural characterization of a toxic protein from the Indian scorpion venom *Buthus tamulus*. *Toxicon*. **32**: 325-338.

Lamoral B.H. (1979). The scorpions of Namibia (Arachnida: Scorpionida). *Ann. Natal Museum* **23**: 498-783.

Lazarovici P., Yana, P., Pelhate M., Zlotkin N. (1982). Insect toxic components from venom of chactoid scorpion, *Maurus palmatus* (Scorpionidae). *J. Biol. Chem.* **257**: 8397-8404.

Le Rü B., Calatayud P.A., Dupas S., Le Gall P., Moyal P. Silvain J.F. (2004). Biodiversity and chemical ecology of gramineous noctuid stemborers in Africa. *ICIPE Annual Scientific Report 2002-2003*, ICIPE Science Press, Nairobi, p39-46.

Loret E.P., Martin-Eauclalire M.F., Mansuelle P., Sampieri F., Granier C., Rochat H. (1991). An anti-insect toxin purified from scorpion *Androctonus australis* (Hector) also acts on the α- and β-sites of mammalian sodium channel: sequence and circular dichroism study. *Biochemistry* **30**: 633-640.

Lourenco W.R. (1994). Diversity and endeminism in tropical versus temperate scorpion communities. *Biogiographica* **70**: 155-160.

Lynch R. E., Wiseman B. R., Plaisted D., Warnick D. (1999). Evaluation of transgenic sweet corn hybrids expressing CryIA(b) toxin for resistance to corn earworm and fall armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **92**: 246-252.

Maes K. (1998). Pyraloidea: Crambidae, Pyralidae. In: Polaszeck A. (ed.). *African Cereal Stem Borers: Economic Importance, Taxonomy, Natural Enemies and Control.* CABI. Wallingford, UK p87-98.

McCormick S. J., Polis G. A. (1990). Prey, predators, and parasites. In: G. A.Polis, (ed.), *The Biology of Scorpions.* Standford University Press, Paolo Alto. p294-320

McCutchen B.F., Hammock B.D., (1994). Recombinant baculovirus expressing an insect-selective neurotoxin. In: Hedin P.A. Menn J.J., Hollingworth R.M. (eds), *Natural and Engineered Pest Management Agents.* American Chemical Society Symp. Ser., Washington, D.C, p348–367.

McCuthen B.F., Choudary P.V., Crenshaw R., Maddox D., Kamita S.G., Palekar N., Volrath S., Fowler E., Hammock B.D.S., Maeda S. (1991). Development of recombinant baculovirus expressing an insect selective neurotoxin: Potential for pest control. *Biotechnology* **9**: 848-852.

Mebs D. (2001). Toxicity in animals. Trends in evolution. *Toxicon* **39**: 87-96

Minton Jr. S.A. (1974). Venomous arachnids and myriapods. In: Kugelmass N. (ed.), *Venomous Diseases*, Charles Thomas Publishers, Illinois, p27-65.

Mohammed A.H. (1942). Preparation of anti-scorpion serum. Use a tropine and ergotine. *Lancet* **2**: 264-365.

Muhyuddin A.I. (1990). Utilization of natural enemies for the control of insect pests of sugarcane . *Ins. Sci. Appl.* **12**: 19-26.

Munkvold G., Hellmich R., Showers W. (1997). Reduced *Fusarium* ear rot and symptomless infection in kernels of maize genetically engineered for European corn borer resistance. *Phytopathology* **87**: 1071-1077.

Newman D.J., Cragg G.M., Snader K.M. (2003). Natural products as a source of new drugs over the period 1981–2002. A detailed analysis and description of current natural-product-derived therapeutic agents. *J. Nat. Prod.* **66**: 1002-1037.

Ngi-Song A.J., Kimani-Njogu S., Overholt W.A., (2001). Multiple parasitism by *Cotesia sesamiae* and *Cotesia flavipes* (Hymenoptera; Braconidae) on *Busseola fusca* (Lepidoptera Noctuidae). *Biocontrol Sci. Technol.* **11**: 38-390.

Ngi-Song A.J., Overholt W.A.(1997). Host location and acceptance by *Cotesia flavipes* (Cameron) and *Cotesia. sesamiae* (Cameron) (Hymenoptera; Braconidae) Parasitoids of African stemborers. Role of frass and other cues. *Biol. Control* **9**: 136-142.

Ngi-Song A.J., Overholt W.A., Njagi P.G.N., Dicke M., Ayertey J.N., Lwande W.(1996). Volatile infochemicals used in host and host habitat location by *Cotesia flavipes* Cameron and *Cotesia sesamiae* (Hymenoptera: Braconidae) larval parasitoids of stemborers on graminae. *J. Chem.Ecol.* **22**: 307-323.

Ngi-Song A.J., Overholt W.A., Njagi P.G.N., Torto B. (2000). Identification of behaviourally active components from maize volatiles for the stemborer parasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae). *Insect Sci. Appl.* **20**: 181-189.

Ngi-Song A.J., Overholt W.A., Stouthamer R. (1998). Suitability of *Busseola fusca* and *Sesamiae calamistis* (Lepidoptera ; Noctuidae) for the development of two populations of *Cotesia sesamiae* (Hymenoptera; Braconidae) in Kenya. *Biol. Control* **12**: 208-214.

Nwanze K.F., Mueller R.A.E., (1989). Management options for sorghum stem borers for farmers in the semi arid tropics. In: Nwanze K.F. (ed). *International Workshop Sorghum Stem Borers* ICRISAT 17-20th November 1987, Patancheru, India.

Nye I.W.B. (1960). The insect pests of graminaceous crops in East Africa. *Colonial Research Studies* No. 31, p1-48.

Omwenga C.O., Kimani S.W., Overholt W.A., Ogol C.K.P.O.(1995). Evidence of the establishment of *Cotesia flavipes* (Hymenoptera: Braconidae) in continental Africa. *Bull. Entomol. Res.* **85**: 525-530.

Overholt W.A. (1998). Biological control. In: Polaszek A. (ed), *African Cereal Stemborers: Economic Importance, Taxonomy, Natural Enemies and Control*. CABI Bioscience UK p349-362.

Pelhate M., Stankiewicz M., Ben Khalifa R. (1998). Anti-insect scorpion toxins: Historical account, activities and prospects .*C. R. Seances Soc Biol. Fil.* **192**: 463-84.

Pickett J.A. (1998). Extended Summaries: SCI Pesticide Group Meeting: Semiochemicals in integrated crop management: commercial prospects. *Pestic. Sci.* **54**: 290-299.

Pickett J.A., Wadhams L.J., Woodcock C.M. (1997). Developing sustainable pest control from chemical ecology. *Agric. Ecost. Environ.* **64**: 149-156.

Pilcher C. D., Rice M. E., Obrycki J.J., Lewis, L.C. (1997). Field and laboratory evaluations of transgenic *Bacillus thuringiensis* corn on secondary lepidopteran pests (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **90**: 669-678.

Pinstrup-Andersen P., Panya-Lorch R., Rosegrant M.W.(1999). *World food prospects: Critical Issues for the Early Twenty First Century*. 2020 Vision Food Policy Report. International Food Policy Research Institute.

Pintar A., Possani L.D., Delepierre M. (1999). Solution structure of toxin 2 from *Centruroides noxius* (Hoffmann), a β-scorpion neurotoxin acting on sodium channels. *J. Mol. Biol.* **287**: 359-367.

Pocock R.I. (1893). Notes upon the habits of some living scorpions. *Nature* **48**:104-107.

Polaszek A., Walker A.K. (1991). The *Cotesia flavipes* species complex parasitoids of cereals stem borers in the tropics. *Redia* **74**: 335-341.

Polis G. A. (1979). Prey and feeding phenology of the desert sand scorpion *Paruroctonus mesaensis* (Scorpionidae: Vaejovidae). *J. Zool.* **188**: 33-346.

Polis G.A. (ed.) (1990). *The biology of Scorpions*. Stanford University Press, Stanford, California.

Possani L.D. (1984). Structure of scorpion toxins. In: Tu A.T. (ed.), *Handbook of Natural Toxins*, Vol. 2. Marcel Dekker Inc., New York, p513-550.

Possani L.D., Selisko B., Gurrola G. (1999). Structure and function of scorpion toxins affecting K^+ - channel. In: Darbon H., Sebatier J.M. (eds.), *Perspective in Drugs Discovery and Design, Vol.* 15/16. Kluwer Academic Publishers, Holland, p15-40.

Primor N. & Zlotkin E. (1980). Penetrability of proteins through the digestive system of Sarcophaga faculata blowfly. *Biochim. Biophys. Acta* **627**:82-90.

Probst P.J. (1973). A review of the scorpions of East Africa with special regard to Kenya and Tanzania. *Acta Tropica* **30**: 312-335.

Quinstad G.B. (1992). Insecticidal activity of spider, centipede, scorpion and snake venom. *J. Econ. Entol.* **85**:19-39.

Quistad G.B., Skinner W.S., Schooley D.A. (1988). Venoms of social Hymenoptera: toxicity to the lepidopteran, *Manduca Sexta. Insect Biochem.* **18**: 511-514.

Rajarajesward G., Sivaprakasara S., Viswanathan J. (1979). Morbidity and mortality in scorpion stings. A review of 68 cases. *J. Indian Med. Assoc.* **73**: 123-60.

Reed L.J., Muench H. (1938). A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**: 493-497.

Reid W.V. (ed). (1993). *Biodiversity Prospecting.* World Resources Institute. Washington, D.C.

Rein J.O. (2003). Prey capture behavior in the East African scorpions *Parabuthus leiosoma* (Ehrenberg, 1828) and *P. pallidus* (Pocock, 1895) (Scorpions: Buthidae). In: *Euscorpius Occasional Publicaltions in Scorpiology.* No. 6, Parkbygget, Trondheim, Norway.

Rice M.E., Pilcher C.D. (1997). Perceptions and performance of Bt corn. In: *Proc. 52nd Annual Corn & Sorghum Research Conf.*, Dec 10-11, 1997, Chicago. p144-156.

Rochat H., Bernard P., Couraud F. (1979). Scorpion toxins: chemistry and mode of action. In: Ceccarelli F. (ed), *Advances in Cytopharmacology.* Raven, New York, p325-334

Rosen J.D., Greth F.M. (1987). Analytical chemistry of pesticides: evolution and impact. In: Marco G.J., Hollingworth R.M., Durhan W.(eds), *Silent Spring Revised.* American Chemical Society, Washington, D.C. p127-143.

Rouhi A.M. (1997). Seeking drugs in natural products. *Chem. Eng. News* **75**: 14-29.

Rudd S. (1996). Investigations into the habit of the bromeliad dwelling scorpion *Chactas raymondhansi.* In: Downie R. Heath M. & Hambly C. (eds.) *Univ. of Glasgow Expedition to Trinidad, unpubl.* Research Report. Univ. of Glasgow Exploration Soc., Glasgow, p79–87.

Saxena R.C. (1989). Insectides from neem. In: Arnason J.T., Philogone B.J.R., Morand T. (eds.), *Insecticides of Plant Origin.* American Chemical Society, Washington D.C, p10-35.

Seshu-Reddy K.V. (1991). Insect pests of sorghum in Africa. *Insect Sci. Appl.* **12**: 653-657.

Seshu-Reddy K.V. (1998). Maize and sorghum: East Africa. In: Polaszek A. (ed.). *African Cereal Stem Borers. Economic Importance, Taxonomy, Natural Enemies and Control,* CABI, Wallingford, p25-29.

Seshu-Reddy K.V., Walker P.T. (1990). A review of the yield losses in graminaceous crops caused by *Chilo* spp. *Insect Sci. Appl.* **11**: 563-569.

Shulov A., Levy G. (1978). Systematics and biology of Buthidae. In: Sergio B. (ed.), *Handbook of experimental Experimental Pharmacology, Vol. 48: Arthropod Venoms.* Springer-Verlag, Berlin, Heidelberg, New York, p309.

Sissom W.D., Polis G.A., Watt D.D. (1990). Field and laboratory methods. In: Polis G.A. (ed.). *The Biology of Scorpions.* Stanford Univ. Press, Stanford, p445–461.

Skovgörd H. Päts P. (1996). Effects of intercropping on maize stem borers and their natural enemies. *Bull. Entomol. Res.* **86**: 599-607.

Smart L.E., Blight M.M., Pickett J.A., Pye B.J, (1994). Development of field strategies incorporating semiochemicals for the control of the pea and bean weevil, *Sitona lineatus* L. *Crop Prot.* **13**: 127-135.

Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke, N.M., Olson B.J., Klenk D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**: 76-85.

Stahnke H.L. (1966). Some aspects of scorpion behavior. *Bull. S. Calif. Acad. Sci.* **65**: 65-80.

Stewart L.M.D., Hirst M., Ferber M.L., Merryweather A.T., Cayley P.J., Posse R.D.(1991).Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature* **352**: 85-88.

Taneja S.L., Nwanze. K.F. (1989). Assessment of yield loss of sorghum and pearl millet due to stem borer damage. In: *Proc Int. Workshop on Sorghum Stem Borers.* ICRISAT, Patancheru, India, 17-20th Nov. 1987, p95-104.

Tytgat, J. Chandy G., Garcia M.L., Gutman G.A., Martin-Eauclaire M.F., Van Der Walt J.J., Possani L.D. (1999). A unified nomeclaure for short chain peptides isolated from scorpion venoms: α-KTx molecular sub-families.*Trends. Pharmacol. Sci.* **8**: 444- 447.

Vachon M. (1952). *Etudes sur les Scorpions.* Institut Pasteur d' Algeriers, p483.

Valdivia H.H., Possani L.D. (1998). Peptide toxins as probes of ryanodine receptor. *Trends Cardiovas. Med.* **8**: 111-118.

Van Beek N.A.M., Hughes P.R. (1998). The response time of insect larvae infected with recombinant baculoviruses. *J. Invertebr. Pathol.* **72**: 338-347.

Waage J.K. (1990). Ecological theory and the selection of biological control agents. In: Mackauer Ehler L.E., Roland J. (eds), *Critical Issues in Biological Control.* Intercept, Andover, UK, p135-158.

Ward A. (1996). Some bromeliad-dwelling scorpions in Trinidad. *Bull. Brit. Arachnol. Soc.* **77**: 10-11.

Ware G.W. (1980). Effects of pesticides on nontarget organisms. *Residue Reviews* **76**:173-201.

Weiss C., Eisner T. (1998). Partnership for value-addition through bioprospecting. *Technol.* Soc. **20**: 481- 498.

Whitman D.W., Blum M.S., Alsop D.W. (1990). Allomones: Chemicals for defense. In. Evans D.L., Schmidt J.O. (eds), *Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators.* State University of New York Press, Albany, p289-351.

Wilkinson C.F. (1987). The science and politics of pesticides. In: Marco G.J., Hollingworth R.M. and Durhan W. (eds), *Silent Spring Revisited. American Chemical Society*, Washington, D.C., p25-46

Williams S.C. (1980). Scorpions of Baja, California, Mexico and adjacent islands. *Occas. Papers Calif. Acad. Sci.* **135**: 1-127.

Wilson W. (1904). The physiological action of scorpion venom. *J. Physiol.* (London) **31**: 48-49

Wudayagiri R., Inceoglu B., Herrmann R., Derbel M., Choudary P.V., Hammock, B.D. (2001). Isolation and characterization of a novel lepidopteran-selective toxin from the venoms of south Indian red scorpion, *Mesobuthus tamulus* (BMC). *Biochemistry* 2: 1471-2091.

WHO (1996). Report of the WHO Informal Consultation on the Evaluation and Testing of Insecticides. WHO, Geneva, p33-37

Zlotkin E., Fraenkel G., Miranda F., Lissitzky S. (1971a). The effect of scorpion venom on blowfly larvae: a new method of evaluation of scorpion venom potency. *Toxicon* **9**: 1-8.

Zlotkin E., Miranda F., Kupeyan C., Lissitzky S. (1971b). A new toxic protein in venom of the scorpion *Androctonus australia* (Hector). *Toxicon* **9**: 9-13.

Zlotkin E., Rochat H., Kupeyan C., Miranda F., Lissitzky S. (1971c). Purification and properties of the insect toxin from the venom of the scorpion *Androctonus australis. Biochemie* (Paris) **53**: 1073-1078.

Zlotkin E., Fraenkel G., Miranda F., Lissitzky S (1972a). Proteins in scorpion venom toxic to mammals and insects. *Toxicon.* **10**: 207-210.

Zlotkin E., Fraenkel G., Miranda F., Lissitzky S. (1972b). A toxic factor to crustacean in the venom of scorpion *Androctonus australis* (Hector). *Toxicon.* **10**: 211-216

Zlotkin E. (1973). Chemistry of animal venoms. *Experimenital (Basel)* **29**: 1453-1466

Zlotkin E., Miranda F., Rochat C. (1978). Chemistry and pharmacology of Buthinae Scorpion venoms. In: Bettini S. (ed), *Arthropod Venoms.* Springer-Verlag, New York, p317-369.

Appendix I: Side chains of most common amino acids

Name	Symbol	Mass (-H₂O)	Side Chain	Occurrence (%)
Alanine	A, Ala	71.079	CH_3-	7.49
Arginine	R, Arg	156.188	$HN=C(NH_2)-NH-(CH_2)_3-$	5.22
Asparagine	N, Asn	114.104	$H_2N-CO-CH_2-$	4.53
Aspartic acid	D, Asp	115.089	$HOOC-CH_2-$	5.22
Cysteine	C, Cys	103.145	$HS-CH_2-$	1.82
Glutamine	Q, Gln	128.131	$H_2N-CO-(CH_2)_2-$	4.11
Glutamic acid	E, Glu	129.116	$HOOC-(CH_2)_2-$	6.26
Glycine	G, Gly	57.052	$H-$	7.10
Histidine	H, His	137.141	$N=CH-NH-CH=C-CH_2-$ $\mid\!\rule{1.5cm}{0.4pt}\!\mid$	2.23
Isoleucine	I, Ile	113.160	$CH_3-CH_2-CH(CH_3)-$	5.45
Leucine	L, Leu	113.160	$(CH_3)_2-CH-CH_2-$	9.06
Lysine	K, Lys	128.17	$H_2N-(CH_2)_4-$	5.82
Methionine	M, Met	131.199	$CH_3-S-(CH_2)_2-$	2.27
Phenylalanine	F, Phe	147.177	$Phenyl-CH_2-$	3.91
Proline	P, Pro	97.117	$-N-(CH_2)_3-CH-$ $\mid\!\rule{1.5cm}{0.4pt}\!\mid$	5.12
Serine	S, Ser	87.078	$HO-CH_2-$	7.34
Threonine	T, Thr	101.105	$CH_3-CH(OH)-$	5.96
Tryptophan	W, Trp	186.213	$Phenyl-NH-CH=C-CH_2-$ $\mid\!\rule{1.5cm}{0.4pt}\!\mid$	1.32
Tyrosine	Y, Tyr	163.176	$4-OH-Phenyl-CH_2-$	3.25
Valine	V, Val	99.133	$CH_3-CH(CH_2)-$	6.48

Appendix II: BSA Standard Curve

BCA PROTEIN ASSAY
Standard Protocol (37 ^{0}C for 30 min)
BSA Standard Curve.

Legend:
- ♦ Y
- ■ Predicted Y
- — Linear (Y)

Y-axis: ABSORBANCE (562nm)

X-axis: PROTEIN (µg BSA)