A TOXICOLOGICAL STUDY OF *MILLETTIA USARAMENSIS* STEM BARK EXTRACT ON *AEDES AEGYPTI* (MOSQUITO), *SCHISTOCERCA GREGARIA* (DESERT LOCUST) AND *MUS MUSCULUS* (MOUSE)

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This thesis is dedicated to my dear husband Kennedy Okeri, son Ellis and daughter Darlene.

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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12L : 12D	Photoperiod of 12 hours light followed by 12 hours darkness
ANOVA	Analysis of Variance
C8, 20	Chemical structure with 8, 20 carbon atoms in the carbon chain
d.f	Degree of freedom
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulphoxide
ED_{50}	Effective dose at which 50% of the desired response is observed
EtOAc	Ethylacetate
LC_{50}	Concentration lethal to 50% of the animals tested
LD_{50}	Dose lethal to 50% of the animals tested
Log	Logarithm
NADH	Reduced Nicotinamide Adenine Dinucleotide
nm	Nanometre
OECD	Organisation for Economic Cooperation and Development
ppm	Parts per million
R&F	Reiche and Fairmaire
RAP	Relative Antifeedant Percentage
TLC	Thin Layer chromatography
v/v	Volume to volume
WHO	World Health Organisation
µg	Microgram
µl	Microlitre

ABSTRACT

Some of the many insects that proliferate in tropical environments due to conduciveness of its weather conditions are crop pests both in the field and in storage. Others transmit diseases and affect the health of both man and livestock. Synthetic insecticides have been used to control them but these have shown pest resistance, they bioaccumulate and are nonbiodegradable. This has led to a search for alternative insecticides and plant insecticides are a promising source. This study investigated the potential of *Millettia usaramensis* subspecies *usaramensis* stem bark extract as a plant insecticide and assessed its toxicological effect on mammals. Shadow dried and powdered *M. usaramensis* stem bark was extracted using dichloromethane/ methanol at the ratio of 1: 1 (v/v). The crude stem bark extract was tested for larvicidal activity against the 4^{th} instar *Aedes aegypti* mosquito larvae. Probit analysis of the results showed a 48 hour activity with a median dose of 50.82 mg/L. The crude extract administered to the locust *Schistocerca gregaria* elicited insecticidal activity as indicated by LD_{50} values of 445.65µg/g through injection at 48 hours, 569.77 µg/g through topical treatment at 72 hours and 504.69 µg/g through oral treatment at 144 hours post exposure. It also showed an anti-feedant activity of ED_{50} 660.71 µg/ml. Pure compounds (+)-12a- epimilletosin, (+)-usararotenoid-A and deguelin were tested in *Aedes aegypti* 4^{th} instar larvae and LC_{50} activities of 2037 mg/L, 4.27 mg/L and 2.63 mg/L respectively were observed at 48 hours post exposure.. The effect of post-treatment temperature on the insecticidal activity of the crude extract was investigated in *S. gregaria*. Increase in temperature significantly increased toxicity of the extract by decreasing LD_{50} from 913.65 µg/g at 25^0C to 323.59 µg/g at 40^0C in a 48 hour post-treatment exposure period. No significant toxicity of the extract was observed in the mouse *Mus musculus* showing that the extract is safe to non-target mammals. The results therefore indicate that *M. usaramensis* is a potential source of botanical insecticide(s).

Key words: *Millettia usaramensis*, plant insecticide, *Aedes aegypti, Schistocerca gregaria, Mus musculus,* toxicological study.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background Information

Many insects proliferate in tropical environments due to conduciveness of its weather conditions. Many of these insects are pests of crops both in the field and in storage and others transmit diseases and affect the health of both man and livestock. This necessitates protection from these insect pests; otherwise they cause food shortage and famine, and spread diseases (Kabaru, 1996).

For many years, insect pests have been controlled with synthetic insecticides, including dichlorodiphenyltrichloroethane (DDT). DDT persists in the environment (non-biodegradable), it is biomagnified in the food chain (bioaccumulates) and it kills non-target organisms. The major classes of insecticidal compounds today are organophosphates and carbamates. Others include organochlorines and pyrethroids (Ware 1982, Dorow 1993). These have problems of pesticide resistance and negative effects on non-target organisms including man and the environment (Rembold 1984, Franzen 1993).

The problems associated with the use of environmentally hazardous insecticides are amplified when they have to be used to control pests like locusts. The areas to be sprayed are very large and the ecosystem is fragile, therefore negative impacts on the environment are enormous. To date locust control methods mainly entail spraying with organochlorines, organophosphates, synthetic pyrethroids and carbamates (Dorow, 1993). There has been a need to develop compounds with less

bioaccumulation potential that are more degradable and applied at lower dose rates than the earlier generation of insecticides. Besides, pyrethroids have been shown to have limited impact on the aquatic and terrestrial environment. They are also readily degraded and have a low dose rate (Yamamoto, 1970). This has led to investigation of alternative methods of insect pest control. Plant insecticides are a promising source of pest control compounds that are environmentally safe (Rembold, 1984).

1.2 Plants as Insect Pest Control Agents

There are several plant genera with potential sources of insect-control agents. These include *Acorus* (Jacobson *et al.*, 1976; Jacobson, 1983) whose roots are used as insect repellent and toxicant; *Mammea* (Crosby, 1971; Jacobson, 1983) whose leaves and seeds are repellent and insecticidal; *Allium* (garlic and onion) that are antibacterial, insecticidal, antifungal, and antithrombotic (Block, 1985); *Tagetes* (Jacobson, 1983; Arnason *et al.*, 1981; Maugh, 1982) whose oil extracts are insecticidal and mosquito larvicidal; *Haplophyton* (Crosby,1971) whose leaves are insecticidal to cockroaches, flies, fleas, lice and mosquitoes; and *Annona* whose seeds are insecticidal and fish poisons (McIndoo, 1945; Harper *et al.*, 1947).

Other plants include poppy, *Colchicum* and *Aconitum* (McIndoo, 1945), *Azadirachta* (Butterworth and Morgan, 1971; Rembold, 1984; Vatandoost and Vaziri, 2001), *Melia* (Kabaru, 1996), white hellebore, bitter lupin, absinthe, bay, cedar, garlic, fig, oak, asafetida, *Cassia* and pomegranate (Smith and Secoy, 1975), tobacco, *Quassia* and pyrethrum (Crosby, 1966; 1971), and certain tropical plants in the family Leguminosae especially *Derris, Lonchocarpus, Tephrosia, Mundulea*, and *Millettia* species (Saxena, 1983).

1.3 *Millettia* - Characteristics and Distribution

The genus *Millettia* (Wight et Arn.) belongs to family Leguminosae (alternative name Fabaceae). The family is divided into three major groups which are viewed as subfamilies (Caesalpiniodeae, Mimosoideae and Papilionoideae) or distinct families (Caesalpiniaceae, Mimosaceae and Papilionaceae) by different scholars. *Millettia* falls in the subfamily Papilionoideae (Yenesew, 1997).

There are 100 known species of *Millettia* worldwide, and in Kenya the genus has six representative species. These are *M. lasiantha* Dunn, *M. oblata* Dunn, *M. leucantha* Vatke, *M. tanaensis* Gillet, *M. dura* Dunn, and *M. usaramensis* Taub (Beentje, 1994).

M. lasiantha is an evergreen coastal forest liana or shrub up to 40m with mauve flowers (Beentje, 1994). In East Africa, *M. lasiantha* is used as an aphrodisiac (Kokwaro, 1993). *M. oblata* is a tree 3-21m with purple-blue flowers. In Kenya it is endemic to Taita hills moist evergreen forest (Beentje, 1994). Its bark is used as a remedy for abdominal pains and coughs as well as treatment for swollen parts of the body. Its roots are used as a remedy for bladder troubles (Kokwaro, 1993). *M. leucantha* is a liana, shrub or tree with white and blue to violet flowers. It is endemic to semi-deciduous forests on rocky hills, or secondary bushland on rocky hills. *M. tanaensis* is a shrub 2-3 m tall with purple flowers. It is endemic to central Kenya (Beentje, 1994).

M. dura is a small tree of moist forest edges or beneath more open forests in Kenya, Uganda and Ethiopia. It is found naturally in Kenya in secondary scrub and forest margins of central province, 1,500-2,000 m but also cultivated successfully elsewhere

as it is drought resistant (Maundu and Tengnas, 2005). It is used as timber, shade or ornamental tree (Yenesew *et al.*, 2003). Its seeds are known in traditional practice for their insecticidal and piscicidal properties (Dagne *et al.*, 1991).

M. usaramensis Taub (Figure 1) is a shrub or small tree 3-7 m tall with rounded buds (Gillet *et al.*, 1971). It is known as *mti chuma* in Swahili, *m'thupa* or *muhawa* in Giriama and *mfutambula* in Sukuma. It has mauve to purple-blue flowers (Maundu and Tengnas, 2005). In East Africa, its roots have been used for snake bite treatment (Kokwaro, 1993). It is found in wooded or bushed grassland and edges of drier coastal forests (Beentje, 1994). There are two subspecies, namely, *usaramensis* and *australis*. Subspecies *usaramensis* occurs in Kenya and Tanzania while subspecies *australis* occur in Mozambique, Malawi and Zimbabwe (Gillet *et al.*, 1971).

Figure 1: A branch of *Millettia usaramensis* Taub. subspecies *usaramensis* showing the characteristic mauve to purple-blue flowers.

1.4 Some Plant Compounds with Insecticidal Activity

In the past, plants have been used as sources and models of insect-control agents. Their bioactive constituents have been used as insecticides, medicine, insect-repellents, anti-feedants, attractants etc. Plant compounds of major commercial importance as sources and models of insect-control agents include rotenoids, rethrins, quassinoids and alkaloids.

Rotenoids are a group of isoflavonoid-type compounds obtained from roots of certain tropical Leguminosae especially species of *Derris* (tuba). Rotenone is the most important of such compounds (Figure 2a). Rotenone is also extracted from the roots of *Lonchocarpus* species in South America (Fukami and Nakajima, 1971). Rotenone and other rotenoids also occur in the genus *Millettia* Wight et Arn (Barron and Ibrahim, 1996; Dewick, 1994). Rotenoid-bearing plants such as *Derris, Lonchocarpus, Tephrosia* and *Mundulea* of the subfamily Papilionoideae of Leguminosae have been used in Asia, Africa and South America as insecticides and fish poison for a long time. Rotenone is the most toxic among rotenoids. It was first isolated from *Lonchocarpus nicou* in 1892, and then in 1912 from *Derris chinensis* called "rotten" where its name rotenone was derived. A similar compound under the name tubotoxin was also isolated from *D. elliptica* and was later shown to be identical with rotenone (Fukami and Nakajima, 1971). Some species of *Derris* genus including *D. elliptica* and *D. chinensis* are commercially cultivated as a source of insecticidal rotenoids (Fukami and Nakajima, 1971). In Kenya, the genus is represented by *Derris trifoliata* Lour (Yenesew *et al.*, 2005; 2006).

Rotenoids (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin (Figure 2 b, c and d respectively) occur in *Millettia usaramensis* subspecies *usaramensis* (Yenesew, 1997).

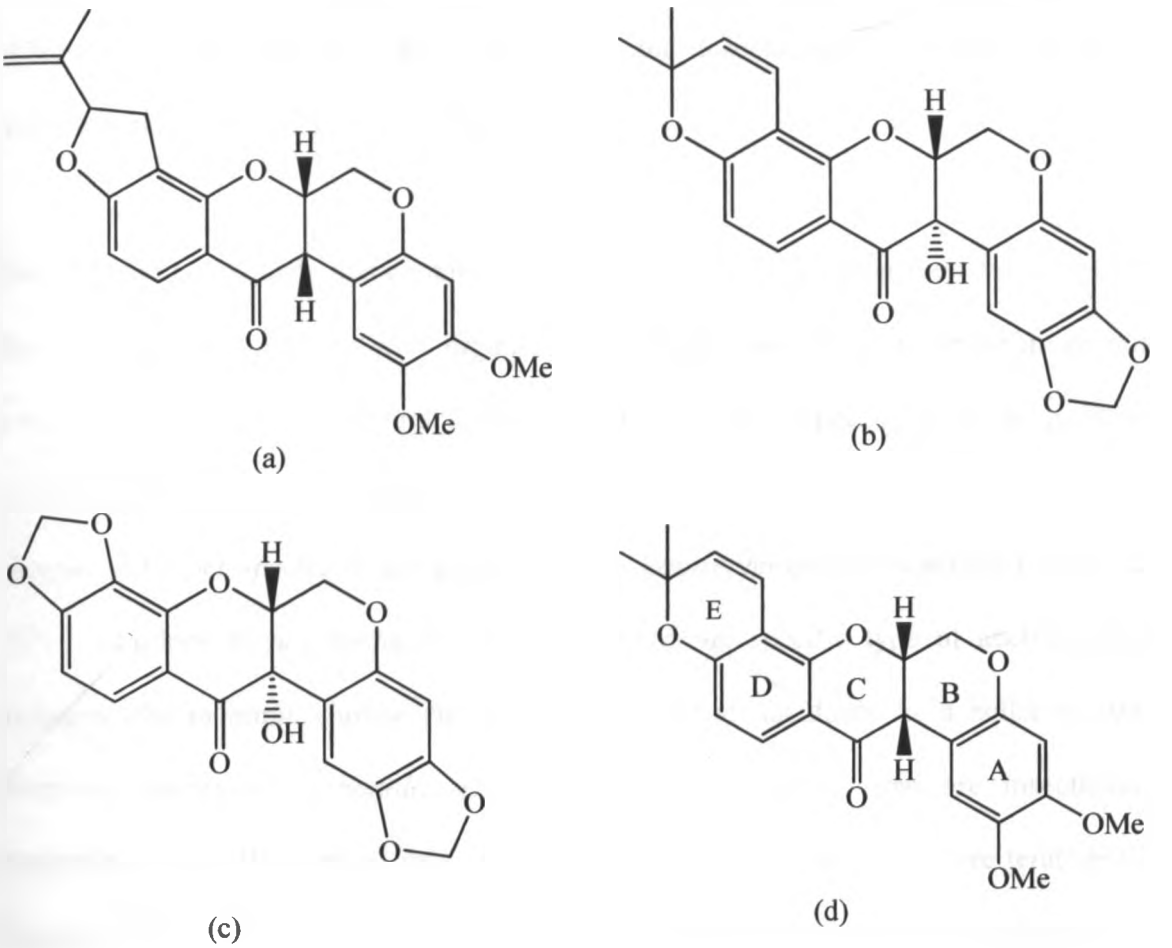

Figure 2: Molecular structures of some rotenoids. (a) Rotenone, (b) (+)-12a-epimilletosin, (c) (+)-usararotenoid-A and (d) deguelin.

Rethrins are a group of six esters that occur in highest concentration in achenes of the pyrethrum plant, *Chrysanthemum cinerariaefolium* and to a lesser extent in *C. coccineum*. These are pyrethrin I and II, cinerins I and II, and jasmolins I and II. They have a rapid paralytic action (termed as knockdown) which is useful against flying insects (also cockroaches and fleas). There are also several synthetic modifications of rethrins (Matsui and Yamamoto, 1971; Saxena, 1983).

Quassinoids are C-20 triterpenoids, for example, quassin and neoquassin from *Quassia amara* and other related plants. They control sawflies and aphids, both as contact and stomach poison. Quassinoids also have antileukemic activity, antiviral, antimalarial, anti-inflammatory, amoebicidal, insect antifeedant and insecticidal properties (McIndoo, 1945; Valenta *et al.*, 1961).

Alkaloids are compounds that contain one or more heterocyclic nitrogen atoms, generally found in the form of salts with organic acids. Many exert their effect upon animal nervous systems (Harborne and Turner, 1984). These include nicotinoids used as insecticides e.g. nicotine from tobacco plant, *Nicotiana* species (*N. tabacum* and *N. rustica*) and other species in the genera *Atropa, Equisetum and Lycopodium* (Schmeltz 1971; Murdock *et al.*, 1985), that act as agonists on specific type of acetylcholine receptors (the nicotinic cholinergic receptors). Veratrum alkaloids from hellebore, the *Veratrum* species (*V. sabadilla* and *V. album*) e.g. veratrine that are insecticidal (louscicidal) as well as strong irritants to mucous membranes and some are teratogenic (McIndoo, 1945; Crosby, 1971). Ryania alkaloids from *Ryania* species (*R. speciosa*) e.g. ryanodine (Casida, 1987) that cause cessation of feeding and flaccid paralysis in insects owing to its poisoning of muscle (knockdown agent). Physostigmine from *Physostigma venenosum* (Leguminosae) seeds that contain a toxic principle, indole alkaloid physostigmine, which reversibly inhibits acetylcholinesterase, and can thereby prolong and exaggerate the effect of acetylcholine (Murdock *et al.*, 1985).

Other plant compounds with potential commercial importance in insect control (as insecticides) include isobutyl amides, N-2-methylpropyl amides of polyunsaturated aliphatic straight chain (C8 to C20) acids isolated from plants of families Asteraceae, Piperaceae, and Rutaceae; and picrotoxinins, a mixture of heterocyclic lactones isolated from seeds of *Anamirta cocculus* L. and other species in the plant family Menispermaceae used as an antidote in barbiturate poisoning (Jacobson, 1971; Su, 1985; Porter, 1967).

Plant compounds are also used in insect-growth regulation, as slow acting substances that specifically affect the growth and development of insects. They are analogues or antagonists of juvenile hormone and molting hormone. Exposure of insects to their own hormones or to synthetic analogues of the hormones at wrong periods of their life cycle or in wrong concentrations, controls growth and development of harmful insects (Turner and Bagnara, 1976).

Juvenile hormone analogues keep insects in their immature stage and are therefore useful in controlling insects that are economically important in their adult stage e.g. mosquitoes and fleas. They prevent the insects from ever attaining maturity. Such compounds include juvabione from *Abies balsamea*, juvocimenes from *Ocimum basilicum*, echinolone from *Echinacea angustifolia* and farnesol found in many plant oils. Synthetic analogues include kinoprene and methoprene used to control mosquitoes, manure-breeding flies and stored-products pests (Menn and Pallos, 1975; Jacobson *et al.*, 1975; Staal, 1982).

Anti-juvenile-hormone compounds include precocene and chromenes (Bowers *et al.*, 1976) isolated from *Ageratum houstonianum* used in controlling insects that are harmful in their immature stage e.g. caterpillars. They promote metamorphosis to the adult stage.

Molting hormone analogues, ecdysteroids like ponasterone, disrupt the normal titres of molting hormone in the insect resulting in abnormalities and death (Hetru and Horn, 1980). However they are rapidly excreted and are too polar to penetrate insect cuticle when topically applied .They are used in sericultural industry for the synchronization of cocoon spinning of silk colonies (Nakanishi, 1977).

Plant compounds are also used as insect-behavior modifiers in the form of attractants, repellents or antifeedants. Dethier *et al.* (1960), defined attractants as "chemicals" that cause insects to make oriented movement towards the source e.g. food, sites for oviposition, to mates, to prey (insect predators) etc. They are obtained from a number of plants e.g. *Geranium* (geraniol), rose, lemongrass, angelica-seed oil, orange oil, castor oil and onion (organo-sulphur compounds). Repellents are those substances which, as stimuli, elicit avoiding reactions (Dethier, 1947). They prevent damage to plants or animals by rendering them unattractive, unpalatable or offensive e.g. oil of citronella (used in candles), cedar wood, eucalyptus, geraniol, pyrethrum and wintergreen.

Antifeedants are substances that, when tasted by insects, result in either temporary or permanent cessation of feeding (Kubo and Nakanishi, 1977). They can be used in insect control e.g. pyrethroids and limnoid azadirachtin (from *Azadirachta indica*).

Plant compounds have other uses in insect control such as acting as synergists or conferring host plant insect resistance. Insecticidal effectiveness of certain insecticides (e.g. pyrethrins and isobutyl amides) can be enhanced by the addition of compounds called synergists or adjuvants, which may or may not be insecticidal themselves, e.g. sesamin (Eagleson, 1940) from oil of sesame (*Sesamum indica*). These compounds inhibit mixed–function oxidase enzymes, thus slowing the detoxification of some insecticides. Host plant resistance is the ability of a plant to reduce infestation or damage, or both, by an insect. This is due to chemicals produced naturally by the plant or produced due to a gene transfer into the plant (Gallun and Khush, 1980).

Wayner *et al.* (1989) reported that plant compounds as insect-control agents have a potential future. This is because plant insecticides are less persistent and more selective insect control chemicals (biorational chemicals). They act by modifying the behavior of specific species of insects (e.g. feeding behavior) or act upon processes peculiar to insects and a few other organisms (e.g. molting). They are relatively innocuous to man and other vertebrates.

1.5 The Mosquito *Aedes aegypti*

Mosquitoes are associated with several public health problems. This includes malaria, filariasis, dengue and Japanese encephalitis, which cause millions of deaths every year (Vatandoost and Vaziri, 2001). *Aedes aegypti* L. (*Stegomyia aegypti*) is a vector for an arbovirus responsible for yellow fever, dengue fever, dengue haemorrhagic

fever and dengue shock syndrome. It has unusual manifestations such as central nervous system involvement (Hendarto *et al.*, 1992; Pancharoen *et al.*, 2002). About two-fifths of the world's populations are at risk of catching dengue (Kautner *et al.*, 1997; Rigau, 1998).

A. aegypti belongs to family Culicidae. The mosquito can be recognized by white markings on legs and a marking in the form of a lyre on the thorax. The mosquito originated from Africa but now is found in the tropics worldwide (Mousson, 2005; Womack, 1993). It prefers breeding in areas of stagnant water such as flower vases, uncovered barrels, buckets, and discarded tyres, as well as wet shower floors and toilet tanks in houses.

Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and, thus, are easy to deal with in this habitat. The use of conventional chemical pesticides has resulted in the development of resistance (Severini *et al.*, 1993; WHO, 1970), undesirable effects on non-target organisms and fostered environmental and human health concerns (Forget, 1989). Therefore, the use of herbal products is one of the best alternatives for mosquito control. The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists in search of alternative vector control (Redwane *et al.*, 2002).

1.6 The Desert Locust *Schistocerca gregaria*

The desert locust, *Schistocerca gregaria* (Forskal) is the most important insect pest of the Old World. During plague periods swarms of it may extend over much of agricultural Africa and Asia, from Morocco and Senegal in the west to Pakistan and India in the east, and southwards to Kenya and Tanzania (Hunter-Jones, 1966).

S. gregaria belongs to family Acrididae together with the African migratory locust, *Locusta migratoria migratoroides* (R&F), both of which are potentially serious pests. *S. gregaria* breeds in low rainfall areas with sufficient moisture for egg development and hatching e.g. Africa, Arabia, Iraq, Iran, Pakistan and India (de Pury, 1973). In 1958, *S. gregaria* caused an estimated loss of 167,000 tons of grain in Ethiopia. Also, in the same year, *S. gregaria* swarms occupied 1000 square kilometers of Somalia consuming an estimated 80,000 tons of food a day (Kumar, 1986). The best strategy of control would be to destroy nymphs at breeding sites. Remote sensing techniques are now being used to identify potential outbreak areas (Voss and Dreiser, 1994).

The fore-going illustrate the locust menace in Africa, a continent which is not self-sufficient in food and therefore cannot afford to lose its fodder and food crops to pests.

1.7 The Mouse *Mus musculus* L.

Mice are the most commonly used animal research model with hundreds of established inbred, outbred, and transgenic strains. Mice are common experimental animals in biology, psychology and medicine primarily because they are mammals, and thus share a high degree of homology with humans.

The mouse genome has been sequenced, and virtually all mouse genes have human homologs. They can also be manipulated in ways that would be considered unethical to do with humans. Mice are a primary mammalian model organism, as are rats.

There are many additional benefits of mice in laboratory research. Mice are small, inexpensive, easily maintained, and can reproduce quickly. Several generations of mice can be observed in a relatively short period of time.

Most laboratory mice are hybrids of different subspecies, most commonly of *Mus musculus domesticus* and *Mus musculus musculus*. Laboratory mice come in a variety of coat colours including agouti, black and albino (white). Many, (but not all) laboratory strains are inbred, so as to make them genetically almost identical. Swiss mice (albino) are therefore one of the laboratory mammalian models for toxicity testing (WHO, 1978).

1.8 Justification of this study

For many years, insect pests have mainly been controlled with synthetic insecticides. However, these have problems of pesticide resistance, pest resurgence and detrimental effects on non-target organisms including man (Rembold, 1984). Plant derived (botanical) insecticides comprise of an array of chemical compounds which act in concert on both behavioural and physiological processes. The chances of pests developing resistance to such substances are less likely (Saxena, 1987). There is need to develop compounds with less bioaccumulation potential and more biodegradability. Botanical insecticides, one of which is rotenone and other rotenoids, offer this potential.

Dagne *et al.* (1991), Ollis *et al.* (1967) and Yenesew *et al.* (1996; 1997), reported that the seeds, seed pods, stem bark and root bark of *Millettia dura* contain rotenoids and isoflavones. Crude chloroform extract of *M. dura* seeds have shown larvicidal activity against *Aedes aegypti* larvae in Kenya (Yenesew *et al.*, 2003). Phytochemical investigation of *M. dura* and *Millettia usaramensis* has resulted in the isolation of a number of isoflavones, rotenoids and chalcones (Yenesew *et al.*, 1996; 1997; 1998). Therefore, *M. usaramensis* stem bark extract is a potential source of insecticidal rotenoids and it was interesting to investigate the activity of the extract on species of insects, and non-target organisms.

It is known that the action of several insecticides is influenced by environmental factors, temperature being one of them and is a critical factor in tropical areas. Some insecticidal activity increase with decrease in temperature (Narahashi and Chambers, 1989; Harris and Kinoshita, 1977), while others increase with increase in temperature (Kabaru, 1996; Mwangi *et al.*, 1997). It was therefore worthwhile to investigate post-treatment effect of temperature on the insecticidal activity of *M. usaramensis* stem bark extract on the locust *S. gregaria.*

This study was intended to investigate the potential of *Millettia usaramensis* stem bark extract as an insecticide and assess its toxic effect on non-target mammals.

1.9 Hypothesis

Extract from *M. usaramensis* stem bark is toxic to *Aedes aegypti* mosquito larvae and *S. gregaria* nymphs but not Swiss mice.

1.10 Main Objective

The main objective of the study was to obtain *M. usaramensis* stem bark extract and determine its toxicity on *A. aegypti, S. gregaria* and Swiss mice in order to establish the safety of use of the extract in the environment as a botanical insecticide.

1.10.1 Specific Objectives

i) To demonstrate the insecticidal effect of *Millettia usaramensis* stem bark extract on *Aedes aegypti* larvae and *Schistocerca gregaria* nymphs.

ii) To determine the larvicidal effect of (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin extracted from *Millettia usaramensis* on *Aedes aegypti* larvae.

iii) To investigate the effect of post-treatment temperature on the insecticidal activity of *Millettia usaramensis* stem bark extract on the locust *Schistocerca gregaria.*

iv) To determine the toxicity of *Millettia usaramensis* stem bark extract on the mouse *Mus musculus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Insecticidal Activities of Rotenoids and Related Compounds

The insecticidal activities of rotenoids against a variety of insect species have been reported. Rotenone is one such compound that has been reported to be less toxic against mammals than against fish and insects, and carcinogenic in rats (Proctor and Hughes, 1978). It has been reported to be a contact and stomach poison. It decreases oxygen uptake by inhibiting NADH-dependent dehydrogenase step of the mitochondrial respiratory chain (O'Brien, 1966; Fukami and Nakajima, 1971).

Rotenone has been found to be extremely potent against many insects. For instance, *Derris* resin (25% rotenone) causes 100% mortality against the larvae of *Bombyx mori* at a concentration lower than 0.7 µg/ml (Fukami and Nakajima, 1971); rotenone and amorphigenin-8'-β-D- glucoside causes very high mortality towards the 4^{th} instar larvae of *Aedes aegypti*, with rotenone causing 100% mortality at 10 ppm (Abe *et al.*, 1985).

Derris trifoliata root and seed extracts have been shown to be larvicidal. Yenesew *et al.* (2005) reported that an acetone extract of the roots of *D. trifoliata* showed high toxicity against the second instar larvae of the mosquito *Culex quinquefasciatus* with LC_{50} value of 1.35± 0.7 µg/ml. Four compounds were isolated from this extract. These were 7a-*O*- methyldeguelol (a modified rotenoid with an open ring-C), (-)-rotenone, (-)-deguelin and (-)-α-toxicarol. The major compound from this extract was

rotenone and it was highly toxic with LC_{50} value of 0.45±0.1 µg/ml against the second instar larvae of *C. quinquefasciatus*. Deguelin was less toxic with LC_{50} values of 1.8±0.6 µg/ml. 7a-O- methyldeguelol and (-)-α-toxicarol were isolated in small quantities and were not tested for larvicidal activity.

Yenesew *et al.* (2006) also reported that a methanol extract of *D. trifoliata* seeds showed potent larvicidal activity against the second instar *Aedes aegypti* with LD_{50} value of 0.74±0.3 µg/ml. Six compounds were isolated from this extract. These were 7a-O-methyl-12a-hydroxydeguelol, spiro-13-homo-13-oxaelliptone, 6, 7-dimethoxy-4-chromanone, rotenone, tephrosin and dehydrodeguelin. Rotenone was the major compound from this extract and it showed a larvicidal activity with LD_{50} value of 0.47±0.2 µg/ml. The larvicidal activity of the crude extract was attributed to rotenone. 6, 7-dimethoxy-4-chromanone and tephrosin were also active with LD_{50} values of 14.8±2.5 µg/ml and 1.6±0.3 µg/ml respectively. However, 7a-O-methyl-12a-hydroxydeguelol and spiro-13-homo-13-oxaelliptone were collected in small quantities and were not tested for larvicidal activities.

The chloroform extract of the stem bark and seeds of *Millettia usaramensis* were tested for larvicidal activities on the second instar larvae of *Aedes aegypti*. The stem bark extract showed a high activity with LC_{50} values of 9.4 µg/ml. The seed extract showed potent larvicidal activity with LC_{50} value of 3.5 µg/ml. The activity of the seed extract was associated with deguelin and tephrosin. Four rotenoids were isolated from the stem bark. These were (+)-12a-epimilletosin, (+)-usararotenoid-A, (+)-12-dihydrousararotenoid-A, and (+)-usararotenoid–B. (+)-usararotenoid-A was the most toxic with LC_{50} values of 9.3 µg/ml within 24 hours. (+)-12a-epimilletosin and (+)-

usararotenoid –B showed activities with LC_{50} values of 35 and 31.7 µg/ml respectively. (+)-12-dihydrousararotenoid-A was not active even at 50 µg/ml (Yenesew, 1997).

Some rotenoids have been found to be potent anti-feedants at low concentration. Rotenone and some of its synthetic derivatives were tested for their insect feeding deterrent activity against the adults of *Sitophilus granarius* and *Tribolium confusum* as well as against the larvae of *Tribolium confusum* and *Trogoderma granarium*. Rotenone showed the highest activity as compared to other anti-feedants belonging to other classes such as sesquiterpenes. Even the anti-feedant azadirachtin is not as effective as rotenone (Nawrot *et al*., 1989).

Tephrosin, a rotenoid isolated from *Tephrosia elata* and related plants, displayed high anti-feedant activity against *Spodoptera exempta*. Rotenone has also been shown to be active against *S. exempta* and *Maruca testulalis* (Bentley *et al*., 1987).

Yenesew (1997) tested the anti-feedant effects of two rotenoids (deguelin and tephrosin) against the 5th instar nymphs of *Locusta migratoria migratoroides*. They showed an average Relative Anti-feedant Percentage (RAP) of 62.7% and 77.7% respectively.

Other isoflavonoids other than rotenoids have also shown insect anti-feedant activities. The activities were also discovered in a number of isoflavonoid phytoalexin structures prompting a suggestion that, such compounds may serve different roles, as antimicrobial agents and as insect anti-feedants (Dewick, 1988). The 6a-

hydroxypterocarpan hildecarpin isolated from the roots of *Tephrosia hildebrandtii* has been reported to have anti-feedant activity towards the larvae of the legume pod-borer *Maruca testulalis* (Hassanali and Lwande, 1989).

The structure-activity relationship in rotenone and analogous compounds against insects is known. In the bean weevil, *Callosobruchus chinensis*, 6a, 12a-dihydrorotoxen-12(6H)-one has been assumed to be the basic structural requirement for toxicity of rotenoids. This is because 6a, 12a-dehydrorotenone is entirely inactive. In addition, a cis-B/C ring fusion is also suggested to be an important requirement for biological activity (Fukami and Nakajima, 1971). This is due to the fact that a novel cis-12a-methylrotenone was found to be much more potent than its trans-epimer as an insecticide and inhibitor of NADH dehydrogenase (Josephs and Casida, 1992). This study also showed that blocking the B/C ring fusion of rotenone with a 12a-methyl or a 6a, 12a-cyclopropyl substituent increased the photo-stability to a great extent.

2.2 Plants with Insect Control Agents

The insecticide application of mangroves has been reported to come mainly from green *Avicennia marina* logs which are very smoky when burnt, keeping away mosquitoes and other biting night insects (Dahdouh-Guebas *et al.*, 2000). Mangrove plant extracts have been shown to have repellency and larvicidal activity against *A. aegypti* (Thangam and Kathiresan, 1992; 1993). Also, the bark, pith and stem of *Rhizophora mucronata* has been reported as a potential plant insecticide for control of locust *Schistocerca gregaria* (Forskal) and mosquito *Aedes aegypti* (L) in Kenya (Kabaru and Gichia, 2001).

Extracts from plants of the Meliaceae family have been found to be sources of a wide range of biological activities including antifeedant, antiovipositant, repellent and growth-regulating activities (Schmutterer, 1995). These include the neem tree *Azadirachta indica*, *Melia volkensii, M. azedarach,* and *M. bombolo.* The active compound in these plants appears to be the limnoid azadirachtin (Butterworth and Morgan, 1971).

2.3 Effect of Post-Treatment Temperature on Biological Activity of Insecticides

It is known that the action of several insecticides is influenced by environmental factors, temperature being one of them and is a critical factor in tropical areas.

The insecticidal activity of DDT, pyrethrins and pyrethroids has been reported to increase with decreasing temperature (Narahashi and chambers, 1989; Harris and Kinoshita, 1977). Temperature in locust prone areas is high. Wilps *et al.* (1993) reported night time temperature of 20 ± 5^{0}C and day time shade temperatures of 45 ± 5^{0}C in Anou Mekkerene, Niger. On the other hand, it has been reported that the insecticidal activity of *Melia volkensii* (Meliaceae) fruit extract increases with increasing temperature (Kabaru, 1996; Mwangi *et al.*, 1997).

The toxicity of deltamethrin, bifenthrin, and endosulfan to the fourth-instar larvae of rice stem borer, *Chilo suppressalis* (Walker), was measured at 17, 27, and 37 ^{0}C. The three insecticides all displayed a positive temperature coefficient between 17 and 37 ^{0}C. The temperature coefficients of deltamethrin, bifenthrin, and endosulfan were 5.59, 1.68, and 2.85, respectively. The inhibition of the above three insecticides to mitochondrial Na^{+}–K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase from the fourth-instar larvae

of rice stem borer was determined at 17, 27, and 37 ^{0}C. The inhibition of deltamethrin to the specific activities of Na^{+}–K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase showed a negative temperature coefficient, but endosulfan exhibited a positive temperature coefficient. Inhibition of bifenthrin exhibited the contrary temperature coefficients between Na^{+}–K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase with a negative temperature coefficient for the former and a positive temperature coefficient for the latter (Li *et al.*, 2006). It was therefore worthwhile to investigate post-treatment effect of temperature on the insecticidal activity of *M. usaramensis* stem bark extract on the locust *S. gregaria.*

2.4 Bioactivity of Rotenoids and Related Compound in Mammals and Other Organisms

Toxicity tests of the rotenoid rotenone on rabbits have shown high lethal dose of 3g/Kg (Fukami and Nakajima, 1971). The studies have also shown rotenone to be considerably more toxic to insects, fish and other invertebrates than to mammals.

Yamamoto *et al.* (1971) has reported relatively high intraperitoneal toxicity of rotenone metabolites, 6aβ, 12aβ-rotenolone and 8-hydroxyrotenone, to mice.

A proprietary fish-toxicant, Pro-Noxfish®, containing as active ingredients 2.5% rotenone and 2.5% sulfoxide, was fed to rats in their drinking water at levels of 100 ppm for a period of 70 weeks. The average weight of the treated animals was less than that of the untreated controls at autopsy. A similar formulation exposed to light and air until no test for rotenone could be obtained was fed to rats in their drinking water at a level of 100 ppm for a period of 53 weeks. The difference between the average weight of the treated animals and the control was very slight at autopsy. No gross abnormalities developed in the animals and histopathologic examination of the tissues

revealed nothing that could be attributed to the action of either formulation (Brooks and Price, 1961).

Two rotenoids (deguelin and millettone) isolated from *Millettia usaramensis* subspecies *usaramensis* were tested for cell growth inhibition of mouse melanoma. They showed cell growth inhibition with ED_{50} values of 0.6 and 2.7 µg/ml respectively (Yenesew, 1997).

Perry and Conway (1977) reported that rotenone induced respiratory changes in the green sunfish, *Lepomis cyanellus*. Experimental animals were treated with rotenone at two dose levels, 0.25 mg/L and 0.5 mg/L, and arterial and venous blood samples were taken when toxicity advanced to the loss of righting reflex stage. The oxygen content, pH, haematocrit and oxygen capacity of blood samples were compared to naive controls. The results indicated that primary rotenone toxicity developed because of reduced oxygen withdrawal from the blood by the tissue capillary beds.

Yenesew *et al.* (2003), reported that a dichloromethane extract of the stem bark of *Millettia usaramensis* subspecies *usaramensis* showed anti-plasmodial activity against the chloroquin-sensitive and chloroquin-resistant strains of *Plasmodium falciparum* with LC_{50} values of 21.1 and 28.0 µg/ml respectively.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Extract

Millettia usaramensis (stem bark) used in this study was collected from Moana Research Station along the Kenyan coast in February 2008. The plant was identified at the University of Nairobi herbarium where a voucher specimen was preserved. The sample was dried in the shade to constant weight and ground to fine powder in a mill.

3.1.1 Preparation of *Millettia usaramensis* stem bark extract

The powdered stem bark of *M. usaramensis* (600g) was extracted with dichloromethane /methanol at the ratio of 1: 1 (v/v) by cold percolation. The slurry of powder and solvent was left standing for 12 hours, after which the supernatant was decanted and filtered. This was repeated three times. The filtrate was combined and dried in a rotary evaporator at 35°C. This afforded 38g solid residue of crude extract, 6.3% (w/w) yield. The dry extract was stored in a desiccator at 4°C.

3.1.2 Compounds isolated from *Millettia usaramensis* subspecies *usaramensis*

Pure compounds isolated from *M. usaramensis* were provided for use in the *Aedes aegypti* bioassays. These were (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin. The compounds were isolated from the stem bark and seeds of *M. usaramensis* by Yenesew *et al.* (1997).

3.2 Experimental Animals

3.2.1 Insects

Larvae for larvicidal bioassays were obtained from an *Aedes aegypti* (L) colony from the School of Biological Sciences, University of Nairobi (Figure 3). The larvae were fed on ground dog biscuit and yeast. The adults were fed on sugar solution and allowed to take blood meals from the blood vessels of immobilized rabbit ears.

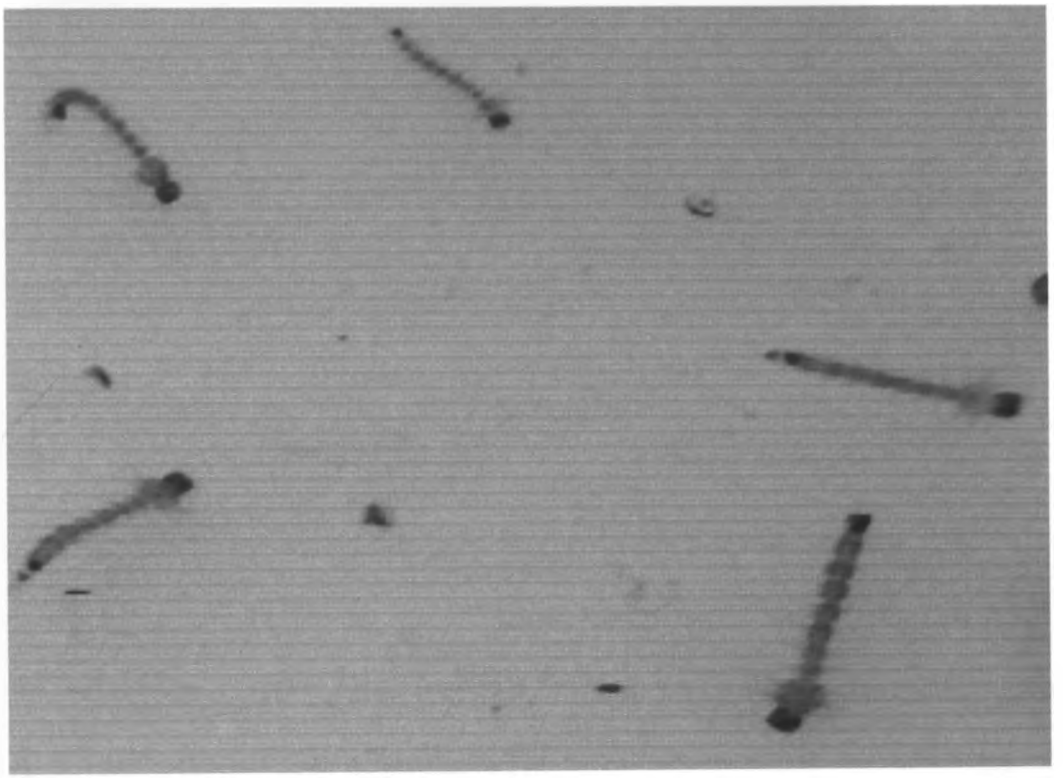

Figure 3: *Aedes aegypti* 4th instar larvae reared for this study.

Schistocerca gregaria Forskal (Figure 4) used in this study were reared in the School of Biological Sciences, University of Nairobi, as described by Hunter Jones (1966). The insects were fed on wheat bran and wheat seedlings. Rearing conditions of 12

hour light: 12 hour dark at a temperature of 29±1°C and relative humidity of 60 % (Kabaru and Gichia, 2001)

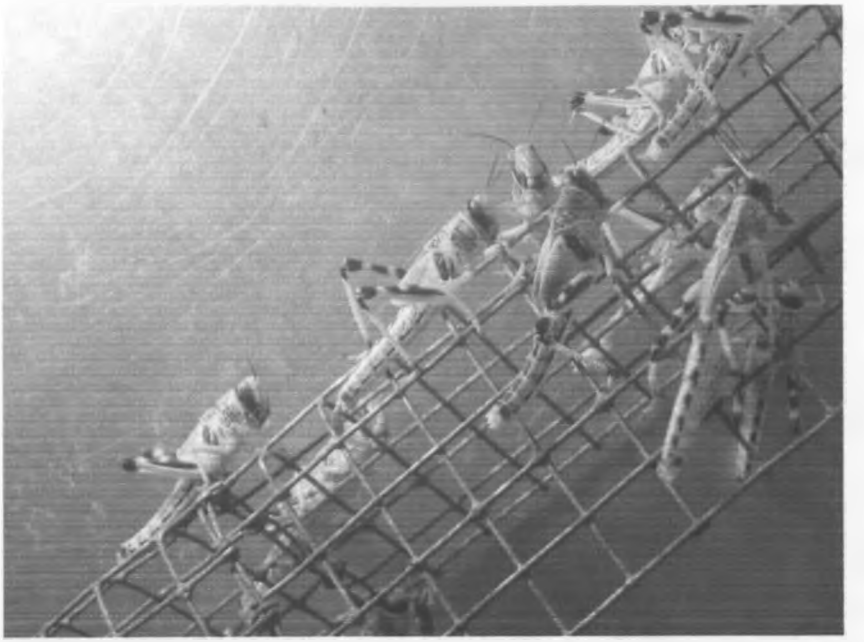

Figure 4: *Schistocerca gregaria* 5th instar nymphs reared for this study.

3.2.2 Mice

Male Swiss mice (Figure 5), body weight 15-30 g, were housed together in groups of four animals under standard conditions with unrestricted access to food and water. All experiments were conducted at the University of Nairobi, School of Biological Sciences Animal House. The animals were housed in a room with a 12/12-hour light/dark cycle at 22 ± 3°C, and relative humidity of 60 per cent. Separate groups of mice were used for the different experiments such that each animal was used on one occasion only. All experiments were performed using age-matched animals.

Figure 5: Some of the mice *Mus musculus* used in this study.

3.3 *Aedes aegypti* Larvae Bioassays

The *Aedes aegypti* larvae bioassays followed the guidelines for laboratory and field testing of mosquito larvicides (WHO, 2005).

3.3.1 Preparation of stock and test solutions

1.6 g of dry *M. usaramensis* stem bark extract was dissolved in 20 ml dimethylsulphoxide (DMSO) to obtain 8% stock solution. The stock solution was kept in a screw-cap vial, with aluminium foil over the mouth of the vial. It was shaken vigorously to dissolve or disperse the material in the solvent. Other strengths of stock

solutions were prepared similarly. Test solutions were then obtained by adding 0.1–1.0 ml (100–1000 µl) of the stock solution to 100 ml tap water (see Table 1). When the series of test solutions were made, the lowest concentration was prepared first. Aliquots of stock solutions were transferred to test cups by means of pipettes.

1% Stock solutions of the pure compounds were prepared from 6 mg of test samples dissolved in 0.6 ml of DMSO. From the stock solution different test solutions were prepared by serial dilution.

Table 1: Aliquots of various strength stock solutions added to 100 ml water to yield final concentration

%	Initial concentration PPM	Aliquots (ml)	Final concentration (PPM) in 100 ml
8	80 000.0	1	800
		0.5	400
7	70 000.0	1	700
		0.5	350
6	60 000.0	1	600
		0.5	300
5	50 000.0	1	500
		0.5	250
4	40 000.0	1	400
		0.5	200
3	30 000.0	1	300
		0.5	150
2	20 000.0	1	200
		0.5	100
1	10 000.0	1	100
		0.5	50
		0.1	10
0.1	1000.0	1	10
		0.5	5
		0.1	1

3.3.2 Exposure of *Aedes aegypti* larvae to *M. usaramensis* crude stem bark extract

Initially, 20 fourth instar mosquito larvae were exposed to 5-1000 mg/L of test solutions of *M. usaramensis* crude stem bark extract and a control. After determining the mortality of larvae in this wide range of concentrations, a narrower range of 8 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h was used to determine LC_{50} values. Batches of 20 fourth instar larvae were transferred by means of droppers to glass jars each containing 100 ml of tap water. The appropriate volume of stock solution was added (see Table 1) to 100 ml water in the glass jars to obtain the desired target dosage. Six replicates were set up for each concentration and an equal number of controls were set up simultaneously with tap water, to which 1 ml DMSO was added. Each test was run three times on different days. Same quantity (20 mg) of larval food was added to each glass jar. The test containers were held at 25–28^{0}C and a photoperiod of 12 h light followed by 12 h dark (12L: 12D). After 24 h and 48 h exposure, larval mortality was recorded.

3.4 *Schistocerca gregaria* Bioassay

S. gregaria nymphs were exposed to *M. usaramensis* crude stem bark extract at a temperature of 28^{0}C. The methods of exposure were through injection, topical application and oral administration. The ability of the extract to deter feeding was also tested.

3.4.1 Injection of locusts

Solutions of plant extract for injection were prepared in DMSO. All the experimental locusts were weighed. Groups of 5 locusts were injected with 10 µl/g of 9 different concentrations. Control locusts were injected with DMSO without test substance. The

locusts were injected with the solution in the intersegmental membrane between the 2nd and 3rd sternite with a microlitre syringe fitted with a hypodermic needle. Mortality was observed and recorded after 24 and 48 hours.

3.4.2 Topical application of extract on locusts

Groups of five locusts were weighed and treated with 10 µl/g of 9 different concentrations of the plant extract prepared in DMSO on the labial, maxillary pulps and the neck membrane beneath the forward projection of the pronotum. Control groups received DMSO only. Mortality was observed and recorded after 48 and 72 hours.

3.4.3 Oral administration of extract to locusts

Potted wheat seedlings, 13cm tall, planted at a density of 200 per pot were sprayed with 6 ml of 5 different concentrations of the plant extract solution in methanol. Control seedlings were sprayed with 6 ml of methanol. The seedlings were dried at room temperature for two hours before being presented to groups of five 24-hour starved locusts in rearing cages in a no choice test. After 24 hours, the treated seedlings were removed and the insects fed on untreated seedling for several days and mortality rate monitored daily for six days.

3.4.4 Anti-feedant test

The plant extract anti-feedant tests were done according to Butterworth and Morgan (1971). Whatman No. 1 filter papers (2x2 cm) were treated with 0.25M sucrose solution and dried. 80µl of six different concentrations of test samples dissolved in methanol were uniformly applied onto the filter papers. The control was also treated

with the solvent. The solvent was left to evaporate at 28^{0}C before the experiments started. The tests were done in duplicates.

Groups of 10 5th instar nymphs which were previously starved for 24 hours were kept in two cages and to each group the treated samples were given in a choice test. The filter papers were removed after 3 hours and the Relative Anti-feedant Percentage (RAP) was calculated.

3.5 Bioassays of Pure Compounds using *Aedes aegypti* Larvae

A. aegypti larvae were exposed to (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin isolated from *M. usaramensis* subspecies *usaramensis*. Batches of 10 fourth instar larvae were transferred by means of droppers to glass jars. The larvae were treated at concentrations of 0-100 mg/L in triplicates. Control larvae in all cases received equal amount of DMSO as in test larvae. Each test was run three times on different days. Same quantity (10 mg) of larval food was added to each glass jar. The test containers were held at 25–28^{0}C and a photoperiod of 12 h light followed by 12 h dark (12L: 12D). After 24 h and 48 h exposure, larval mortality was recorded.

3.6 Determination of the Insecticidal Activity of *M. usaramensis* Stem Bark Extract in *S. gregaria* Nymphs at 25^{0}C and 40^{0}C

The toxicity of injected *M. usaramensis* crude stem bark extract to *S. gregaria* 5th instar nymphs was tested at 25^{0}C and 40^{0}C post-treatment temperatures. Two groups of insects were treated with 0-1000 µg/g concentrations of *M. usaramensis* crude extract. One group was kept at 25^{0}C and the other at 40^{0}C. The insects were placed in well ventilated plastic cages and had unrestricted access to wheat seedlings (food) throughout the study period. The relative humidity was maintained at 55 to 60%. The

tests were done in triplicates and there were 5 nymphs per replicate. Mortality was observed and recorded after 24 and 48 hours.

3.7 *Mus musculus* Bioassays

The mice *Mus musculus* were also exposed to *M. usaramensis* crude stem bark extract through injection, oral and topical application. All the mice used in the tests were weighed before the test. Same quantity per body weight of 5 different concentrations of the plant extracts was administered. The control group received the solvent without test substance. Organization for Economic Co-operation and Development (OECD) guidelines for toxicological studies were observed.

3.7.1 Injection of mice

A single dose of 50 µl of 0-1600 µg/g concentrations of the plant extract dissolved in DMSO were injected intraperitoneally into groups of four mice randomly chosen. Observations were made after every 24 hours for 2 weeks and mortality recorded.

3.7.2 Topical application of extract on mice

Approximately 24 hours before the test, fur was removed from the dorsal area of the trunk of the test animals in groups of four by shaving. 200 µl of 0-2000 mg/g concentrations of the test substance were applied uniformly over the area which was approximately 10 per cent of the total body surface area. Test substances were held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site was further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals did not ingest

the test substance. Observations were made after every 24 hours for 2 weeks and mortality recorded.

3.7.3 Oral administration of extract to mice

A single dose of 100 µl of 0-8000 mg/kg concentrations of the plant extract dissolved in DMSO were administered orally by intubation to mice in groups of four. Observations were made after every 24 hours for 2 weeks and mortality recorded.

3.8 Data Analysis

In the *A. aegypti* bioassays, since larvae that pupate during the test period negate the test, when more than 10% of the control larvae pupated in the course of the experiment, the test was discarded and repeated. When the control mortality was between 5% and 20%, the mortalities of treated groups was corrected according to the formula,

$$\text{Mortality (\%)} = \frac{X - Y}{X} 100,$$

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample (Abbort, 1925).

Data from all bioassay replicates was pooled for log- probit analysis. This analysis enables fitting probit and logit sigmoid dose/stimulus response curves and for calculating confidence intervals for dose-response quantiles such as ED_{50}, LC_{50} or LD_{50}. When biological responses are plotted against their causal stimuli (or logarithms of them) they often form a sigmoid curve. Sigmoid relationships can be linearized by transformations such as logit, probit and angular. For most systems the probit (normal sigmoid) and logit (logistic sigmoid) give the most closely fitting

result. In biological assay work, probit analysis is preferred. Probits or probability units are used in transforming the sigmoid dose-mortality curve to a straight line (Finney, 1952; Bliss, 1935).

Data are entered as dose levels, number of subjects tested at each dose level and number responding at each dose level. At the time of running the analysis a control is entered for the number of subjects responding in the absence of dose/stimulus; this provides a global adjustment for natural mortality/responsiveness. Automatic log transformation of the dose levels is also specified at run time if appropriate. StatsDirect® gives the effective/lethal levels of dose/stimulus with confidence intervals at the quantiles specified, e.g. ED_{50}, LD_{50} or LC_{50}

The concentrations were transformed into logarithm to the base 10 and percentage mortality into probits. Median doses (LC_{50} or LD_{50}) were calculated using StatsDirect® statistical software. Log dosage–probit mortality was plotted with Minitab® statistical software. The p-values and 95% confidence intervals of the means of median doses were calculated and recorded.

The potency of the crude extract against *A. aegypti* larvae and S. *gregaria* nymphs, as well as that of pure compounds against *A. aegypti* was evaluated. Data on the effect of post-treatment temperature of *M. usaramensis* crude stem bark extract on *S. gregaria* 5^{th} instar nymphs was subjected to log-probit analysis as well as ANOVA (Zar, 1984).

Relative Antifeedant Percentage (RAP) for anti-feedant test on *S. gregaria* 5th instar nymphs was calculated using the formula:

$$RAP = \frac{\text{Consumed control paper - Consumed treated paper}}{\text{Consumed control paper + Consumed treated paper}} \times 100$$

(Yenesew, 1997).

The anti-feedant test data was also subjected to correlation analysis. This is a linear relationship between two variables but neither is assumed to depend on the other (no cause-effect relationship). A correlation coefficient, *r*, measures the linear relationship between two attributes or columns of data. The correlation coefficient is also known as the Pearson product-moment correlation coefficient. The value of *r* can range from -1 to +1 and is independent of the units of measurement. A value of *r* near 0 indicates little correlation between attributes; a value near +1 or -1 indicates a high level of correlation.

When two attributes have a positive correlation coefficient, an increase in the value of one attribute indicates a likely increase in the value of the second attribute. A correlation coefficient of less than 0 indicates a negative correlation. That is, when one attribute shows an increase in value, the other attribute tends to show a decrease. The r measures the intensity of association between two variables, i.e. the strength of the straight line relationship. The correlation index, $r^{2,}$ is calculated by simply squaring r. It's described as the amount of variability in one of the variables accounted for by correlating that variable with the 2^{nd} variable (Zar, 1984).

CHAPTER FOUR

RESULTS

4.1 Insecticidal effect of *M. usaramensis* stem bark extract on *A. aegypti* larvae and *S. gregaria* nymphs

4.1.1 Mortality of *A. aegypti* 4th instar larvae exposed to *M. usaramensis* stem bark extract

The results on mortality of *A. aegypti* larvae 24 and 48 hour after exposure to *M. usaramensis* stem bark extract at 0-800 mg/L is shown in Table 2. The regression equations of probit mortality versus log dosage plot at 24 hour and 48 hour period are presented in Figure 6. The plot shows a positive linear relationship between concentration and probits. As concentration increases so does probits. The R^2 values indicate that concentration accounts for 96.3% and 91.2% of the variation in probit for the 24 and 48 hour period respectively.

The regression analysis (probit versus log concentration) of the 24 and 48 hour bioassay showed that mortality significantly increased with the increase in extract concentration. Log probit analysis of the results showed a 48 hour median dose of 50.82 mg/L.

A summary of the Log probit analysis of the mortality results of *A. aegypti* 4th instar larvae exposed to the crude extract 24 and 48 hours after treatment is shown in Table 3.

Table 2: The toxicity of *M. usaramensis* crude stem bark extract on the 4th instar *A. aegypti* larvae a, 24 hours; b, 48 hours post-exposure; mean mortality from 18 replicate experiment (No. of larvae per assay=20)

Conc (mg/L)	0	5	10	25	50	100	200	400	800
%Mortality[a]	3.24	5.29	9.12	16.76	25.00	35.00	48.24	64.41	87.94
%Mortality[b]	4.71	15.59	21.18	27.94	40.59	56.47	71.47	83.82	98.53

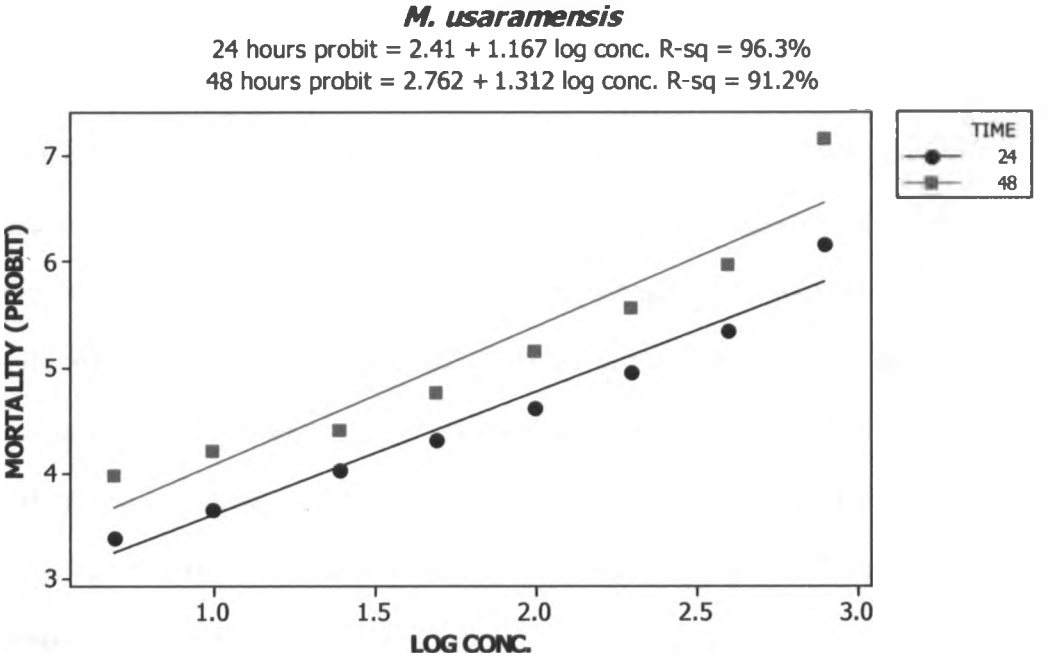

Figure 6: Mortality of *A. Aegypti* 4th instar larvae exposed to *M. usaramensis* crude stem bark extract.

Table 3: Summary of Log probit analysis of the larvicidal activity of *M. usaramensis* crude stem bark extract on the 4th instar *A. aegypti* larvae

Bioassay period (hours)	Regression equations	R^2 (%)	LC_{50} values (mg/L)	95% confidence Limits (mg/L)	
				Lower	Upper
24	PROBIT=2.41+1.17LOG CONC.	96.3	167.00	142.82	196.28
48	PROBIT=2.76+1.31LOG CONC.	91.2	50.82	49.91	67.72

LC, lethal concentration; *R*, coefficient of regression equations; Calculated Log LC_{50} transformed to LC_{50}.

4.1.2 Mortality of *S. gregaria* 5th instar nymphs exposed to *M. usaramensis* stem bark extract

Time taken for the manifestation of activity of *M. usaramensis* stem bark extract in *S. gregaria* 5th instar nymphs differed with the method of administration. While the activity was manifested at 24 hours in the injection treatment, it took 48 hours to be manifested in the topical treatment and 72 hours in the oral treatment. For each of these methods of administration, observations were made from the time of manifestation of activity. Table 7 is a summary of Log probit analysis of the insecticidal activity of the crude stem bark extract in *S. gregaria* 5th instar nymphs exposed to *M. usaramensis* stem bark extract.

4.1.2.1 Mortality of *S. gregaria* 5th instar nymphs injected with *M. usaramensis* crude stem bark extract

The mortality over a period of 48 hours in *S. gregaria* 5th instar nymphs injected with *M. usaramensis* crude stem bark extract at concentrations of 0-800µg/g is shown in Table 4. The regression equations of probit mortality versus log dosage plot at 24 and 48 hour period are presented in Figure 7. The log-probit plots show a positive linear

relationship between concentration and probits. From R^2 values, concentration accounts for 91.8% and 80.9% of variation in observed probits for 24 and 48 hour period respectively.

The LD_{50} values of mortality in *S. gregaria* 5th instar nymphs injected with *M. usaramensis* crude stem bark extract 24 and 48 hours after treatment were 897.55 µg/g and 445.85 µg/g respectively.

Table 4: The toxicity of injected *M. usaramensis* crude stem bark extract on *S. gregaria* 5th instar nymphs a, 24 hours; b, 48 hours post-exposure; mean mortality from triplicate experiment (No. of nymphs per assay=5)

Conc (µg/g)	0	100	200	300	400	500	600	700	800
% Mortality[a]	0.00	13.40	20.00	26.00	26.00	33.00	40.00	47.70	53.30
% Mortality[b]	0.00	13.40	20.00	26.00	26.00	47.70	66.70	73.30	86.70

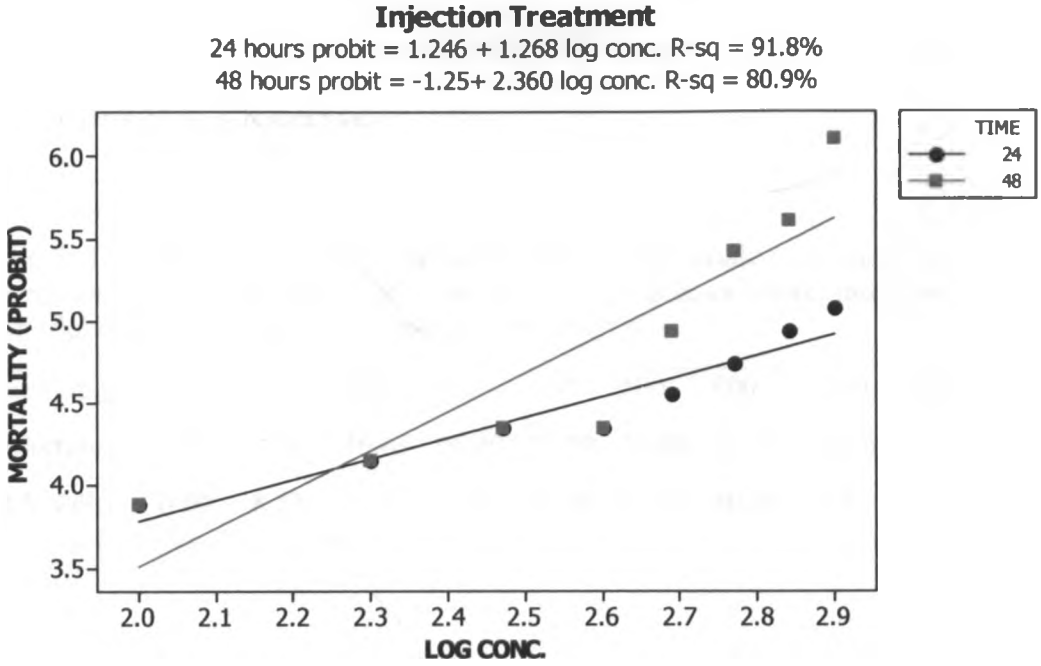

Figure 7: Mortality of *S. gregaria* 5th instar nymphs injected with *M.usaramensis* crude stem bark extract.

4.1.2.2 Mortality of *S. gregaria* 5th instar nymphs topically applied with *M. usaramensis* crude stem bark extract

The 72 hour period mortality in *S. gregaria* 5th instar nymphs topically applied with *M. usaramensis* crude stem bark extract at concentrations of 0-800 µg/g is shown in Table 5. The regression equations of probit mortality versus log dosage plot at 48 and 72 hour period are presented in Figures 8. The log-probit plots show a positive linear relationship between concentration and probits. Concentration accounts for 73.9% and 90.2% (R^2 values) of variation in observed probits for the 48 and 72 hour period respectively.

The LD_{50} values of mortality in *S. gregaria* 5th instar nymphs topically applied with *M. usaramensis* crude stem bark extract 48 and 72 hours after treatment were 896.75 µg/g and 569.77 µg/g respectively.

Table 5: The toxicity of topically applied *M. usaramensis* crude stem bark extract on *S. gregaria* 5th instar nymphs a, 48 hours; b, 72 hours post-exposure; mean mortality from triplicate experiment (No. of nymphs per assay=4)

Conc (µg/g)	0	100	200	300	400	500	600	700	800
% Mortality[a]	0.00	0.00	16.67	25.00	25.00	25.00	33.33	41.67	50.00
% Mortality[b]	0.00	8.33	16.67	25.00	25.00	41.67	50.00	58.33	75.00

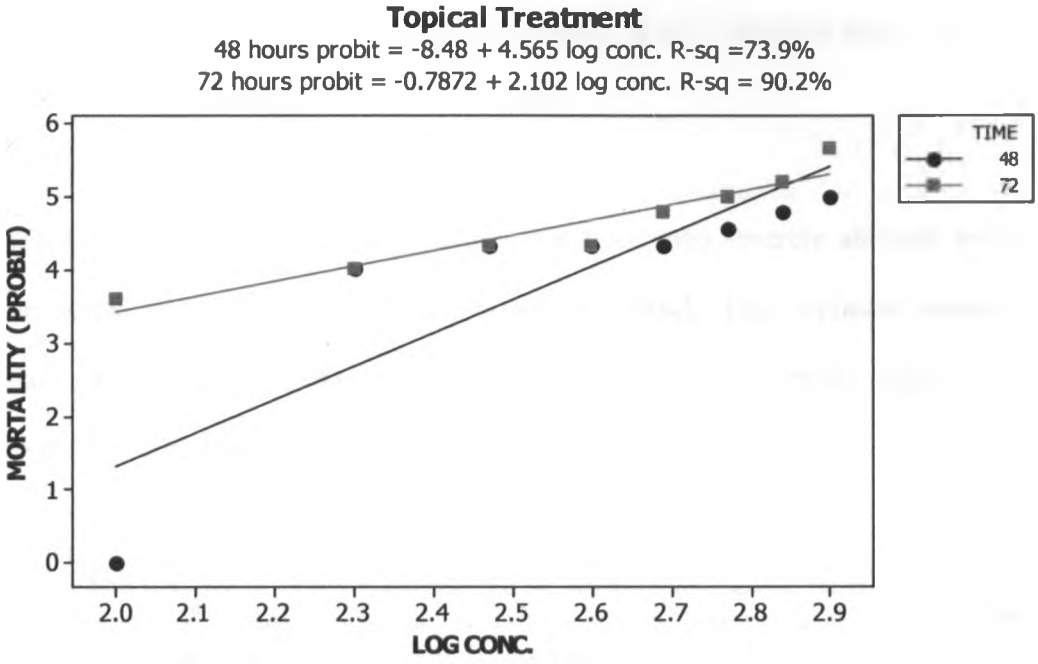

Figure 8: Mortality of *S. gregaria* 5th instar nymphs topically treated with *M.usaramensis* crude stem bark extract.

4.1.2.3 Mortality of *S. gregaria* 5th instar nymphs orally administered with *M. usaramensis* crude stem bark extract

The results of mortality observed in the *S. gregaria* 5th instar nymphs exposed to seedlings treated with 0-800 µg/g concentration of *M. usaramensis* crude stem bark extract are shown in Table 6. The regression equations of probit mortality versus log dosage plot at 72 and 144 hour period are presented in Figures 9. The log-probit plots show a positive linear relationship between concentration and probits with concentration accounting for 70.2% and 89.8% (R^2 values) of the observed variation in probits for the 72 and 144 hour period respectively.

The LD_{50} values of mortality in *S. gregaria* 5th instar nymphs that ingested seedlings treated with *M. usaramensis* crude stem bark extract 72 and 144 hours after treatment were 1093.24 µg/g and 504.69 µg/g respectively.

Seedlings treated with low concentrations of extract were severely attacked while those treated with high concentrations showed less attack. This implicated an anti-feedant effect of the extract on the *S. gregaria* 5th instar nymphs, which was subsequently investigated.

Table 6: The toxicity of orally administered *M. usaramensis* crude stem bark extract on *S. gregaria* 5th instar nymphs a, 72 hours; b, 144 hours post-exposure; mean mortality from triplicate experiment (No. of nymphs per assay=5)

Conc (µg/g)	0	100	200	400	800
% Mortality[a]	0.00	0.00	20.00	20.00	40.00
% Mortality[b]	0.00	20.00	40.00	40.00	60.00

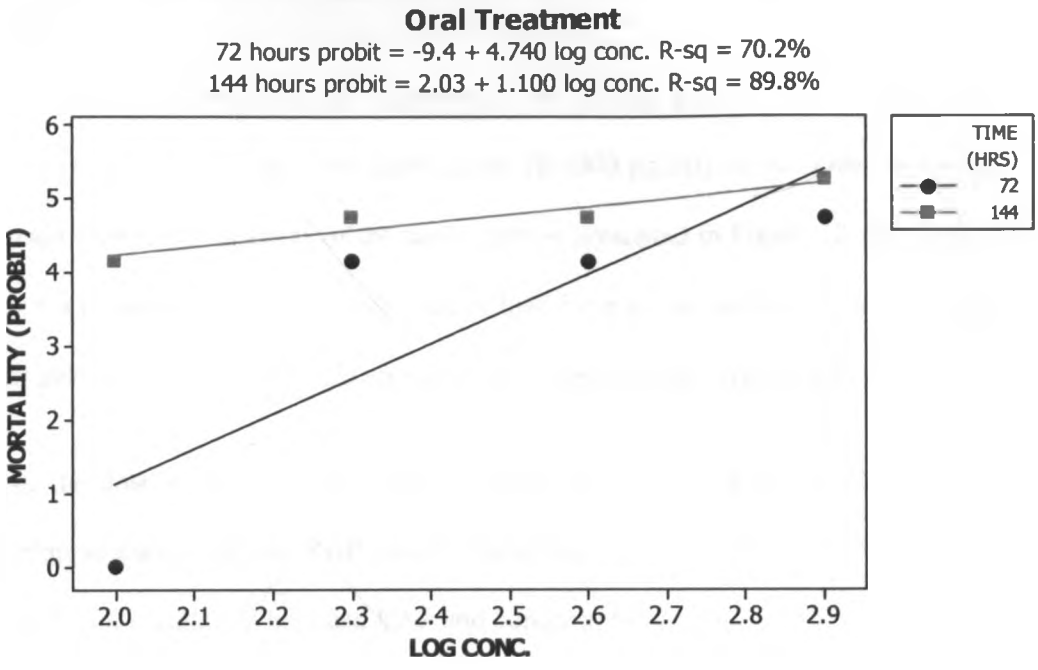

Figure 9: Mortality of *S. gregaria* 5th instar nymphs exposed to *M.usaramensis* crude stem bark extract through oral treatment.

Table 7: Summary of Log probit analysis of the insecticidal activity of *M. usaramensis* crude stem bark extract on *S .gregaria* 5th instar nymphs

***M. usaramensis* Crude stem bark extract**	**Bioassay period (hours)**	**Regression equations**	**R^2 (%)**	**LD_{50} values (µg/g)**	**95% Confidence Limits (µg/g)**	
					Lower	**Upper**
Injection	24	P = 1.25 + 1.27 X	91.8	897.55	559.10	6986.64
	48	P = - 1.25 + 2.36 X	80.9	445.85	350.00	579.69
Topical	48	P = - 8.48 + 4.57 X	73.9	896.75	604.85	3675.53
	72	P = - 0.787 + 2.10 X	90.2	569.77	429.57	918.28
Oral	72	P = - 9.4+ 4.74 X	70.2	1093.24	940.48	1246
	144	P = 2.03 + 1.10 X	89.8	504.69	502.86	506.52

P, probit; X, log conc; *R*, coefficient of regression equations; LD, lethal dose; Calculated Log LD_{50} transformed to LD_{50}.

4.1.2.4 RAP values of *M. usaramensis* crude stem bark extract on *S. gregaria* nymphs

The results of 3 hour paper anti-feedant tests on 24-hour starved *S. gregaria* 5th instar nymphs of *M. usaramensis* stem bark extract (0-1000 µg/ml) are presented in Table 8. Pearson correlation analysis of the test results is presented in Figure 10. The analysis shows a positive correlation. This implies that for a given increase in concentration, RAP also increases. R^2 (85.6%) measures the strength of the straight line relationship.

ED_{50}, the dose at which 50% of the test paper is protected was 660.71 µg/ml. The correlation analysis (mean RAP versus concentration) of the anti-feedant test showed a significant correlation between RAP and concentration.

Table 8: Anti-feedant test results of *M. usaramensis stem* bark extract on *S. gregaria* nymphs

Conc. (ppm)	1000	800	600	400	200	0
Area consumed (cm^2)[a]	0.37	0.54	0.58	0.66	1.20	1.77
Area left (cm^2)[a]	11.63	11.46	11.42	11.34	10.80	10.23
RAP	65.42	53.24	50.63	45.67	19.19	

a, mean of duplicate experiment (No of filter papers per conc =3); 8µl of sample applied per 4.00 cm^2 of filter paper.

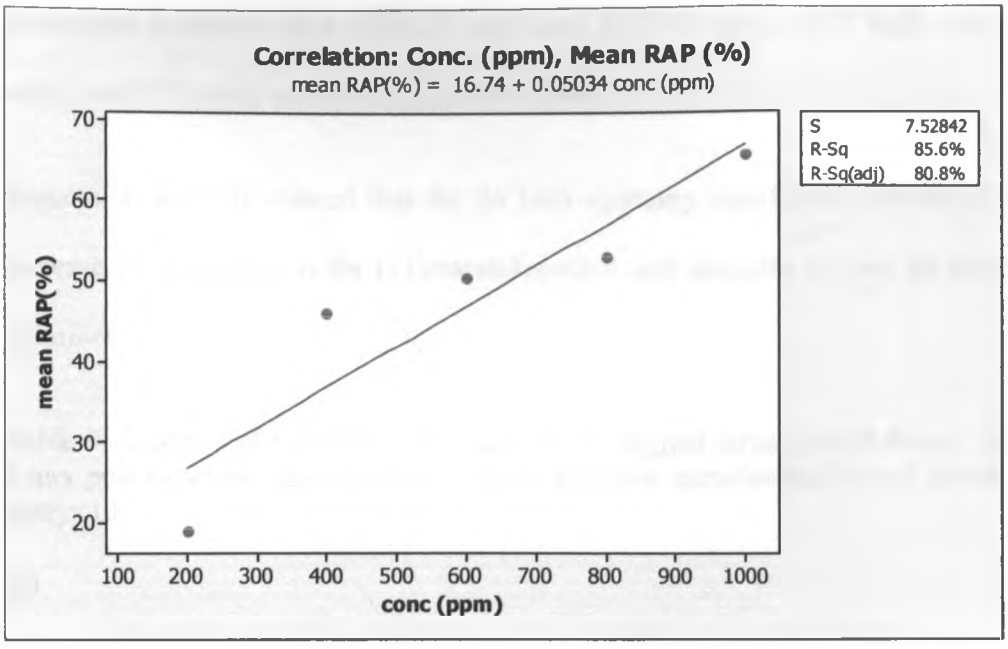

Figure 10: Plot of correlation of Relative Anti-feedant Percentage (RAP) and concentration of *M. usaramensis* crude stem bark extract.

4.2 Larvicidal Effect of Pure Compounds on *A. aegypti* Larvae

The 24 and 48 hour mortality of *A. aegypti* larvae exposed to the pure compounds (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin at concentrations of 0-100 mg/L are shown in Table 9. The regression equations of probit mortality versus log dosage plot at the 24 and 48 hour period are presented in Figures 11-13. All the log-probit plots show a positive linear relationship between concentration and probits. Corresponding R^2 values indicate the magnitude of variation in probits accounted by concentration.

Probit analysis of the pure compounds *A. aegypti* bioassay results 24 and 48 hours after treatment are shown in Table 10. The LC_{50} values of mortality in *A. aegypti* larvae exposed to (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin 24 and 48

hours after treatment were 6350.31 mg/L and 2037.00 mg/L; 31.77 mg/L and 4.27 mg/L; and 5.75 mg/L and 2.63 mg/L respectively.

Regression analysis showed that the 24 hour mortality significantly increased with increase in concentration for (+)-usararotenoid-A and deguelin but not for (+)-12a-epimilletosin.

Table 9: Toxicity of the pure compounds to *A. aegypti* larvae (a) 24 hours, (b) 48 hours post-exposure; mean mortality from triplicate experiment (No. of larvae per assay=10)

(a)

	Conc (mg/L)	0.00	6.25	12.50	25.00	50.00	100.00
(+)-12a-epimilletosin	% Mortality	0.00	3.33	3.33	3.33	6.00	13.30
(+)-usararotenoid-A	% Mortality	0.00	30.00	36.67	40.00	46.67	76.67
Deguelin	% Mortality	0.00	63.33	63.33	66.67	96.67	100.00

(b)

Compound	Conc (mg/L)	0.00	6.25	12.50	25.00	50.00	100.00
(+)-12a-epimilletosin	% Mortality	3.33	5.00	10.00	15.00	15.00	20.00
(+)-usararotenoid-A	% Mortality	3.30	60.00	73.33	76.67	83.33	100.00
Deguelin	% Mortality	3.33	80.00	83.33	96.67	100.00	100.00

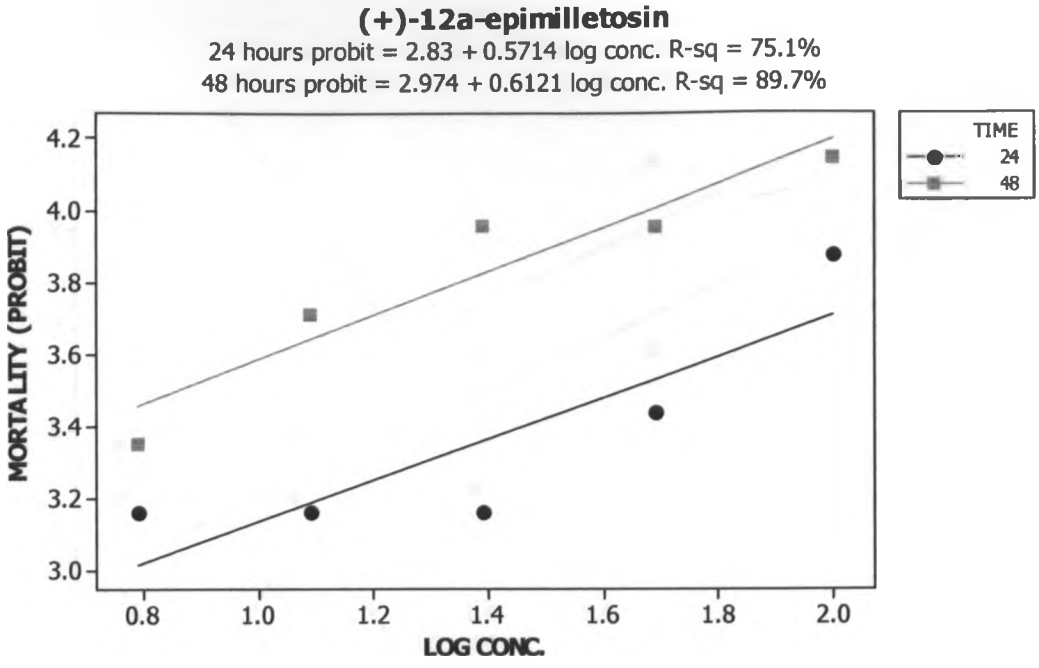

Figure 11: Mortality of *A. aegypti* 4th instar larvae exposed to (+)-12a-epimilletosin.

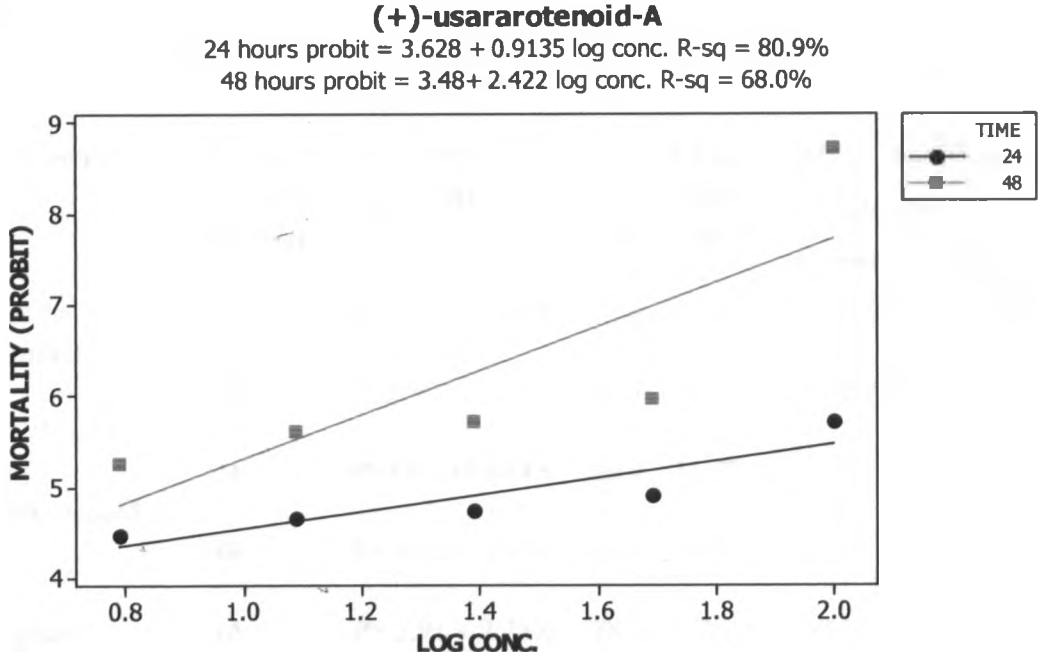

Figure 12: Mortality of *A. aegypti* 4th instar larvae exposed to (+)-usararotenoid-A.

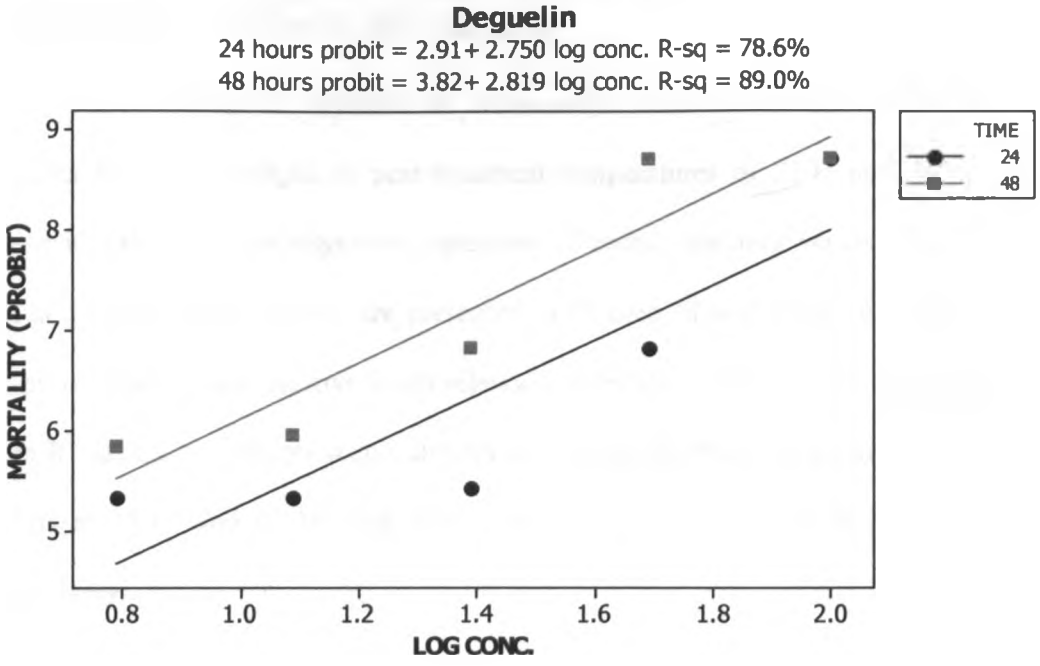

Figure 13: Mortality of *A. aegypti* 4th instar larvae exposed to deguelin.

Table 10: Summary of Log probit analysis of the larvicidal activity of some pure compounds on the 4th instar *A. aegypti* larvae

Compound	Bioassay period (hours)	Regression equations	R^2 (%)	LC_{50} values (mg/L)	95% confidence Limits (mg/L)	
					Lower	Upper
(+)-12a-epimilletosin	24	P=2.83 +0.571X	75.0	6350.31	6348.76	6351.86
	48	P=2.97+ 0.612X	89.7	2037.00	2035.68	2038.32
(+)-usararotenoid- A	24	P=3.63 +0.914X	80.9	31.77	18.79	70.16
	48	P= 3.48 + 2.42X	68.0	4.27	0.799	8.05
Deguelin	24	P= 2.91 + 2.75X	78.6	5.75	2.15	9.21
	48	P=3.82+ 2.82X	89.0	2.63	0.25	5.02

P, probit; X, log conc; *R*, coefficient of regression equations; LC, lethal concentration; Calculated Log LC_{50} transformed to LC_{50}.

4.3 Mortality of *S. gregaria* 5th Instar Nymphs Injected with *M. usaramensis* Crude Stem Bark Extract at 25°C and 40°C

The mortality results of injected *M. usaramensis* crude stem bark extract to *S. gregaria* 5th instar nymphs at post-treatment temperatures of 25°C and 40°C are shown in Table 11. The regression equations of probit mortality versus log dosage plot at 24 and 48 hour period are presented in Figures 14 and 15 respectively. The log-probit plots show a positive linear relationship between concentration and probits. From R^2 values, concentration accounts for 83.4% and 88.3% of variation in observed probits at 25°C; and 92.3% and 65.0% at 40°C for the 24 and 48 hour period respectively.

Probit analysis of mortality results 24 and 48 hours after treatment at 25°C and 40°C are shown in Table 12. The LD_{50} values of mortality at 25°C was 1466.11 µg/g and 913.65 µg/g for 24 and 48 hours respectively while LD_{50} values of mortality at 40°C was 540.92 µg/g and 323.59 µg/g respectively.

Table 11: The toxicity of injected *M. usaramensis* crude stem bark extract on *S. gregaria* 5th instar nymphs at 25°C and 40°C (a) 24 hours, (b) 48 hours post-exposure, mean mortality from triplicate experiment (No. of nymphs per assay=5).

(a)

Post-treatment Temperature	Conc (µg/g)	0	200	400	600	800	1000
25°C	% Mortality	0.00	0.00	6.67	26.67	26.67	26.67
40°C	% Mortality	0.00	12.00	33.30	40.00	73.30	86.67

(b)

Post-treatment Temperature	Conc (µg/g)	0	200	400	600	800	1000
25°C	% Mortality	0.00	13.33	20.00	26.67	46.67	60.00
40°C	% Mortality	6.67	40.00	46.67	60.00	93.33	100.00

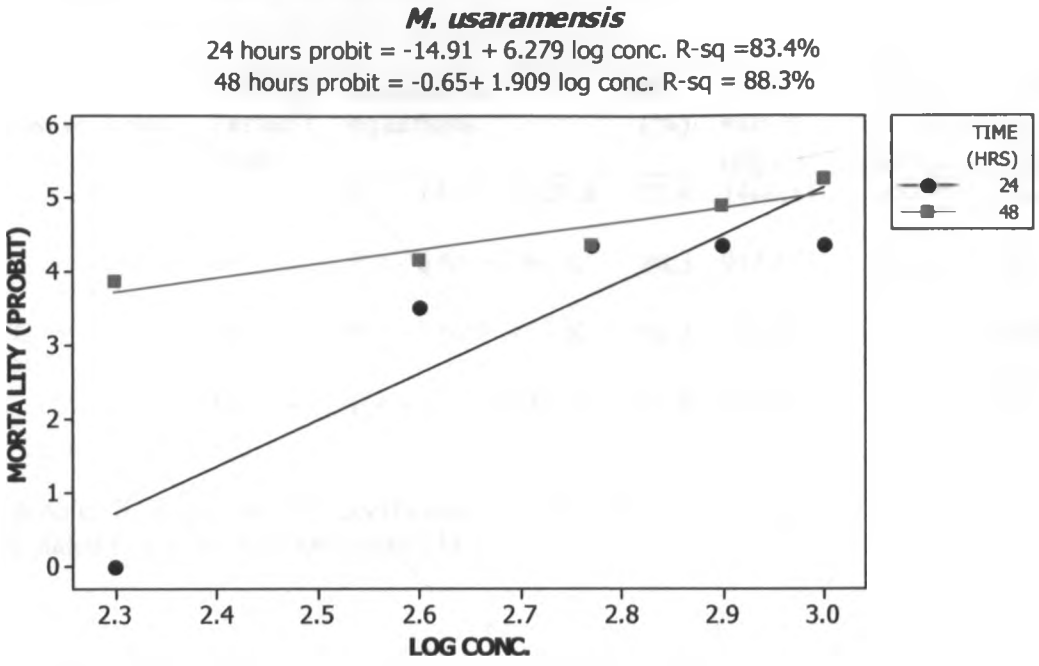

Figure 14: Mortality of *S. gregaria* 5th instar nymphs injected with *M.usaramensis* crude stem bark extract at 25^{0}C post-exposure temperature.

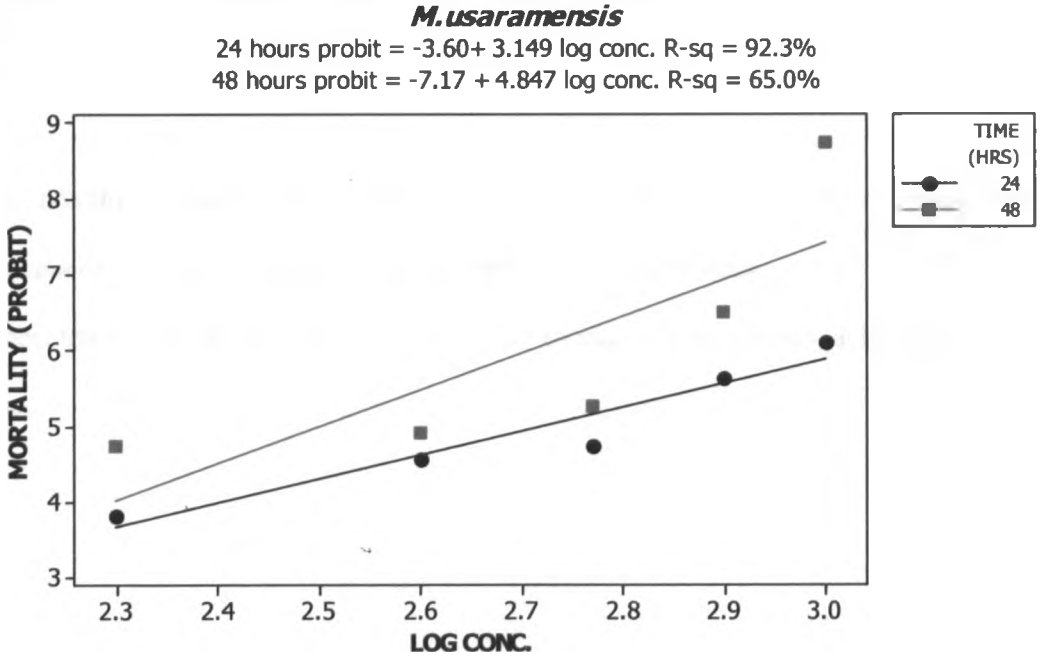

Figure 15: Mortality of *S. gregaria* 5th instar nymphs injected with *M.usaramensis* crude stem bark extract at 40^{0}C post-exposure temperature.

Table 12: Summary of log probit analysis of results of mortality in the 5th instar *S. gregaria* nymphs exposed to *M. usaramensis* crude stem bark extract at 25^{0}C and 40^{0}C.

Post-treatment exposure Temp.	Bioassay Period (hours)	Regression equations	R^2 (%)	LD_{50} values (µg/g)	95% Confidence Limits (µg/g)	
					Lower	Upper
25^{0}C	24	P = - 14.91 + 6.28 X	83.4	1466.11	950.90	34223.74
	48	P = - 0.65 + 1.91 X	88.3	913.65	639.10	3009.77
40^{0}C	24	P = - 3.60 + 3.15 X	92.3	540.92	410.72	698.88
	48	P = - 7.17 + 4.85 X	65.0	323.59	194.85	426.11

P, probit; X, log conc; *R*, coefficient of regression equations; LD, lethal dose; Calculated Log LD_{50} transformed to LD_{50}

ANOVA for the entire 48 hour period, the 24 hour period only and the 48 hour period only were also calculated. ANOVA for all the time periods showed p-values of p= 0.000 for 25^{0}C and 40^{0}C. Their mean intervals also did not overlap. This is strong evidence of difference in means.

The boxplot (Figure 16) compares mortality in *S. gregaria* injected with *M. usaramensis* crude stem bark extract with temperature, time period and concentration. It shows that at 40^{0}C, peak mortality was already achieved in 24 hours.

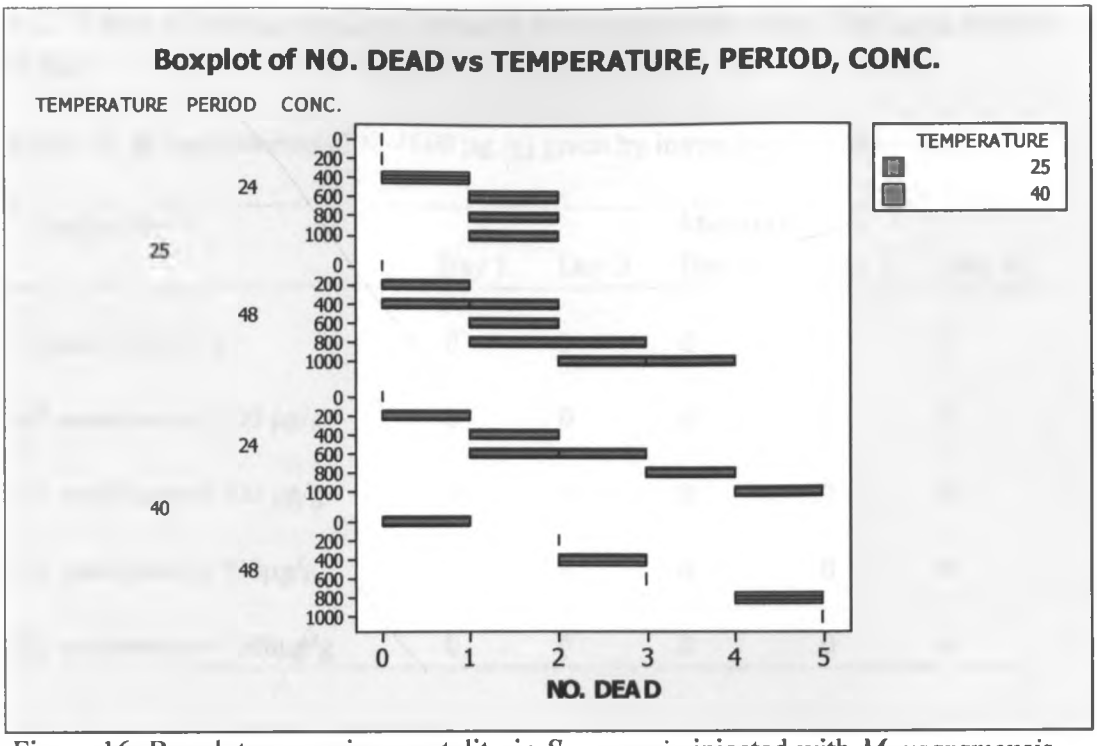

Figure 16: Boxplot comparing mortality in *S. gregaria* injected with *M. usaramensis* crude stem bark extract with temperature, time period and concentration.
No. Dead = mean of triplicates(N=5); Temperature ^{0}C; Period (hours);Conc.(µg/g).

4.4 Effect of *M. usaramensis* Stem Bark Extract on Mouse *Mus musculus*

The results of acute toxicity in mice are shown in Tables 13-15. In all the three methods of administration, there was no mortality observed in a 2 week post exposure period. No adverse signs of toxicity were observed during and after treatment.

4.4.1 Effect of intraperitoneally injected *M. usaramensis* crude stem bark extract in mice

Table 13: *M. usaramensis* (200-1600 µg /g) given by intraperitoneal administration

Sample (n=4)	Mortality				
	Day 1	Day 2	Day 3	Day 7	Day 14
Control (DMSO)	0	0	0	0	0
M. usaramensis 200 µg/g	0	0	0	0	0
M. usaramensis 400 µg/g	0	0	0	0	0
M. usaramensis 800µg/g	0	0	0	0	0
M. usaramensis 1600µg/g	0	0	0	0	0

4.4.2 Effect of topically administered *M. usaramensis* crude stem bark extract in mice

Table 14: *M. usaramensis* (250-2000 mg/g) administered topically

Sample (n=4)	Mortality				
	Day 1	Day 2	Day 3	Day 7	Day 14
Control (DMSO)	0	0	0	0	0
M. usaramensis 250mg/g	0	0	0	0	0
M. usaramensis 500mg/g	0	0	0	0	0
M. usaramensis 1000mg/g	0	0	0	0	0
M. usaramensis 2000mg/g	0	0	0	0	0

4.4.3 Effect of orally administered *M. usaramensis* crude stem bark extract in mice

Table 15: *M. usaramensis* (200-8000 mg/kg) given by oral administration

Sample (n=4)	Mortality Day 1	Day 2	Day 3	Day 7	Day 14
Control (DMSO)	0	0	0	0	0
M. usaramensis 200mg/kg	0	0	0	0	0
M. usaramensis 2000mg/kg	0	0	0	0	0
M. usaramensis 4000mg/kg	0	0	0	0	0
M. usaramensis 8000mg/kg	0	0	0	0	0

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 Discussion

In the present study, the toxicity of dichloromethane/methanol (1:1, v/v) stem bark extract of *Millettia usaramensis* subspecies *usaramensis* to *Aedes aegypti* 4th instar larvae and *Schistocerca gregaria* 5th instar nymphs was investigated. Toxicity of pure compounds extracted from *M. usaramensis* subspecies *usaramensis* to *Aedes aegypti* 4th instar larvae was also determined. The relationship between temperature and toxicity of the extract was investigated in *S. gregaria* 5th instar nymphs. Swiss mice *Mus musculus* were used in toxicity testing as non-target organisms.

5.1.1 The larvicidal potential of *M. usaramensis* stem bark extract on *A. aegypti* 4th instar larvae

In this study the crude dichloromethane/methanol stem bark extract showed a larvicidal activity of LC_{50} 50.82 µg/ml in a 48 hour period to *A. aegypti* 4th instar larvae. The results are in agreement with those of other workers. In a similar study, Yenesew (1997) reported that *M. usaramensis* crude chloroform extract showed larvicidal activities of LC_{50} 9.4 µg/ml (stem bark) and LC_{50} 3.5 µg/ml (seeds) to *A. aegypti* 2nd instar larvae in a 24 hour period. He noted that the extract was less active to *A. aegypti* 4th instar larvae causing only 43 % mortality on the fourth day. The results of the present study indicate that the insecticidal compounds in *M. usaramensis* bark are extractable by dichloromethane/methanol. Therefore *M. usaramensis* crude stem extract has larvicidal effect against *A. aegypti.*

5.1.2 The insecticidal potential of *M. usaramensis* stems bark extract on *S. gregaria* 5th instar nymphs

This study showed that *M. usaramensis* stem bark extract causes mortality in *S. gregaria* 5th instar nymphs, but the duration taken for insecticidal activity to be manifested differed with method of administration. Administration of the extract through injection caused significant mortality in 24 hours, while mortality after treatment through the topical route was observed after 48 hours. The onset of toxic effects after administration of the extract orally was slow, with mortality being observed after 72 hours.

The highest activity was shown in the injection treatment with LD_{50} of 445.85µg/g at 48 hour period. Topical treatment had an LD_{50} of 569.77 µg/g at the 72 hour period while oral treatment had an LD_{50} of 504.69 µg/g at the 144 hour period. This showed a substantial insecticidal activity of the crude stem bark extract.

The mode of action of rotenoids is thought to be inhibition of the mitochondrial electron transport chain, the oxidative phosphorylation (Dewick, 1988). An injected dose could have taken a shorter time to initiate a response due to the topographical proximity of the dose to internal organs. Injection also ensured that the entire dose was delivered without any loss.

A topically applied dose of insecticide however does not readily penetrate the insect and the minor fraction that does so is largely retained in the body wall. This results in a small amount that actually passes into the haemolymph. This is supported by Kabaru (1996) who reported that incorporating olive oil in topically applied *Melia*

volkensii fruit extract to *Locusta migratoria* nymphs and adults enhanced its activity. Also, Ware (1982) reported that poor cuticle penetrating properties of a contact insecticide may be improved by incorporating lipophilic solvents.

A dose administered through the oral route could have taken a longer time to initiate activity due to the fact that the insecticide only formed a thin layer on the surface of the treated seedlings resulting in the actual amount ingested being small. This could have been coupled with poor penetration due to retention in the lining of the gut, and loss through excretion in feaces.

Anti-feedant activity of *M. usaramensis* stem bark extract is evident in *S. gregaria* 5^{th} instar nymphs with an ED_{50} value of 660.71 ppm. This is equivalent to $13mg/cm^2$ of treated paper in the paper anti-feedant test (see Appendix I). This strongly suggests an anti-feedant effect of *M. usaramensis* stem bark extract on *S. gregaria* 5^{th} instar nymphs.

5.1.3 The larvicidal potential of (+)-12a-epimilletosin, (+)-usararotenoid-A and deguelin on *A. aegypti* 4^{th} instar larvae

Phytochemical studies of *M. usaramensis* stem bark extract has resulted in isolation of isoflavones, rotenoids and chalcones (Yenesew *et al.*, 1996; 1997; 1998). The biological activity of the extracts in the Leguminosae family especially in species *Derris*, *Lonchocarpus*, *Tephrosia*, *Mundulea* and *Millettia* is attributed to the rotenoids (Wayner *et al.*, 1989).

In this study, some rotenoids isolated from *M. usaramensis* showed a higher larvicidal activity against *A. aegypti* 4^{th} instar larvae than the crude extract. Deguelin (from

seed extract) showed the highest activity of LC_{50} 2.63µg/ml followed by (+)-usararotenoid-A (from stem bark) with LC_{50} of 4.27 µg/ml in a 48 hour period. (+)-12a-epimilletosin (also from stem bark) however showed very low activity of 2037 µg/ml in same time period. This suggests a high insecticidal potential in deguelin (*M. usaramensis* seeds). It also suggests that the moderate activity (LC_{50} 50.82 µg/ml) manifested by the crude stem bark extract is attributed to the pure compound (+)-usararotenoid-A. The fact that (+)-usararotenoid-A showed a much higher A. *aegypti* larvicidal activity than the crude extract suggests that purification of the crude extract could improve its potential.

The activity of rotenoids against insects is associated with their chemical structure. The main structural unit of all the rotenoids and associated compounds is a fused four-ring system- a chromanochromanone known as 6a, 12a-dihydrorotoxen-12 (6H)-one . The B/C ring junction in all of the active rotenoids is also cis. Compounds with modified rings and a trans- B/C ring junction are less insecticidal (Fukami and Nakajima, 1971; Joseph and Casida, 1992). (+)-12a-epimilletosin has the B/C ring junction with a trans-stereochemistry. This explains its low insecticidal activity. Despite (+)-usararotenoid-A also having a trans-B/C ring junction, its activity is relatively high. It could be an exception to the rule or its mechanism of action could be different (Yenesew, 1997). However, the structure-activity relationship is not entirely clear as less structurally complex isoflavonoids have shown some activity against insects (Bowers, 1983).

5.1.4 Effect of post-treatment temperature on the toxicity of *M. usaramensis* crude stem bark extract on *S. gregaria* 5th instar nymphs

Investigations into the effect of post-treatment temperature on toxicity of *M. usaramensis* crude stem bark extract in *S. gregaria* 5th instar carried out in this study showed that there is a positive temperature coefficient. Increase in temperature from 25^0C to 40^0C lowered LD_{50} from 913.65 µg/g to 323 µg/g in a 48 hour period. This was supported by ANOVA results which showed p-values of p=0.00 for 25^0C and 40^0C, indicating significant differences in means of mortality between the two temperatures.

The positive temperature coefficient of the crude extract would be of great advantage as locust- prone areas are generally hot. This may also lead to further clues on the mode of action of the extract. Kabaru (1996) suggested that high temperatures may lead to the acceleration of catabolic reactions which could lead to accumulation of lethal end-products such as ammonia and carbon dioxide. Another possibility would be temperatures causing changes in the fluid structure of cell membranes, accentuating the toxic effects of the extract. Such membrane structure changes may allow better penetration of the extract into cells resulting in accelerated disruption of certain metabolic pathways. Disruption of the cell membrane integrity would also result in cell death.

5.1.5 Toxicity of *M. usaramensis* stem bark extract on the mouse *Mus musculus*

Acute toxicity testing of effects of *M. usaramensis* stem bark extract to the mouse *Mus musculus* through intraperitoneal injection (200-1600 µg/g), oral administration (200-8000mg/kg) and topical application (250-2000 mg/g) showed no mortality in 2

weeks. In a similar study, Brook and Price (1961) showed that a fish toxicant, pro-Noxfish® containing the rotenoid rotenone, was harmless to rats. Fukami and Nakajima (1971) also showed that rotenone had a high lethal dose (3g/kg) to rabbits. This suggests that rotenoids are not toxic to mammals and therefore *M. usaramensis* stem bark extract is safe to mammalian non-target organisms. The insecticide will be safe to handlers in its preparation, application and transport. It will also be safe to mammals in the environment where the pests are to be eradicated.

5.2 Conclusions

The conclusions drawn from this study are summarized below:-

1. *Millettia usaramensis* crude stem bark extract showed larvicidal activity against *Aedes aegypti* and insecticidal as well as anti-feedant activity against *Schistocerca gregaria.*
2. The moderate *Aedes aegypti* larvicidal activity and *Schistocerca gregaria* insecticidal activity observed in *Millettia usaramensis* crude stem bark extract can be attributed to (+)-usararotenoid-A, one of the major compounds in the extract.
3. *Millettia usaramensis* offers promise as potential bio-control agent against *Aedes aegypti,* particularly the markedly larvicidal effect of deguelin isolated from its seeds. Plant extracts or isolated bioactive phytochemicals (deguelin and (+)-usararotenoid-A) could be used in stagnant water bodies for the small scale control of the mosquitoes acting as vector for many communicable diseases
4. The acute toxicity of *Millettia usaramensis* crude stem bark extract on *Schistocerca gregaria* has a positive temperature coefficient.

5. *Millettia usaramensis* crude stem bark extract is safe to mammalian non-target organisms.

5.3 Recommendations

1. *M. usaramensis* subspecies *usaramensis* stem bark and some of its constituents, (+)-usararotenoid-A and deguelin, have potential uses as larvicidal agents. Preliminary field trials are recommended to assess efficacy and photo-stability.
2. Toxicity testing of *M. usaramensis* extracts should be extended to other non-target organisms especially soil indicator organisms (e.g. earthworms) as soils may be irrigated with larvicide-treated water. This should also include organisms in water bodies especially crustaceans (e.g. daphnia and shrimps), mollusks and fish. Phytotoxicity testing should also be carried out on aquatic flora (e.g. algae) as well as selected terrestrial plant crops.
3. *M. usaramensis* seeds are the best candidate for larvicidal compounds as they contain deguelin which has shown to be highly larvicidal. Harvesting seeds is a non-consumptive way of using the trees instead of harvesting other plant parts which, if done on a large scale, poses a danger of destroying the plants to an extent of not being able to regenerate.
4. It is worthwhile to test the insecticidal activity of deguelin and (+)-usararotenoid-A against other insect pests and vectors.

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APPENDIX I

FORMULA FOR CONVERSION OF ED_{50} TO MICROGRAMS OF *MILLETTIA USARAMENSIS* STEM BARK EXTRACT PER CM^2 OF FILTER PAPER USED IN PAPER ANTIFEEDANT TESTS

$$\text{Micrograms of } M.\ usaramensis \text{ extract per } cm^2 \text{ of test paper} = \frac{(ED_{50} \times 80\mu l)/4cm^2}{1000\mu l}$$

Where;
ED_{50} = Effective dose in ppm at which 50% of the test paper is protected.

80µl =Volume of extract solution applied to test paper of area $4cm^2$

1000µl = Volume of extract containing the weight of extract in micrograms numerically corresponding to the ED50 expressed in ppm.

APPENDIX II

A: Results of regression analysis of the data on the toxicity of *M. usaramensis* crude stem bark extract on *A. aegypti* 4th instar larvae.

Analysis of Variance for 24 hour period

Source	DF	SS	MS	F	P
Regression	1	5.70757	5.70757	155.64	0.000
Error	6	0.22003	0.03667		
Total	7	5.92760			

Analysis of Variance for the 48 hour period

Source	DF	SS	MS	F	P
Regression	1	7.21547	7.21547	62.46	0.000
Error	6	0.69308	0.11551		
Total	7	7.90855			

B: Results of regression analysis of data on the toxicity of the pure compounds on *A. aegypti* 4th instar larvae

Analysis of Variance for (+)-12a-epimilletosin 24 hours

Source	DF	SS	MS	F	P
Regression	1	0.29784	0.29784	9.03	0.057
Residual Error	3	0.09896	0.03299		
Total	4	0.39680			

Analysis of Variance for (+)-12a-epimilletosin 48 hours

Source	DF	SS	MS	F	P
Regression	1	0.34167	0.34167	26.12	0.014
Residual Error	3	0.03925	0.01308		
Total	4	0.38092			

Analysis of Variance for (+)-usararotenoid-A 24 hours

Source	DF	SS	MS	F	P
Regression	1	0.76114	0.76114	12.72	0.038
Residual Error	3	0.17946	0.05982		
Total	4	0.94060			

Analysis of Variance for (+)-usararotenoid-A 48 hours

Source	DF	SS	MS	F	P
Regression	1	5.3484	5.3484	6.38	0.086
Residual Error	3	2.5143	0.8381		
Total	4	7.8627			

Analysis of Variance for Deguelin 24 hours

Source	DF	SS	MS	F	P
Regression	1	6.8960	6.8960	11.03	0.045
Residual Error	3	1.8761	0.6254		
Total	4	8.7721			

Analysis of Variance for Deguelin 48 hours

Source	DF	SS	MS	F	P
Regression	1	7.2476	7.2476	24.26	0.016
Residual Error	3	0.8964	0.2988		
Total	4	8.1439			

APPENDIX III

Results of correlation and regression analysis of data on the Anti-feedant tests of *M. usaramensis* stem bark extract on *S. gregaria* 5th instar nymphs.

Correlations: conc (ppm), mean RAP(%)

Pearson correlation of conc (ppm) and mean RAP(%) = 0.925
P-Value = 0.024

Regression Analysis: mean RAP(%) versus conc (ppm)

The regression equation is
mean RAP(%) = 16.74 + 0.05034 conc (ppm)

S = 7.52842 R-Sq = 85.6% R-Sq(adj) = 80.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1013.44	1013.44	17.88	0.024
Error	3	170.03	56.68		
Total	4	1183.48			

APPENDIX IV

A: Results of Analysis of Variance on data on the effect of post-treatment temperature (25^0C and 40^0C) on toxicity of *M. usaramensis* crude extract on *S. gregaria* nymphs in the entire 48 hour period.

Two-way ANOVA: NO. DEAD versus TEMPERATURE, CONC.

Source	DF	SS	MS	F	P
TEMPERATURE	1	36.125	36.1250	68.81	0.000
CONC.	5	97.903	19.5806	37.30	0.000
Interaction	5	11.458	2.2917	4.37	0.002
Error	60	31.500	0.5250		
Total	71	176.986			

S = 0.7246 R-Sq = 82.20% R-Sq(adj) = 78.94%

Individual 95% CIs For Mean Based on Pooled StDev

```
TEMPERATURE    Mean ----+---------+---------+---------+-----
25°C         1.05556 (----*----)
40°C         2.47222                    (---*----)
               ----+---------+---------+---------+-----
                 1.00      1.50      2.00      2.50
```

Individual 95% CIs For Mean Based on Pooled StDev

```
CONC.    Mean ---+---------+---------+---------+------
   0  0.08333 (---*--)
 200  0.83333      (---*--)
 400  1.33333         (--*---)
 600  1.91667           (---*--)
 800  3.00000                (--*--)
1000  3.41667                  (--*---)
           ---+---------+---------+---------+------
              0.0       1.2       2.4       3.6
```

No. Dead = mean of triplicates(N=5). Conc.(µg/g).

B: Results of Analysis of Variance on data on the effect of post-treatment temperature (25^0C and 40^0C) on toxicity of *M. usaramensis* crude extract on *S. gregaria* nymphs for the 24 hour period only.

Two-way ANOVA: NO. DEAD versus TEMPERATURE, CONC.

Source	DF	SS	MS	F	P
TEMPERATURE	1	16.0000	16.0000	52.36	0.000
CONC.	5	39.5556	7.9111	25.89	0.000
Interaction	5	9.6667	1.9333	6.33	0.001
Error	24	7.3333	0.3056		
Total	35	72.5556			

S = 0.5528 R-Sq = 89.89% R-Sq(adj) = 85.26%

Individual 95% CIs For Mean Based on Pooled StDev

TEMPERATURE	Mean	-+---------+---------+---------+--------
25^0C	0.72222	(----*-----)
40^0C	2.05556	(----*----)
		-+---------+---------+---------+--------
		0.50 1.00 1.50 2.00

Individual 95% CIs For Mean Based on Pooled StDev

CONC.	Mean	-----+---------+---------+---------+----
0	0.00000	(----*----)
200	0.33333	(---*----)
400	1.00000	(----*----)
600	1.66667	(----*---)
800	2.50000	(----*----)
1000	2.83333	(---*----)
		-----+---------+---------+---------+----
		0.0 1.0 2.0 3.0

No. Dead = mean of triplicates(N=5). Conc.(µg/g).

C: Results of Analysis of Variance on data on the effect of post-treatment temperature (25^0C and 40^0C) on toxicity of *M. usaramensis* crude extract on *S. gregaria* nymphs for the 48 hour period only.

Two-way ANOVA: NO. DEAD versus TEMPERATURE, CONC.

Source	DF	SS	MS	F	P
TEMPERATURE	1	20.2500	20.2500	48.60	0.000
CONC.	5	60.4722	12.0944	29.03	0.000
Interaction	5	3.5833	0.7167	1.72	0.168
Error	24	10.0000	0.4167		
Total	35	94.3056			

S = 0.6455 R-Sq = 89.40% R-Sq(adj) = 84.54%

Individual 95% CIs For Mean Based on Pooled StDev

TEMPERATURE	Mean	--+---------+---------+---------+-------
25^0C	1.38889	(----*----)
40^0C	2.88889	(----*----)
		--+---------+---------+---------+-------
		1.20 1.80 2.40 3.00

Individual 95% CIs For Mean Based on Pooled StDev

CONC.	Mean	---+---------+---------+---------+------
0	0.16667	(---*---)
200	1.33333	(---*---)
400	1.66667	(---*---)
600	2.16667	(--*---)
800	3.50000	(--*---)
1000	4.00000	(---*--)
		---+---------+---------+---------+------
		0.0 1.5 3.0 4.5

No. Dead = mean of triplicates(N=5). Conc.(µg/g).