A COMPARATIVE STUDY OF THE EFFECTS OF *MELIA VOLKENSII* (GURKE) EXTRACTS AND AZADIRACHTIN ON GROWTH AND OVIPOSITION OF *AEDES AEGYPTI* L. (DIPTERA: CULICIDAE)

BY

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DECLARATION

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

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Signature .....

Date 27th July, 1994

This thesis has been submitted for examination with our approval as University supervisors.

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Signature .....

Date 27/7/94

DEDICATION

This thesis is dedicated to
My parents and relatives
Who needed my attention
But could not get it
During the course
Of this study
And had to remain
Patient with me;
My success is
Theirs.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ac	$COCH_3$.
ACN	acetonitrile.
AF	Abbott's formula.
cf.	compare.
$CHCl_3$	chloroform.
cm	centimetre.
D	dark.
DDT	dichloro-diphenyl-trichloroethane.
ED_{50}	dose effective to 50 percent of the animals tested.
EE	ethyl acetate.
e. g.	*exempti gratia.*
etc.	*et cetera.*
et al.	*et alia (alibi).*
F_p	an arbitrary multiplication factor.
g	grams.
H_2O	water.
Hex	hexane.
HPLC	High Performance Liquid Chromatography.
IC_{50}	concentration inhibiting the growth of the parasites tested by 50 percent.
ID	internal diameter.
i. e.	*id est.*
kg	kilograms.
km	kilometres.
L	light.

LC	Liquid Chromatography.
LC_{50}	concentration lethal to 50 percent of the animals tested.
LD_{50}	dose lethal to 50 percent of the animals tested.
Log.	logarithms.
Me	CH_3.
MeOH	methanol.
mg	milligrams.
mg/ml	milligrams per millilitre.
ml	millilitres.
mm	millimetres.
MV-1, 2, 3	*Melia volkensii* extracts 1, 2, 3.
N_tS	total number of all stages living.
n_tS_p	the number of a particular developmental stage living at that time of observation.
pers. commun.	personal communication.
ppm	parts per million.
rDS	relative developmental stage.
RP	reverse phase.
t	day of observation.
U. S. A.	United States of America.
µl	microlitres.
µM	micromolar.
µg	micrograms.
µg/ml	micrograms per millilitre.
v:v	volume to volume.

WHO	World Health Organisation.
wt.	weight.
wt./v	weight per volume.
%	percent.
^{o}C	degrees Celsius.
$	American dollar.
>	greater than.
<	less than.

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Abstract

Three fractions and one compound from *Melia volkensii* (Gurke) were tested for toxicity and growth inhibition effects against the second instar larvae of *Aedes aegypti* L. in the laboratory. Their effects were compared with that of a natural growth inhibitor, azadirachtin A. The dosage-mortality test data revealed that all the test substances from *M. volkensii* had a higher acute toxicity on the larvae than azadirachtin A after 48 hours, though azadirachtin A gave higher percentage total mortality rates on the earlier developmental stages in the long run. Both azadirachtin A and *M. volkensii* test substances showed good growth inhibition effects. The increasing order of LC_{50} (ppm) in 48 hours was as follows: fraction 4:6&1:1 = 9.4; fraction B = 18.2; fraction A = 44.5; compound D_2 = 55.3; and azadirachtin A = > 200 (282.9). In the limited field trial studies, fraction B from *M. volkensii* at concentrations of 50 and 100ppm regulated the pre-imaginal stages of *Ae. aegypti*.

When the above substances were tested for any effect on hatchability of *Ae. aegypti* eggs and any influence on the oviposition behaviour of the adult females of the same mosquito, the two less toxic test substances (fraction A and compound D_2) from *M. volkensii* and azadirachtin A increased the rate of hatching of the eggs but the two toxic fractions

(4:6&1:1 and B) from *M. volkensii* slightly increased hatchability and then caused drastic inhibition on hatching after reaching their critical levels. At the highest concentration tested (200ppm), *M. volkensii* test substances deterred oviposition of the adult *Ae. aegypti* females and had an ovicidal effect on the eggs.

The effect of pellets formulated from the acetone extract fraction of *M. volkensii* on third- and fourth-instar larvae of *Ae. aegypti* was also investigated. The results showed that the pellets caused a total percentage mortality of 95% on the larvae when applied on water as larval food.

Since the active products from *M. volkensii* fruit extracts have higher acute toxic action to *Ae. aegypti* larvae than azadirachtin A and may also be potent as insect growth inhibitors, they can possibly be used in the control of mosquitoes.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Since the 1940's, mosquitoes have been the most widely and extensively studied arthropods of medical and veterinary importance. They are widely distributed throughout the world and practically no part of the world, where there is human existence is free of them. Man has suffered from their activities since time immemorial. They have influenced and still influence man's living, working and playing environment (Gomma, 1966).

Of nearly thirty-three genera of Culicine mosquitoes (Service, 1986), *Aedes aegypti* L. has been the most extensively studied, and it is currently popular as a laboratory animal and often a domestic pest and vector of pathogens in domestic and peridomestic foci in and near the tropics (William *et al.*, 1973). *Ae. aegypti* females are among the most prominent of the numerous kinds of blood sucking arthropods that annoy man, other mammals and birds (Harwood and James, 1979). These females are the sole vectors of the viruses causing yellow fever, dengue fever and viral encephalitids of man, and they are of prime importance in the transmission of bancroftian and brugian filariases (Phillip, 1962). The males do not bite, but their probing after moisture from sweat may be almost as

annoying as biting (Hermes, 1961).

Losses resulting from lowered productivity of industries concentrating on outdoor activities are frequently considerable because of mosquito annoyance. Such losses alone would justify the great sums of money that are spent on mosquito abatement, yet these losses must be added to the widespread suffering and death due to the mosquitoes as vectors of the pathogens causing disease. Numerous accounts report that mosquito attacks affect weight gains in livestock significantly and milk production in cattle; of some $25 million loss in the U.S.A. in 1965, $10 million of this was due to reduction in milk production (Steelman, 1976). An estimate of 166 millilitres of blood loss per animal each night was recorded in parts of Queensland, Australia (Standfast and Dyce, 1968).

The basic purpose of controlling medically important arthropods is to preserve the health and well-being of man, whereas control of arthropods affecting livestock is fundamentally guided by economic principles. The frequency and intensity of infection in the human population must always be the ultimate criteria of success in controlling mosquito-borne diseases. Diseases are not just a collection of signs and symptoms calling for treatment by a physician. There is a more cheerful point of view than the purely clinical one, because it inspires the hope, not merely of curing diseases when they occur but of preventing

their occurrence and even, ultimately abolishing them altogether.

Mosquito-borne diseases are especially complicated because they involve an additional component, the mosquito vector by which the disease is carried from the human host to another, or from one animal to the other. While the mosquito is bringing the pathogen to maturity, it has to pass through at least one reproductive cycle, usually several mosquitoes will take the risk of attendant of blood meal, oviposition and location of the host. Such a vector is itself subjected to environmental hazards which determine whether it will live long enough for the pathogens it is carrying to mature and become more infective. It is therefore necessary to seek the most effective ways of understanding vector biology in order to avoid further transmission of the pathogens. It is essential to know for instance where and how mosquitoes breed, factors favouring their breeding, their flight range, their biting time and resting sites. These measures can be taken to control mosquitoes depending on particular circumstances which may be directed against the immature stages by using insect growth inhibitors (regulators), or the adult mosquito by using oviposition deterrents.

One way of achieving the above measures is through the use of natural organic chemicals from plants which are plentiful and varied. Phytochemicals derived from various botanical sources have provided numerous

beneficial uses ranging from pharmaceuticals to insecticides (Matsumura, 1975). Applied entomologists are in the process of developing safe and long lasting control measures by using such chemicals as growth, feeding and oviposition inhibiting compounds. Since nature constantly produces secondary substances in large amounts and because of their natural origin, the environmental hazards of their use are probably less than with synthetic pesticides (Schoonhoven, 1982).

Man first used botanical (organic) insecticides to control insect pests and disease vectors in his environment around the 17th century. The first botanical (organic) insecticides to be used were nicotine, in the form of tobacco extracts which were mainly obtained from the leaves of *Nicotiana tabacum* and *Nicotiana rustica* (family Solanaceae); pyrethrum powders from the flowers of plants belonging to the family Compositae and the species which possess a high toxic content called pyrethrin are *Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum*; and rotenone from the roots of plants belonging to the family Leguminosae, namely *Derris malaccensis* and *Derris ellipta* both from Malaya and East Indies where the dry product is called derris or tuba. Other sources of rotenone are *Lonchocarpus utilis* and *Lonchocarpus urucu* from South America where it is called timbo or cube (Matsumura, 1975). The East African species, *Tephrosia* have also been shown to contain rotenone and related compounds (O'Brien, 1967).

Some of the natural plant compounds that also contain effective insecticidal properties come from the family Meliaceae (Chopra, 1928) which is widely distributed in Asia, Africa, and other tropical parts of the world (Siddiqui *et al.*, 1988). Such compounds are sometimes used in the indigenous methods of insect control (Mwangi and Mukiama, 1988). The Chinaberry tree, *Melia azedarach* L. has been found to be reputedly immune to attack by virtually all insects, and this rare occurrence creates curiosity (Thorsteinson, 1960). Recent work which has been done on *Melia volkensii* (Gurke), a member of the Meliaceae family, which often grows widely in dry areas of Eastern Africa upto a height of 20 metres, show that it has some of the most effective insecticidal activities (Mwangi and Mukiama, 1988). It contains potent insect growth regulators, antifeedants, and acute toxic activity which seem to be specific to arthropods (Mwangi, 1989). The leaves of *M. volkensii* are used in traditional methods of insect control. The leaves are meshed up and an aqueous extract obtained which is sprinkled on animals or huts to eliminate fleas (Mwangi, 1989). Insecticidal activities of the other two members of the Meliaceae family, namely the neem tree, *Azadirachta indica* A. Juss, and *M. azedarach* which are the well known sources of a tetranortriterpenoid (limonoid) compounds called azadirachtins (azadirachtin A, B, C, D, H and I) (Rembold *et al.*, 1984; Goviandacharia *et al.*, 1991) have

been well studied and documented (Warthen, 1979). Azadirachtin A is the most active compound in the group and is simply referred to as azadirachtin (Kabaru pers. commun). In terms of activity, it has efficient insect growth regulator (IGR) and feeding inhibition activity (Schoonhoven and Jermy, 1977; Blaney, 1981; Simmonds and Blaney, 1984).

It is apparent that very little information is available as regards the control of mosquitoes using phytochemicals from *M. volkensii* extracts although the earliest reports of the use of plant extracts against mosquitoes was recorded by Campbell *et al.*, (1933).

1.2 LITERATURE REVIEW

Mosquitoes have four larval instars. The relative length of the various instars may be a specific characteristic, but in general the second and third instars are the shortest than the first, and the fourth is the longest of all. The rate of growth of mosquito larvae seems to depend largely on three classes of factors: the environmental temperature, the inherent (genetic) characteristics of the species and the nature of culture medium especially in relation to availability of food (Bates, 1949). Under optimum conditions at 28^{o}C, the larvae of *Ae. aegypti* reach the fourth stage in four days (Shannon and Putman, 1934) but the complete larval period lies between six to eight days (Christophers, 1960). Where man-made containers are the principle breeding sites, number of emerging adults seem to be mainly determined by larval mortality (Southwood *et al.*, 1972).

The essential requirement for oviposition in *Ae. aegypti* females is water. This behaviour was investigated by Kennedy (1941). He found that actual contact with water was the chief stimulus to oviposition. No eggs were laid by *Ae. aegypti* in the absence of such contact. *Ae. aegypti* females breed by preference in artificial containers of rain-water for example in barrels, tanks, cisterns, tin cans, old tyres and tree-holes. These breeding sites are susceptible to drying, therefore the adaptation of the eggs to

withstand desiccation to a very marked degree even up to a year's time is of advantage to this mosquito species but creates a problem in its control. *Ae. aegypti* at full maturation of the ovaries, under almost any condition deposits its eggs singly on any water or wet surface that may be available like glass, earthenware, filter paper, sponge or the floor of the cage wet with seeped water or spilled water (Christophers, 1960). In the laboratory *Ae. aegypti* females lay their eggs on filter papers on clean tap water in a tray. Wallis (1954a), studying the oviposition activity of mosquitoes including *Ae. aegypti* found that chemoreceptors used for examining water before oviposition were located in the tarsal segments on the leg. It is doubtful indeed if there is any very marked selection of the character of water for oviposition if other conditions are suitable, except in respect to strong salinity (Christophers, 1960). Salt or saline solution has effects on the growth of *Ae. aegypti* larvae (Macfie, 1914, 1922; Wigglesworth, 1933c) while change in pH has very little marked direct effect upon the larvae (MacGregor, 1921, 1929; Senior White, 1928).

Urban form of yellow fever is transmitted by *Ae. aegypti*. Like the main vector, *Ae. aegypti*, yellow fever originated in Africa and along with this vector, it was taken to the New World in slave trade ships in the 1500s (Harwood and James, 1979). Service (1986) has shown that infected *Aedes* mosquitoes can pass on yellow

fever virus through their progeny so that the next generation of adults are already infected even before they have bitten a host. *Ae. aegypti* can also transmit the yellow fever virus upto 168 days after an infective blood meal (Phillip, 1962). These two conditions therefore make the control of the yellow fever mosquito a continuing concern with even their presence and increase in many urban centres of the world, and the absence of the disease in Asia (Harwood and James, 1979).

Control measures of the nature of anti-*Ae. aegypti* campaigns have been carried out more especially in those parts of the New World where yellow fever was liable to occur in epidemic form. The first attempt at all on the onslaught on *Ae. aegypti* eradication seem to have started in north eastern Brazil in 1927-1929 following the yellow fever epidemic. Most of these anti-*Ae. aegypti* control campaign measures pre-date the discovery and use of DDT and other synthetic insecticides in residual spraying. These insecticides were initially very effective and hence described as "Classical methods of control" (Christophers, 1960).

In the last two decades increasing resistance of mosquitoes to inorganic insecticides was noticed during observations of the world wide occurrence and evolution of mosquito borne-diseases (Zebitz, 1986), and has now been reported in 19 species of *Aedes*; of which 17 of them show resistance to DDT and 12 to one

or more of the organophosphorous compounds: *Ae. aegypti* has now shown resistance to DDT in numerous locations throughout the world and several early studies showed a good correlation of cross-resistance in it (Hemingway *et al.*, 1989). Prassitsuk and Busvine (1977) , and Halliday and Georghiou (1984) observed that an unknown mechanism associated with DDT resistance in *Ae. aegypti* and *Anopheles gambiae* Giles conferred low level cross tolerance to even more recently developed synthetic pyrethroids. This problem of resistance together with environmental destruction has made the control of disease vectors using inorganic (synthetic) insecticides a drawback and therefore becoming unpopular with entomologists and environmental conservationists.

Interest in the naturally occurring pesticides has enjoyed a renaissance during the past 20 years. Materials such as nicotine, rotenone, and pyrethrum have long been used in the control of insect pests and disease vectors control, but suffered a decline following the discovery of inorganic and some synthetic pesticides, especially DDT and pyrethroids respectively, with lower costs, greater toxicity, and more durability to effects such as weathering (Perkins, 1985). Historically, the commercial development of botanical insecticides is credited to a lady of Ragusa, Dalmatia, who noticed dead insects on a discarded bouquet of pyrethrum flowers. She began milling pyrethrum into powder thus the pyrethrin industry was born (Hartzell

and Wilcoxon, 1941).

Nicotine as a crude tobacco extract has been used as an insecticide as early as 1763. The pure alkaloid was isolated in 1828, and was synthesised in 1904 by Pictet and Rotschy (Metcalf, 1955), and still ranks as an excellent contact insecticide and stomach poison (Matsumura, 1975); but this naturally occurring compound is both hazardous in use and lacks persistence (Corbett *et al.*, 1984). It is especially effective against aphids and other soft bodied insects. It is highly toxic to mammals, with an acute oral lethal dose 50 (LD_{50}) in rats of 50-60mg/kg, and beneficial insects like the silkworm, *Bombyx mori* at an oral LD_{50} of 4mg/kg (Matsumura, 1975).

The earliest recorded use of the rotenoids as insecticides was against leaf-eating caterpillars in 1849 (Metcalf, 1955). Rotenone can act either as a contact or a stomach poison. The mammalian toxicity of rotenone varies greatly with animal species, method of administration, and type of formulation. The acute oral LD_{50} of crystalline rotenone to rats is 132mg/kg, to rabbits 300mg/kg, and to guinea pigs is 60mg/kg. When administered intravenously in Olive oil, it is more toxic. It is very toxic to fish since it acts through the gills (Matsumura, 1975). It is also toxic to beneficial insects such as the honey bee, *Apis mellifera* L. at an oral LD_{50} of 3mg/kg, and the silkworm at the same LD_{50} value. Its paralytic effect is more pronounced in vertebrates than in insects (O'Brien, 1967). It is

readily detoxified by the action of air and light and therefore almost all toxicity may be lost after 2-3 days of summer exposure. Deterioration also takes place with heat (Matsumura, 1975).

The natural pyrethrins found in pyrethrum flowers of which pyrethrin I is the most active constituent, are potent insecticides (Perring and Mellan, 1976). Pyrethrum powders were first used as an insecticide around 1800, and by 1851 their use was world-wide. This natural insecticide was used in a limited scale to kill mosquitoes resting in houses. Pyrethrum is essentially non-toxic to mammals (acute oral LD_{50} in rats is about 200mg/kg). It is very fast acting towards insects thus killing beneficial insects such as the honey bees. It rapidly paralyses insects especially house-flies, and is commonly used in fly sprays and in the control of domestic insects. Often DDT or other synthetic insecticides are mixed with pyrethrins because insects may later recover from pyrethrum alone (Corbett *et al.*, 1984). For full action to occur, it is synergised with pipronyl butoxide. Pyrethrum is also toxic to fish and fish food (aquatic insects and crustaceans) (Casida, 1973). The pyrethrins are contact insecticides and have no stomach poisoning action because they are readily hydrolysed to non-toxic products. Their primary action is on the insect nervous system since they produce rapid paralysis. They are highly unstable in the presence of light, moisture and air (Matsumura, 1975).

The synthetic pyrethroids have emerged from prolonged efforts to improve the biological activity and chemical stability of the natural pyrethrins, which comprise a mixture of insecticidal esters obtained from the flowers of *C. cinerariaefolium*. The natural pyrethrins are unstable to light and air, and early synthetic analogues such as allethrin and bioallethrin were expensive, other than being chemically metabolised rapidly by insects. They were therefore mainly used in mixtures with a synergist as household insecticides (Corbett *et al.*, 1984). However, recent research, both by Elliott and his colleagues (1974), and in industry has led to the discovery of simpler synthetic compounds such as bioresmethrin, cypermethrin, permethrin and deltamethrin which are more stable under field conditions (Corbett *et al.*, 1984). Bioresmethrin was not only more active than pyrethrin I against insect species, but also even safer to mammals. On the criterion of relative acute toxicity, it is the safest insecticide known and virtually harmless to mammals. This advantageous selectivity has been retained, although less dramatically, in subsequent synthetic analogues such as permethrin and decamethrin which is the most potent insecticide of any class yet discovered (Elliott *et al.*, 1974). Pyrethroids interfere with the insect's normal nervous activity. Insects treated with pyrethroids show restless behaviour, become uncoordinated and are then paralysed; flying insects

are, in general rapidly knocked down. They are insoluble in water but can be absorbed by insect's nervous tissue. An intriguing feature of action of pyrethroids is that they become toxic to insects as the temperature is lowered and this effect is reversible (Corbett *et al.*, 1984) but this is not applicable within the tropics where temperatures are high throughout.

Although the synthetic pyrethroids are also relatively expensive, they can often be applied at lower rates than other insecticides (Worthings, 1979) and their use in agriculture is already widespread (Corbett *et al.*, 1984). But only recently, several insect species are known to have exhibited resistance to pyrethroids at least in the laboratory, and several instances of resistance serve to corroborate the conclusion that the primary site of action is in the nervous system. A major type of DDT resistance (knockdown resistance) is a cause for great concern since insects with this type of resistance are cross resistant to pyrethroids (Corbett *et al.*, 1984).

Since insect behaviour is known to be influenced by plant-emitted substances (Gupta and Thorsteinson, 1960; Fraenkel, 1969; Yamamoto *et al.*, 1969), chemists working in conjunction with pest control scientists have now opened a whole panoply of new materials for experimentation and use in the control of insecticide resistant species of insects. Among such plants are *A. indica*, *M. azedarach* and *M. volkensii*. Leaves and

fruits of *A. indica* and *M. azedarach* contain at least three active chemical structures which are tetranortriterpenoids, i. e. azadirachtin (Butterworth and Morgan, 1968), salannin (Henderson *et al.*, 1964), and meliantriol (Levie *et al.*, 1967). Extracts of *Melia toosendan* Sieb et Zucc. have been found to contain a compound called toosendanin which has antifeedant effect (Chiu *et al.*, 1984). The idea of using the neem tree to control insect pests originated in Asia while the use of various parts of neem tree for medicinal and crop protection is part of the Indian folkore (Indian Inst. Res. Bull., 1983). Pradhan *et al.*, (1962) first reported the antifeedant properties of neem kernels on the adult desert locust, *Schistocerca gregaria* (Forks).

It is claimed that extracts of *M. azedarach* and *A. indica* are successful in protecting fruits, vegetables and cereal crops in China. When leaves are placed with food, there is protection from insect attack. Also when the leaves are cut and placed in books, the books are protected from attack by book mites. These are known to be practised in the Eastern countries (Parmar, 1986). Neem powder at 0.25, 0.50, 1.00 and 2.00g per 25.0g of dried *Tilapia* has been shown to protect the fish against oviposition by *Dermestes maculatus* Deeger (Okorie, *et al.*, 1990). In India, the leaves of *A. indica* are fed on by cattle (Indian Res. Bull., 1983). Flowers and leaves can be eaten by man

(Irvine, 1961). Ketkar and Ketkar (1989) reported that there is no toxicity to man and other domestic animals, and no or relatively negligible side effects on beneficial organisms by neem products. Almost every part of the neem tree has long been used in folkoric and traditional systems of medicine for the treatment of a variety of human ailments, particularly against diseases of bacterial and fungal origin (Siddiqui *et al.*, 1988). In Northern Nigeria, the bark of *M. azedarach* decoction is used as a wash for syphilitic condition (Dalziel, 1937), while in Kenya almost all parts of the neem tree are used for treating about forty different kinds of ailments hence the local name "muarubaine" (Local people pers. commun.).

Fagoonee (1983) found that the third and fourth instars of the cabbage webworm *Crocidolomia binotalis* are capable of detoxifying the antifeedants like azadirachtin to a limited extent, but at the cost of poor weight gain and disruption in larval and pupal development. Ethanol extract of the petroleum ether extracted kernel oil (from *A. indica*) was found to be an oviposition deterrent at 2.5% or higher concentrations against *Dacus cucurbitae* (Coq.) and at 20% or higher against *Dacus dorsalis* (Hendel) (Singh and Srivastava, 1983). Yadav (1985) observed that treating green gram, *Vigna radiata* Wlcz. with 50mg neem oil/10g seed was antiovipositional and 30mg/10g was ovicidal in *Callosobruchus maculatus* F., and that 40mg/10g was

antiovipositional in *Callosobruchus analis* F. and C. and *Callosobruchus chinensis* L. Rovesti and Deseoe (1991) showed that aqueous neem seed kernels (20g kernels per litre) reduce hatching rate in the eggs of leafminer, *Leucoptera malifoliella* by 60% and slowed the growth rate of hatched larvae with no pupation occurring.

Sankaram *et al.*, (1986) developed a highly biologically active fraction from fresh neem kernels which showed remarkable antiviral and antimicrobial properties. These biologically active compounds they obtained had growth inhibition and antifeedant effects. These new compounds were designated as vepaol which is closely related to azadirachtin and the two other known compounds, isoverpaol and nimbin. Khalid *et al.*, (1989) have isolated and characterised an antimalarial agent, gedunin which has an IC_{50} of 1µM after 48 hours of exposure to *Plasmodium falciparum* in vitro (roughly equivalent to quinine) from *A. indica* and *M. azedarach.* An amorphous bitter principle, nimbidin, which is antiarthritic and anti-infamatory in action and possessing significant antiulcer activity has been also isolated from *A. indica*, whereas various other fractions from the same plant extracts have been found to have antipyretic and antitumour properties (Siddiqui *et al.*, 1988). A new limonoid, Mahmoodin, has also been isolated from *A. indica* (neem) oil which has showed significant antibacterial activity against various Gram-negative and positive bacteria at 10mg in laboratory

culture media (Siddiqui *et al.*, 1992). From the above reports, it can be seen that the effect of extracts from fruits, seeds and leaves of *M. azedarach* and *A. indica* range from invertebrates down the evolutionary line to the micro-organisms. This can also be true for the extracts of *M. volkensii* but a thorough investigation is still required.

Effects of azadirachtin have been widely investigated on insect pests of agricultural importance (In reviews by: Gill, 1972; Warthen, 1979; Schmutterer and Ascher, 1984) while very little work has been done on the effects of the active principles of *M. volkensii* (Mwangi, 1982, 1989; Rajab *et al.*, 1988) on the same pests. Only a few scientists have paid attention to the potential of neem tree (Gill, 1972; Chavan *et al.*, 1979; Chavan, 1984; Attri and Pasad, 1980; Warthen, 1979; Steffens and Schmutterer, 1982; Zebitz, 1984, 1986) and *M. volkensii* (Mwangi and Rembold, 1987, 1988; Mwangi and Mukiama, 1988, 1989; Al-Sharook *et al.*, 1991) derived pesticides in mosquito control. Chavan *et al.*, (1979) found petroleum ether, ether, chloroform and alcohol extracts of neem leaves to be toxic to fourth instar larvae of *Culex fatigans* Wied. Zebitz (1984) found that permanent exposure of fourth instar larvae of *Ae. aegypti* to water treated with aqueous and organic extracts of neem seed kernel result in conspicuous growth disruption, mainly during imaginal development. The effectiveness of the extracts was increasing with

decreased polarity of the solvent used during extraction.

Further investigations by Zebitz (1986) on growth regulating effects of different neem seed kernel extracts and azadirachtin on fourth instar mosquito larvae showed susceptibility in a decreasing order as follows: *Ae. tongoi* (Theobald) = *An. stephensis* Liston, *Culex quinquefasciatus* Say and *Ae. aegypti*. The LC_{50} data of a simple extract from neem seed kernels (AZT-VR-K-E) ranged from 1.19ppm for *Ae. tongoi* to 18.1ppm for *Ae. aegypti*. He also found in semi-field trials with *Culex pipiens* L. that the good insect growth regulator effect of neem seed kernel fruit extract was demonstrated. His work on oviposition deterrency showed that in cage tests with females of *Ae. aegypti*, enriched extract had no effect, and neem oil weakly influenced the oviposition behaviour of female *Cx. quinquefasciatus*, but females of *Ae. aegypti* were not affected.

The crude extract of dried fruits of *M. volkensii* has been shown to be toxic, and to have antifeedant activity and growth inhibiting effects on the desert locust *S. gregaria* (Mwangi, 1982). A standard bioassay for the active fractions has been developed (Mwangi and Rembold, 1987) and methods of purifying active fractions have been established (Mwangi and Rembold, 1987, 1988; Mwangi and Mukiama, 1988; Rajab *et al.*, 1988). An azadirachtin-free fraction from *M. volkensii* seed

kernel extracts has been shown by Mwangi and Rembold (1987, 1988) to have greater acute toxic and growth inhibiting effects on *Ae. aegypti* larvae than azadirachtin containing fraction from neem seed kernel extracts. The lethal effects for larvae exposed to *M. volkensii* extracts also occurred earlier than for larvae exposed to neem seed kernel extracts (Mwangi and Mukiama, 1988). According to Mwangi (1989) the substances with more biological activity from *M. volkensii* are more lipophilic than azadirachtin.

An aqueous extract from fruit kernels of *M. volkensii* has been found to show strong growth inhibiting and insecticidal activities (LC_{50} 20-30ppm) on the last instar larvae of *Culex pipiens*. It had also strong repellent effect on egg laying behaviour of *Cx. pipiens* females which hardly laid eggs on the treated water (100ppm) for about one week (Jiang', unpublished paper).

Al-Sharook *et al.*, (1991) when comparing the larvicidal effect of acetone extracts from *M. volkensii* and *M. azedarach* seeds with the pure natural growth inhibitor, azadirachtin A in their morphogenetic effects against *Culex pipiens molestus* Forskal larvae found that there was significant differences between the insecticidal activity of both the crude extracts. They found that *M. volkensii* acetone extract resulted in equal toxicity for larvae and pupae with LD_{50} of 30 µg/ml while *M. azedarach* acetone crude extract was exclusively

larvicidal with LD_{50} of 40µg/ml and had no growth inhibitory effect on the pupal stage. Their work also showed that pure azadirachtin A, like the crude *M. volkensii* extract, was equally toxic to both the larvae and pupae with LD_{50} of 1-5mg/ml. They also found the bioactive compounds from *M. volkensii* thermostable and partially soluble in water.

The available scientific publications (Al-Sharook *et al.*, 1991; Mwangi, 1982; Mwangi and Rembold, 1987, 1988; Mwangi and Mukiama, 1988, 1989; Rajab *et al.*, 1988) have now shown that derivatives from *M. volkensii* contain the most effective natural insecticides at present time. The derivatives from the other two members of the family (*A. indica* and *M. azedarach*) have been reported to be non-toxic to man and other mammals (Irvine, 1961; Ketkar and Ketkar, 1989), are neither mutagenic nor cancerogenic, and do not cause any skin irritations or organic alterations to mice and rats at high concentrations (Jacobson *et al.*, 1984; Schmutterer, 1983). A study by Vollinger (1986) showed that there is no sign of resistance or cross-resistance between deltamethrin, neem seed kernel extract and diflubenzuron (Dimilin, a synergistic moulting inhibitor) in the diamondback moth, *Plutella xylostella* (L.). This therefore, shows that naturally derived plant insecticides especially from the Meliaceae family offer promising possibilities for the third world countries.

Several other botanical derivatives have also

been found to offer great promise as sources of phytochemicals for the control of mosquitoes (Kumuda *et al.*, 1991).

This study therefore presents results of investigations on potential products of *M. volkensii* for mosquito control.

1.3 Objectives of the study.

This study was undertaken to determine the effects of one pure compound coded D_2 (C D_2), three fractions coded A (F_A), B (F_B), and 4:6&1:1 ($F_{4:6\&1:1}$) that are all natural products from *M. volkensii* on *Ae. aegypti* which was used as a laboratory model. The above effects were compared with those of a natural growth regulator, azadirachtin A (Aza. A), which is known to have a low effective dose 50 (ED_{50}) on mosquitoes. The specific objective of this study were:-

1. To evaluate and compare the toxicity levels of compound D_2, fractions A, B and 4:6&1:1, and azadirachtin A on the second instar larvae of *Ae. aegypti* in the laboratory.

2. To determine the effects of the above test substances on growth and development of the second instar larvae of *Ae. aegypti* until the emergence of adults in the laboratory.

3. To formulate pellets from the acetone extract fraction of *M. volkensii* and study their effects on third and fourth larval instars of *Ae. aegypti* when used as larval food.

4. To determine the effect of fractions and a compound from *M. volkensii*, and azadirachtin A on

hatchability of *Ae. aegypti* eggs.

5. To investigate if the *M. volkensii* test substances have any oviposition deterrency effect on adult *Ae. aegypti* females.

6. To find out if there was any possibility of ovicidal effect on the eggs laid in the oviposition deterring concentration (200.0ppm) of the *M. volkensii* test substances.

7. To carry out limited field control studies within Chiromo campus on the effect of fraction B from *M. volkensii* on growth and development on any species of mosquito larvae that occurred during the study period.

CHAPTER TWO

MATERIALS AND METHODS

2.1 TEST SUBSTANCES

2.1.1 Fractions and a compound from *M. volkensii.*

Fractions A and B were provided by Mr. J. M. Kabaru of Zoology Department (Insect Physiology Section), University of Nairobi. These fractions were prepared as follows: Ripe fruits of *M. volkensii* were obtained from Embu, approximately 150km north of Nairobi, Kenya. The whole fruits were dried at 40^{o}C in an oven to a constant weight (Mwangi and Rembold, 1987) and pulverised to fine powders by means of a hammer-mill until the powder passed through 1mm mesh sieve. The powder was extracted three times with equal volumes of methanol: water 80:20 (v/v) (Mwangi and Rembold, 1988). Each round of extraction consisted of ratios of 1:1, powder:liquid (wt./v). The solution was filtered and evaporated to dryness (lipholysed) in a rotary evaporator under vacuum at 40^{o}C. The dry residue was extracted with cold methanol at a ratio of 10ml of methanol/g of extract. The supernatant was dried and the procedure repeated with acetone.

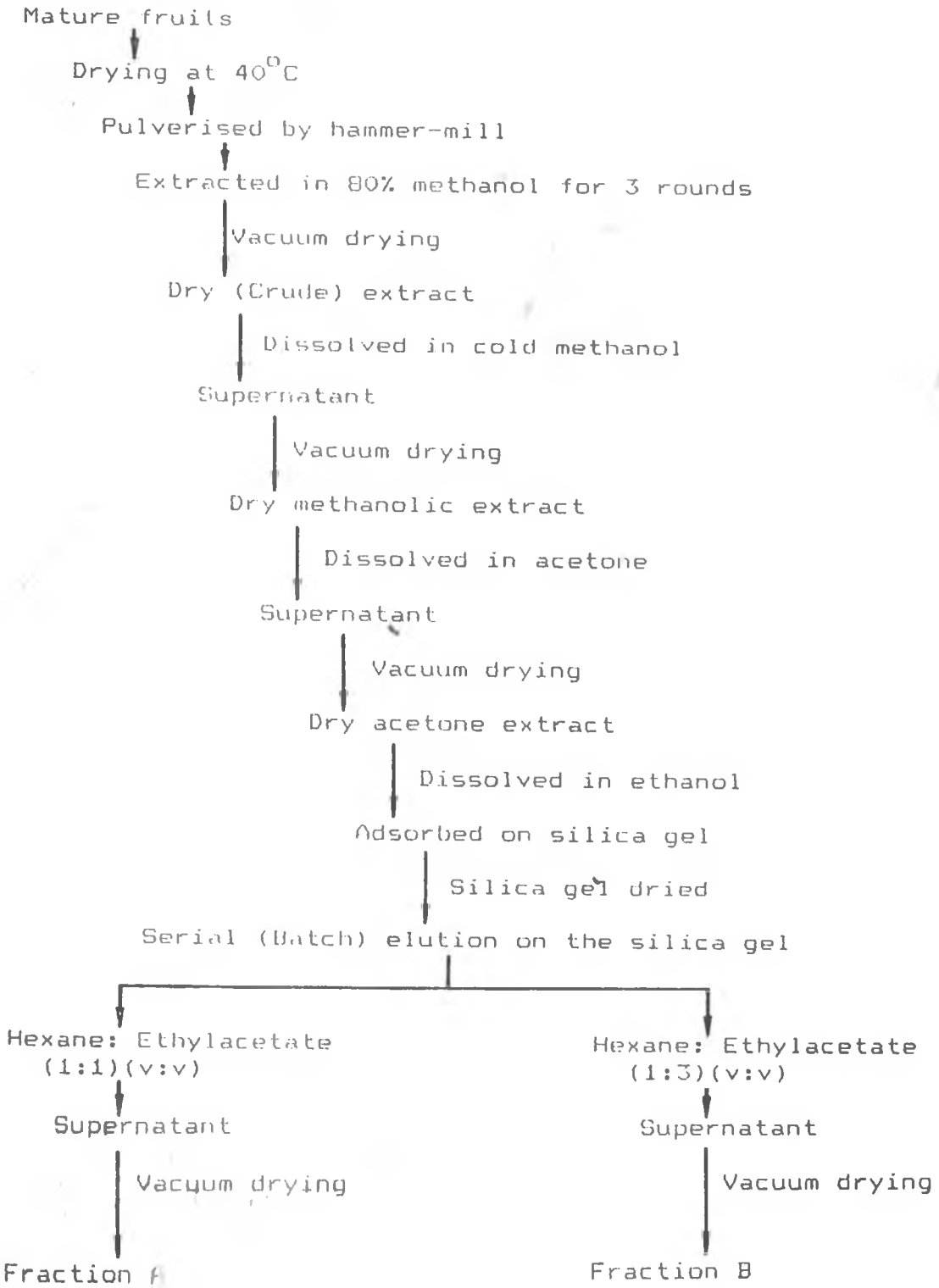
The dry acetone-soluble fraction was dissolved at a ratio of 10ml ethyl acetate/g of material. The material was adsorbed onto silica gel at a ratio of 200mg of extract per gram of silica gel. Portions of 5g of the silica gel were applied on top of a glass column

(4cm ID x 30cm) packed with silica gel. The material was isocratically with 500ml of hexane:ethyl acetate (1:1) (v:v). This fraction was dried to give fraction A. Another isocratic elution with hexane:ethyl acetate (1:3) (v:v) was done to give fraction B (Mwangi and Rembold, 1988) (Figure 1).

Fraction 4:6&1:1 and compound D_2 were prepared as follows: Fine powders from *M. volkensii* seeds (prepared as outlined above) were extracted with 80% methanol to give, *M. volkensii* extract 1, MV-1, which was further extracted with 100% methanol to yield, MV-2. The MV-2 was again further extracted with 100% acetone. The resulting acetone extract, MV-3 was then adsorbed onto silica gel. It was then eluted serially with hexane: ethyl acetate in the ratio of 7:3 (v:v), 1:1 (v:v), 4:6 (v:v), 0:1 (v:v), and methanol alone to give fractions 7:3, 1:1, 4:6, 0:1, and a methanol extract.

The active hexane: ethyl acetate fractions (1:1 and 4:6) were pooled together to form fraction 4:6&1:1 (standard). This fraction was adsorbed onto the silica gel and Liquid Chromatography (LC) was performed whereby the fraction was eluted with a solvent system of chloroform: acetone (7:3) (v:v) and three fractions, 4:6:1, 4:6:3, and 4:6:5 were obtained of which the active ones were 4:6:1 and 4:6:3. These two fractions were then passed through High Performance Liquid Chromatography (HPLC) with RP-8 gel and eluted with different solvent systems. Fraction 4:6:1 was eluted

Figure 1: A flow chart showing the procedure used in the preparation of fractions A and B from *M. volkensii* fruits.



(Mwangi, 1989)

with 50% acetonitrile and two compounds, salannin and ohchinin-3-acetate were obtained while elution of fraction 4:6:3 with 65% methanol yielded four compounds namely D_1, D_2, D_3, and D_4 (Figure 2) (Balan, pers. commun.).

2.1.2 Azadirachtin A.

Pure azadirachtin A (96%) (Al-Sharook *et al.*, 1991) extracted from the neem seeds using the method developed by Rembold (1988) was supplied by Prof. H. Rembold of Max Planck Institute for Biochemistry, Munich, Germany and kept in the Insect Physiology laboratory, Department of Zoology, University of Nairobi.

2.1.3 Olive oil.

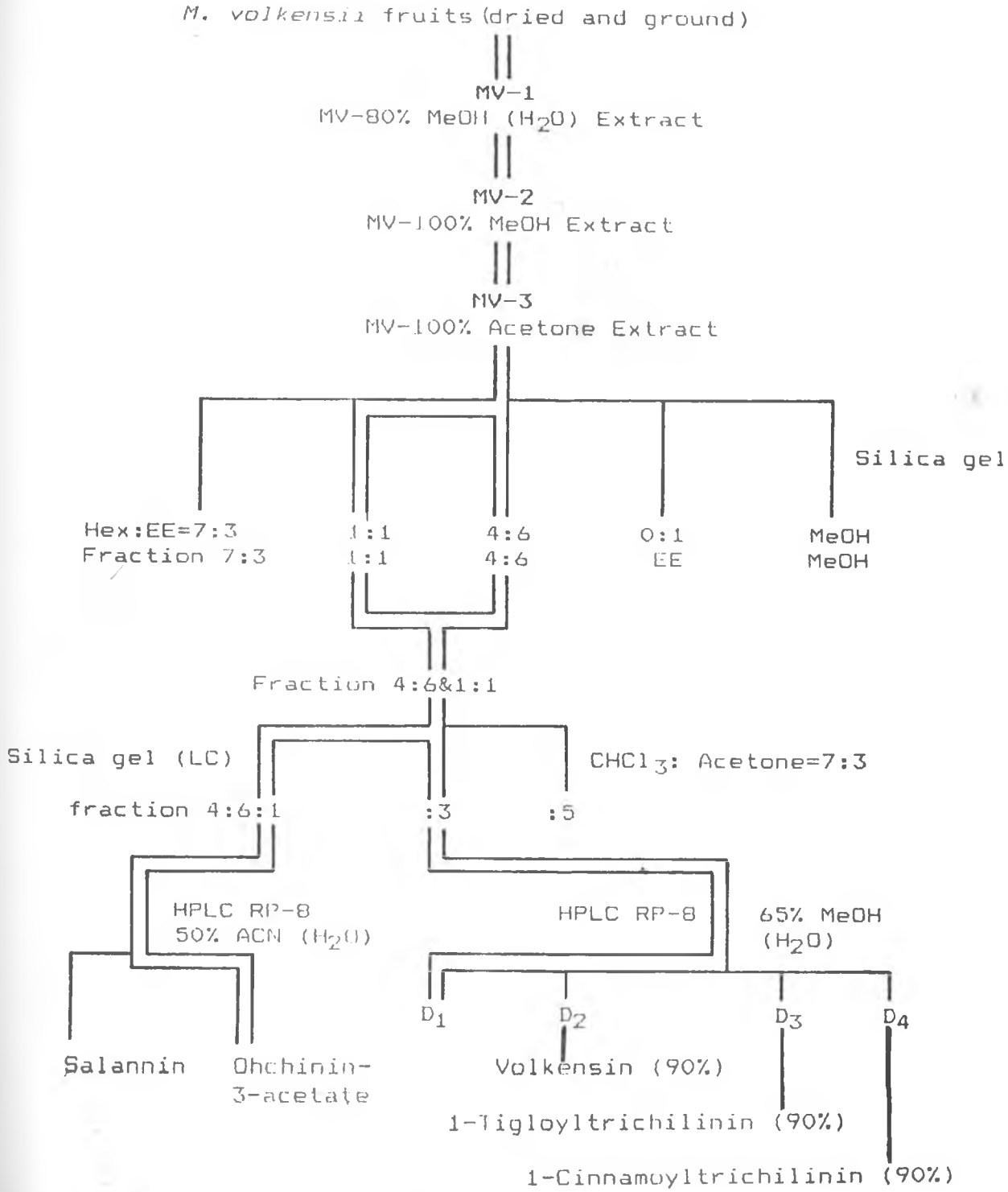
Pure olive oil used was of a brand name Rusco and was purchased from a recognised pharmaceutical dealer in Nairobi City, Kenya.

2.2 REARING OF EXPERIMENTAL INSECTS

Aedes aegypti colony.

Eggs of *Ae. aegypti* were obtained from a desiccator in the insectary, Department of Zoology, University of Nairobi. The eggs were flooded with 300ml of water in a metallic tray measuring 27x35x5cm to hatch. Finely ground yeast (100mg) was given daily as food for the hatched larvae. Second instar larvae (48 hours old) were used for the tests on toxicity, and growth and development. The larvae which were not used

Figure 2: A flow chart showing the procedure used in the preparation of fraction 4:6 & 1:1 and compound D_2 from *M. volkensii* fruits.



‖ represents active fractions or compounds.

(Balan, pers. commun.)

for the tests were reared until the emergence of adults. The emerged adults were maintained as a colony to provide more eggs.

During the rearing period of the larvae, the rearing media was changed after every three days and the trays were washed to avoid scum formation and mould growth which could produce toxic substances and kill the larvae. After pupation, the pupae were removed from the rearing tray using a pasteur pipette and put into a 250ml beaker with 100ml of tap water in a rearing net cage measuring 30x30x30cm so that the emerging adults were enclosed in the cage. A cotton wool wick soaked in a flask containing 10% sucrose solution (Angerilli, 1980) was put inside the net cage for the emerged adults to act as a source of energy. This solution was changed after every three days and a fresh one was provided to avoid fermentation and mould growth. The adult females were also provided with a live rabbit (to act as a source of blood meal which was necessary for the development of eggs) enclosed in a 20x35x15cm wooden box with the exposed head inserted into the cage so that the mosquitoes could feed on the ears. Three days after giving the females a blood meal, a metallic tray (measuring 20x15x6cm) containing 15cm Whatman No. 1 filter papers soaked in tap water was put inside the cage to act as an oviposition substrate. The filter papers containing eggs were removed after every three days and then placed in another tray measuring 21x26x6cm

with slightly wet sheets of cotton wool spread on the bottom to absorb water so that they can dry. After drying, the filter papers with eggs were kept in the desiccator for future use.

In all cases (i.e. in rearing and in experiments) the larvae and adults were kept at a room temperature of 28 $\pm$ 2°C and L14 - D10 photoperiod (Mwangi and Rembold, 1988).

2.3 PREPARATION OF TEST SOLUTIONS

2.3.1 *M. volkensii* fractions and compound D_2 test solutions.

Standard test substances from *M. volkensii* were prepared by dissolving 100mg of the test material into 1ml of absolute ethanol so that the resulting (stock) solutions had a concentration of 100mg/ml. Mixtures of *M. volkensii* standard test substances were prepared by mixing equal volumes of the stock solutions (100mg/ml) of the test substances required to make stock solution mixtures. From the stock solutions (stock solution mixtures) 0.4, 2.0, 4.0, 6.0, 8.0, 20.0, 40.0 and 80.0 µl were applied into sets of eight marked 250ml glass jars containing 40ml of distilled water to make final concentrations (test solutions) of 1.0, 5.0, 10.0, 15.0, 20.0, 50.0, 100.0 and 200.0ppm respectively. For the controls 80.0 µl of absolute ethanol was applied into other similar marked jars containing the same volume of distilled water. 80.0 µl of absolute ethanol was used in

the controls because it was equivalent to the maximum volume of the solvent present in the highest concentration (200.0ppm) of the test solution.

2.3.2 Azadirachtin A test solutions.

A standard test material was prepared by dissolving 10mg of azadirachtin A into 1ml of absolute ethanol so that the resulting (stock) solution had a concentration of 10mg/ml. From this stock solution 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 50.0, 100.0 and 200.0µl were applied into a set of eight marked 50ml beakers containing 10ml of distilled water to make final concentrations (test solutions) of 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 50.0, 100.0 and 200.0ppm respectively. A concentration of 2.5ppm was prepared for azadirachtin A test material only. For the controls 200µl of absolute ethanol was used. All the test solutions were then thoroughly mixed by swirling the containers.

2.3.3 Olive oil test solutions.

A standard test solution of olive oil was prepared by mixing 0.1ml of pure olive oil, weighing approximately 100mg with 1ml of absolute ethanol and then stirring the mixture to make a homogeneous solution (stock solution). From the stock solution, volumes equivalent to the ones used for *M. volkensii* test substances were applied into a set of seven marked 250ml glass jars containing 40ml of distilled water. It was then assumed that these test solutions had the same

concentrations as the *M. volkensii* and azadirachtin A test substances. 15ppm test solution was not prepared.

2.3.4 A mixture of fraction B and Olive oil test solutions.

A standard (stock) solution of a mixture of fraction B and Olive oil was prepared by mixing equal volumes (1ml) of each stock solution and then stirring the mixture thoroughly to make a homogeneous solution (stock solution). From this stock, volumes equivalent to the ones used in section 2.3.1 were applied into a set of seven marked 250ml glass jars with 40ml of distilled water to make the desired concentrations as above. Controls in sections 2.3.3 and 2.3.4 were prepared in the same way as indicated in section 2.3.1 above.

2.4 TESTS

2.4.1 Evaluation of toxicity levels of compound D_2, fractions A, B, and 4:6&1:1, and azadirachtin A on the second instar larvae of *Ae. aegypti* in the laboratory.

Twenty second instar larvae were counted and introduced into each concentration of the test solutions for each test substance prepared as indicated in section 2.2. About 25mg of finely ground yeast was added into each container daily as larval food. The containers were then plugged with cotton wool to ward-off dust from the test solutions. The test larvae were continuously exposed to the test solutions (Hseich and

Steelman, 1974). Dead and/or moribund larvae were removed and counted after every 24 hours but death of moribund larvae was confirmed after 24 hours the next day before including them in the count. Change in size (instars) in the experimental and control groups were observed and noted. The number of emerging adults was also recorded.

Dead larvae were those that could not be induced to move when probed, whereas moribund ones were moving but incapable of rising to the surface or showing characteristic movements when the test solutions were agitated.

When the control mortalities above 20% were recorded, the tests were discarded. However, if control mortalities ranged between 5 and 10%, such control percentage mortalities were included in probit analysis for corrections by Abbott's formula (AF) as follows:

$$AF = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

For every data set, values between 0 and 100% were analysed by computer probit analysis regression program on IBM computer adapted by Finney (1971).

During the dosage - mortality tests, dead intermediate stages of development such as moulting larvae, larval-pupal intermediates, pupal-adult intermediates and dead pupae resulting from each concentration of the test substance were separated from the dead larvae and recorded after every 24 hours.

Percentage total mortality for each test substance was also calculated by taking into consideration the mortalities in the dosage-mortality tests for all the stages which occurred during the experiment until the emergence of normal adults. Whenever the volume of the test solutions in the glass jars was lower than 40ml and 10ml for *M. volkensii* test substances and azadirachtin A respectively, it was adjusted by addition of distilled water to the required level. A minimum of four replications were done for each concentration of the test solutions and the control batch.

2.4.2 Determination of the effects of *M. volkensii* test substances and azadirachtin A on growth and development of the second instar larvae of *Ae. aegypti* until the emergence of adults.

To determine if any of the test substances had any effect on the rate of larval development upto the emergence of adults, results of the concentrations of the test substances in the above dosage-mortality tests (section 2.4.1) showing growth inhibition effects were identified. The tests were then repeated at the same time using the concentrations which showed some growth inhibition effects, with the second instar larvae of the same age and from eggs of the same batch. A control was also set up for comparison.

Each particular developmental stage was attributed an arbitrary multiplication factor (F_p),

representing it, namely, second instar larvae 2; third instar larvae 3; fourth instar larvae 4; and the pupal stage 5. The relative development stage (rDS) of the population at each day of observation (t) was then calculated daily for each test substance and the control until the emergence of adults by the following formula:

$$\frac{(n_t S_p \times F_p)}{N_t S} = rDS$$

where $n_t S_p$ is the number of a particular developmental stage living at that time of observation, $N_t S$ means the total number of all stages living on the day of observation t and F_p means the particular arbitrary multiplication factor mentioned above.

Example: (Day X) 10 second instars x 2 = 20
80 third instars x 3 = 240
10 fourth instars x 4 = 40
0 pupal stage x 5 = 0

100 live instars divided into 300 = 3

or the mean developmental stage in this population is the third instar (Zebitz, 1984). A minimum of four replicates was done.

2.4.3 Formulation of pellets from acetone extract fraction of *M. volkensii* and studying their effects on the third and fourth instar larvae of *Ae. aegypti*.

Five grams of jelly crystals which is usually used for preparing pudding was poured into a

100ml beaker containing 80ml of hot water. The mixture was stirred to make a hot jelly solution. The hot solution was then transferred into a 10cm (diameter) glass petri-dish. While the solution was still hot in the petri-dish, 1.0g of acetone extract fraction powder from *M. volkensii* was sprinkled on top of the solution and left to settle. After about 48 hours, there were some solid/crystal like particles formed above the solution. The solid/crystal like particles were removed with aid of a plastic spatula and put in another glass petri-dish of the same size to harden. The thick jelly like solution was then discarded. After hardening, the solid/crystal like particles were ground into fine particles by the use of a blender. The active ingredient (acetone extract fraction) in these fine particles was partly coated with jelly. The coating procedure above was repeated by sprinkling the fine partly jelly coated particles on top of freshly prepared hot jelly solution to ensure that coating was fully done. The resulting solid/crystal like particles were then mixed with 3.5g yeast granules and ground again so that the final wholly jelly coated acetone extract fraction fine particles were having a second thin coat of yeast powder around them. The resulting fine double coated particles were now mixed with some free yeast particles (acting as a spreading agent) thus resulting into newly formulated pellets ready for testing. Control pellets were prepared by mixing the jelly crystals with the yeast

granules and then ground to give fine particles which could be easily applied on water.

The newly formulated acetone extract fraction pellets and the control pellets were then applied as larval food on alternate days on top of 200ml of water in two different marked metallic trays measuring 12x18x4cm, with each having one hundred larvae starved for twenty four hours.

Dead larvae, pupae or larval-pupal intermediates were removed from the trays and counted after every 24 hours. Normal pupae were removed from the trays and put into marked glass jars containing 50ml of tap water and plugged with cotton wool. The number of adults emerging from each glass jar was recorded daily. Dead pupal-adult intermediates were removed from the jars and their number recorded. Any abnormal observation in behaviour (e.g. resting position and movements) of the larvae was also recorded. A minimum of four replications were done for both the control and the test pellets.

During formulation, acetone extract fraction was used because it was found to form solid/crystal like particles easily when sprinkled on top of the hot jelly solution and also from the bioassay tests on the second instar larvae of *Ae. aegypti*, it has been shown to contain an active fraction which is toxic against the larvae (Mwangi and Rembold, 1988; Al-Sharook *et al.*, 1991). Though fractions B and 4:6&1:1 are very toxic, they were not miscible with the hot jelly solution

easily to form solid/crystal like particles as was in the case with acetone extract fraction. This might be due to the high lipophilic nature of these toxic fractions (Mwangi, 1989). The acetone extract had to be coated with the jelly to prevent the antifeedant effect present in the fruit extracts of *M. volkensii* (Mwangi, 1982), which was likely to repel the larvae.

Yeast was used as a spreading agent in this case because it was found to be the material which could spread and settle in water best. Also the thin yeast powdery layer which formed around the jelly coated pellet was looking brighter in water and attractive to the hungry larvae hence leading to quick feeding and swallowing of the pellets in the process.

2.4.4 Determination of the effect of fractions and compound D_2 from *M. volkensii*, and azadirachtin A on hatchability of *Ae. aegypti* eggs.

From the Whatman No. 1 filter papers containing three month old eggs, two hundred eggs were counted under a dissecting microscope and lines were then drawn with a pencil to mark their boundaries. Sections of the filter papers containing the eggs were then cut off using a pair of scissors and placed into marked randomly selected 9.0cm (diameter) sterile plastic petri-dishes containing 40ml of 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0ppm of the test solutions from *M. volkensii* test substances. For azadirachtin A, sections

of the filter papers containing the same number of eggs as above were placed into marked randomly selected 5.5cm (diameter) glass petri-dishes containing 10ml of the test solutions having the same concentrations as indicated above. Control batches were also set up for each test substance. The glass and plastic petri-dishes were then covered with their respective lids to avoid evaporation of the test solutions. All the test solutions and controls were prepared as indicated in section 2.2 above.

Hatched larvae were removed from the different concentrations of each test substance and the control and counted after every two hours for the first twelve hours. For the second day (after 24 hours), the hatched larvae were removed and counted after every eight hours, and for the third day (72 hours) removal and counting was done after 24 hours. After the third day (72 hours), there were no more larvae hatching. This removal and counting procedure was adapted because there was reduction in the number of larvae hatching with increasing time.

The hatching rate was then recorded with the increasing concentration of each test substance. In all cases, after counting, the hatched larvae were placed into different marked metallic tray measuring 27x35x5cm containing 300ml of tap water and reared as indicated in section 2.3 above until the emergence of adults so that their survival rate could be determined. Dead pupae,

larval-pupal and pupal-adult intermediate stages were removed and counted. Two replicates were done for each of the test substances and for the control.

2.4.5 Investigation of oviposition deterrency effect in the *M. volkensii* test substances on adult *Ae. aegypti* females.

In order to find out if any of the test substances from *M. volkensii* had oviposition deterrency effect on adult *Ae. aegypti* females, twenty adult females and ten adult males, laboratory reared (as indicated in section 2.3) from the same batch of eggs and seven to ten days old (Angerilli, 1980), were released into the five marked mosquito net cages measuring 30x30x30cm. Exposed head of a live rabbit enclosed in a 20x35x15cm wooden box was inserted into each of the net cages for three hours as a source of bloodmeal for the females. Thereafter a flask with a cotton wool wick soaked in a 10% sucrose solution was made available in each of the net cages to act as a source of energy. After three days, two 9.0cm (diameter) sterile plastic petri-dishes containing 8.5cm Whatman No.1 filter papers soaked in 50.0ppm solutions of *M. volkensii* test substances were placed into each of the corresponding marked net cages to act as an oviposition substrate for mature females. The petri-dishes containing the eggs were removed after every two days and new ones placed in the cages.

The marked filter papers containing the eggs were

then removed from the petri-dishes and placed in different marked trays with slightly wet sheets of cotton wool spread on the bottom to absorb the test solutions so that the filter papers can dry. After drying the filter papers, the number of eggs laid on each filter paper for each test substance were counted with the aid of a dissecting microscope and recorded. The tests were then repeated using higher concentrations (i.e. 100.0 and 200.0ppm) of the *M. volkensii* test substance until a reduction in oviposition by 50% or more was observed in all the test substances. A control batch was also set up for comparison.

After finding out the concentration at which all the solutions of the *M. volkensii* test substances reduces oviposition by 50% or more, a choice test was then performed as follows: In each marked net cage, two marked petri-dishes were placed in the middle. One of the petri-dishes was containing a filter paper soaked in 15ml of distilled water mixed with 80µl of absolute ethanol (control) while the other had a filter paper soaked in 15ml of the concentration of the test substance which deterred oviposition by 50% or more. Whenever the petri-dishes were removed after every two days from the net cages in order to dry the filter papers and then count the number of eggs laid, the positions of the newly placed control and test substances petri-dishes were reversed inside the net cages to avoid oviposition selection behaviour of the

laying female mosquitoes. After counting, the number of eggs laid in the control and the experimental batches was recorded. All the test solutions and the controls were prepared as indicated in section 2.2 above. For each test substance and control, four replicates were done. Chi-square (X^2) test was performed to find out if there was any significant difference between the experimental and control batches.

2.4.6 Determination of any ovicidal effect on the eggs of *Ae. aegypti* laid in the highest (200.0ppm) concentration of *M. volkensii* test substances which deterred oviposition by 50% or more.

From the marked dry Whatman No. 1 filter papers containing the eggs which were laid in the highest concentration, 200.0ppm (oviposition deterrent solution) of the *M. volkensii* test substances in section 2.4.5 above, four hundred eggs were counted and sections of the filter papers containing the eggs were cut off following the procedure described in section 2.4.5 above. The portions of the filter papers containing eggs from different *M. volkensii* test substances were then soaked in different marked 27x35x5cm metallic trays containing 300ml of tap water to hatch.

The number of hatched larvae were counted after every twenty four hours and recorded until there were no more larvae hatching. The hatched larvae were then reared as indicated in section 2.3 above until the

emergence of adults. Dead pupae, larval-pupal and pupal-adult intermediate stages resulting during the rearing period were removed and counted. A control was also set up for comparison. A minimum of two replicates were done for each test substance and the control.

2.4.7 Limited field trial studies within Chiromo campus.

For this study, fraction B from *M. volkensii* was used as a test substance of choice. Four marked five litre tin cans were set up and into each tin, one litre of rain water was poured. From the stock solution (100mg/ml) of fraction B, 250, 500 and 1000µl were applied into the corresponding marked tins to make final concentrations of 25, 50 and 100ppm respectively. For the control 1000µl of absolute ethanol was applied since it was equivalent to the maximum volume of the solvent present in the highest concentration (100ppm) of the test solution.

After mixing each test solution thoroughly, the tins were then transferred to the field trial study area situated in the eastern side of Chiromo campus (Figure 3). Tins with each concentration of the test substance solutions and the control were placed in different sites under the banana plants. This area was chosen for the experiment because the preliminary studies which were carried out earlier on, revealed that it was a favourable site for mosquito breeding in Chiromo campus.

The tins were sampled after every four days and

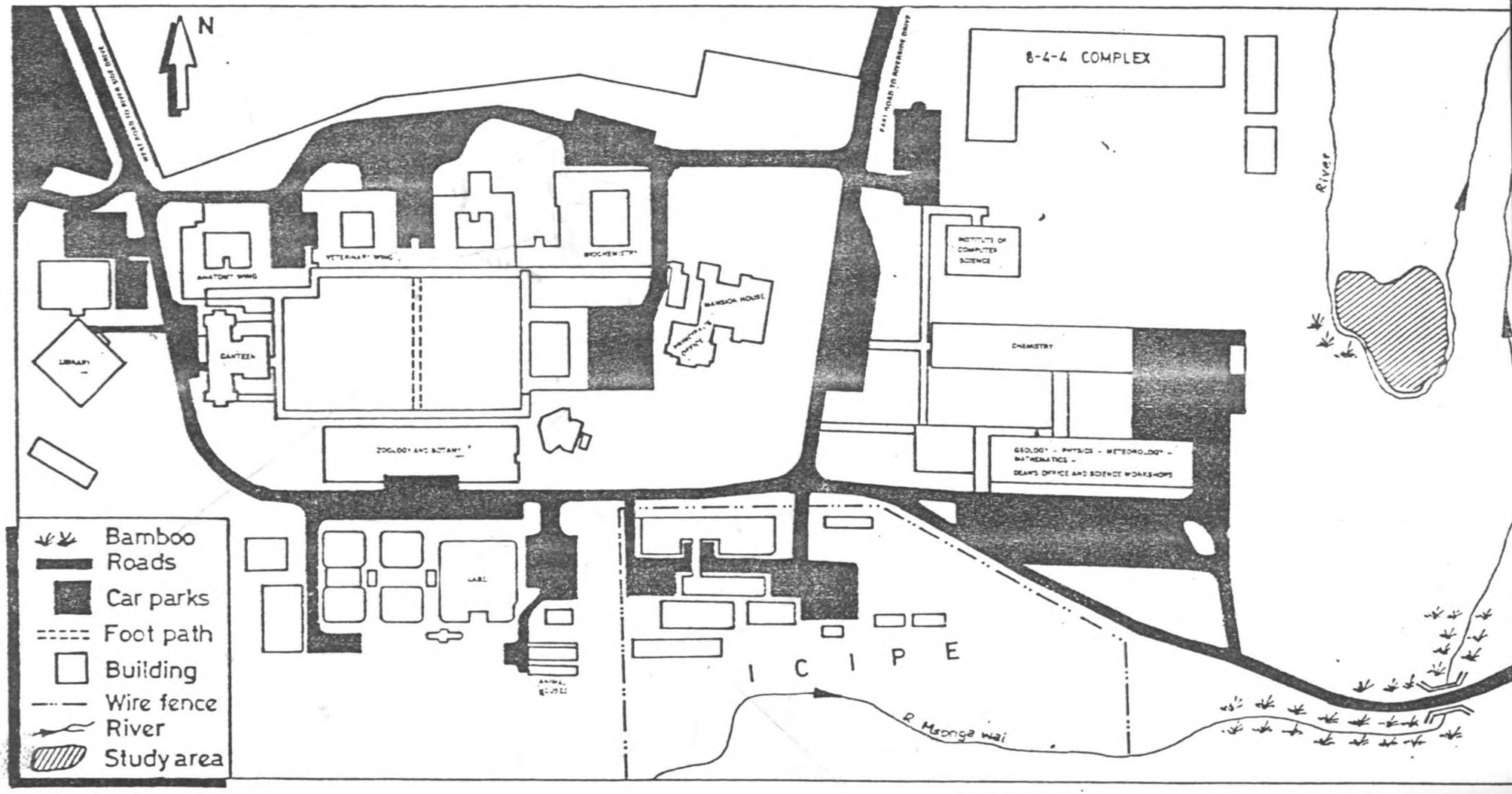


Figure 3: Chiromo Campus (after Kiongo, 1976).

the number of different developmental stages (instars) present was estimated. The estimation of different developmental stages was done by using a small soup ladle of 9cm in diameter and holding 70ml of water, and similar to the one developed by Service (1968). The soup ladle was dipped into each tin and the number of various larval developmental stages present in 70ml of water in it was counted and recorded. This procedure was repeated five times per tin and the mean number of each developmental stage was calculated. The number of larval developmental stages present in 1000ml of the test solutions in each tin was then approximated as indicated below.

Assuming that the larvae in the tins were uniformly distributed and the mean number in 70ml of water in the soup ladle for the first, second, third, and fourth instar larvae is X_1, X_2, X_3 and X_4 respectively; their corresponding number in each tin (1000ml of the test solution) will therefore be $1000/70 \times X_1$, $1000/70 \times X_2$, $1000/70 \times X_3$, and $1000/70 \times X_4$ respectively. The number of pupae in each tin was determined directly by physically counting the pupae present on each sampling day. The number of emerged adults was determined by removing and counting of the exuviae (pupal skin) left in the tins by the emerging adults. The above method for estimating the number of various larval developmental stages present in each concentration was adapted because there were numerous

larvae in most of the tins therefore making physical counting impossible. For each concentration and the control, four replicates were done.

In order to identify the mosquito species which were available during the study time, separate tins with rain water only were placed in different sites for the mosquitoes to oviposit in. The tins were then checked also after every four days for the presence of larvae. After noticing the larvae, the tins were taken to the laboratory and the larvae reared as indicated in section 2.3 until the emergence of adults. The different species of emerged adults were preserved by pinning in a 14x14x5cm insect box for identification.

The inner surfaces of all the tin cans used in this experiment were painted to avoid rusting which was likely to cause corrosion and therefore result into leaking of the test solutions. Also the toxic substances resulting from rusting could have killed the larvae.

The biodegradation period of fraction B was also determined by finding out after how long a particular concentration would take to give results similar to the ones of the control or a lower concentration on any day of sampling.

CHAPTER THREE

RESULTS

3.1 The effects of the solutions of *M. volkensii* test substances and azadirachtin A on the second instar larvae of *Ae. aegypti* upto the emergence of adults.

3.1.1 Evaluation of toxicity levels.

3.1.1.1 Toxicity levels after 48 hours.

Table 1 presents results of toxicity bioassays against second instar larvae of *Ae. aegypti* after 48 hours. 100% mortality was attained in fractions B and 4:6&1:1 at concentrations 100 and 200ppm. Fraction A and compound D_2 caused 100% mortality at only 200ppm while at 100ppm they caused mortality rates of 89% and 53% respectively. At 50ppm the mortality rates of fractions A, B, 4:6&1:1, and compound D_2 were 37%, 95%, 90%, and 47% respectively. There was no concentration of azadirachtin A which caused 100% mortality after 48 hours. Mortality rates in higher concentrations of azadirachtin A test solutions were lower than 50% and were as follows: 32% in 20, 50 and 100ppm, and 37% in 200ppm. Some mixtures of *M. volkensii* fractions were bioassayed in order to investigate for any cases of synergism. A mixture of fractions (A+B) caused

Table 1. Percentage mortality (after correction by Abbott's formula) of the second instar larvae of *Ae. aegypti* in various concentrations of the test substances after 48 hours. Mixtures used were always in a ratio of 1:1 (v:v) (n=20).

Conc. (ppm)	Percentage mortality								
	F_A	F_B	F_{A+B}	$F_{4:6\&1:1}$	$F_{4:6\&1:1+B}$	CD_2	Olive oil	Olive oil + B	Azad. A
1	0	0	0	5	5	0	0	0	0
5	0	5	0	30	10	0	0	0	0
10	0	10	0	55	35	0	0	0	5
15	0	30	11	55	50	5	-	-	21
20	11	65	5	75	90	11	5	5	32
50	37	95	53	90	100	47	5	16	32
100	89	100	63	100	100	53	0	47	32
200	100	100	100	100	100	100	10	74	37

Conc. - Concentration
Cont. - Control
\- — Concentration not tested
Azad. A - Azadirachtin A

F_A - Fraction A
F_B - Fraction B
F_{A+B} - Fractions (A+B)
Olive oil+B - Olive oil+fraction B

$F_{4:6\&1:1}$ - Fraction 4:6&1:1
$F_{4:6\&1:1+B}$ - Fractions (4:6&1:1+B)
CD_2 - Compound D_2

100% mortality at 200ppm, 63% mortality at 100ppm and 53% mortality at 50ppm. However, a mixture of fractions (4:6&1:1+B) caused higher mortality rates of 100% at concentrations of 50ppm and above. With the exception of fraction A, a mixture of fractions (A+B), compound D_2 and azadirachtin A, mortality rates of above 50% were achieved at 20ppm in the other test substances as follows: 65% in fraction B, 75% in fraction 4:6&1:1, and 90% in a mixture of fractions (4:6&1:1+B). With fraction 4:6&1:1, 50% mortality was obtained at a concentration of 10ppm while in a mixture of fractions (4:6&1:1+B), 50% mortality was achieved at a concentration of 15ppm.

Presented in Table 2 and Figures 4-7 are probit analysis results of susceptibility of the second instar larvae of *Ae. aegypti* to various concentrations of the test substances after 48 hours, for the purpose of ascertaining the lethal concentrations 50 (LC_{50}) and 95 (LC_{95}). The values of the slope (b) and its standard error (S. E.) are given for each test substance and are components of the regression equation $y = a + bx$. Student's, t, values for the slopes are also given. Y = the probit value which is a measure of percentage mortality at concentration x, expressed on logarithmic scale. From the equation it was possible to calculate the concentration of a test substance corresponding to particular percentage mortality of the mosquito larvae.

This concentration was the lethal concentration (LC). Specific percentage mortalities, for example, at 50% and 95% corresponded to LC_{50} and LC_{95} respectively. Table 2 also shows 95% confidence limits (C. L.) attached to LC_{50} at 95%, the calculated LC_{95} from the regression equation of the probit line from the intercept, (a) and (b) values.

The LC_{50} values of *M. volkensii* test substances ranged from moderate to high toxicity when compared with that of azadirachtin A which was very low. Fraction 4:6&1:1 was the most toxic with LC_{50} value of 9.4ppm (95% C. L. range 6.8-13.0ppm) while the LC_{95} value was 68.7ppm. The next toxic fraction was B which had LC_{50} value of 18.2ppm (95% C. L. range 15.1-22.0ppm) while the LC_{95} value was 49.1ppm. Fraction A and compound D_2 were fairly toxic with LC_{50} values of 44.5ppm (95% C. L. range 33.3-59.3ppm) and 55.3ppm (95% C. L. range 38.9-78.7ppm) respectively. Their LC_{95} values were very high, i. e. greater than 200ppm (267.9ppm for fraction A and 497.6ppm for compound D_2). The LC_{50} value of azadirachtin A was higher than 200ppm (282.9ppm) and had a very wide 95% C. L. (Table 2).

A mixture of fractions (A+B) had high LC_{50} value of 48.6ppm with 95% C. L. range 35.1-67.1, greater than that of either fractions A or B separately showing lowered activity of fraction B. This lowering of the activity (toxicity) of fraction B by fraction A might

Table 2: Susceptibility of the second instar larvae of *Ae. aegypti* to various concentrations of the test substances after 48 hours (n = 20).

Test substance	LC_{50}(ppm)	95% confidence limit for LC_{50}(ppm)	LC_{95}(ppm)	Intercept	Slope ± S.E.	t value	d.f.
Fraction A	44.5	33.3 - 59.3	>200(267.9)	1.5	2.1 ± 0.3	7.202*	6
Fraction B	18.2	15.1 - 22.0	49.1	0.2	3.8 ± 0.7	5.892*	6
Fractions (A+B)	48.6	35.1 - 67.1	>200(386.5)	1.9	1.8 ± 0.3	6.894*	6
Fraction 4:6 & 1:1	9.4	6.8 - 13.0	68.7	3.2	1.9 ± 0.3	6.687*	6
Fractions (4:6 & 1:1 + B)	11.2	8.8 - 14.2	44.9	2.2	2.7 ± 0.5	5.924*	6
Compound D_2	55.3	38.9 - 78.7	>200(497.9)	2.0	1.7 ± 0.3	6.667*	6
Olive oil	>200($3.1x10^7$)	-	>200	3.3	0.2 ± 0.2	1.040	5
Fraction B + Olive oil	114.3	63.5 - 205.6	>200	2.4	1.3 ± 0.3	5.134*	5
Azadirachtin - A	>200(282.9)	66.8 - >200	>200	3.4	0.7 ± 0.2	3.692*	6

* Significant at probability, p less than 0.05

d.f. = degrees of freedom.

have been due to the oily nature of fraction A which could have reduced solubility of fraction B thus preventing homogeneous dissolution in water. This assumption was confirmed when fraction B was mixed with a neutral oil, Olive oil, and LC_{50} value of 114.3ppm was obtained which shows lowered activity of fraction B. Olive oil alone caused a very low mortality rate of only 10% in the highest test concentration, 200ppm, after 48 hours (Table 1). It had a very high LC_{50} value, 3.1×10^{7}ppm, which was far much greater than 200ppm. Its slope, b, was 0.2 and did not differ significantly from 0 statistically, $t = 1.040$; d.f. = 5; $p > 0.05$ (Table 2). When a statistical test, analysis of covariance (Zar, 1984) was performed to find out if there was any significant difference between the slopes of fraction B, Olive oil, and a mixture of fraction B + Olive oil (Figure 4), the results revealed a significant difference between the slopes, $F = 5.876$; d.f. = 2, 16; $p < 0.05$. When a multiple comparison range test, the Tukey test, was performed to determine which slope(s) were different from the others, it was found that the slope of fraction B (3.8) was significantly different from that of Olive oil (0.2), q (Studentized range) = 3.701; d.f. = 3,16; $p < 0.05$. But there was no significant difference statistically between the slopes of fraction B and a mixture of fraction B + Olive oil, and Olive oil and a mixture of fraction B + Olive oil, q

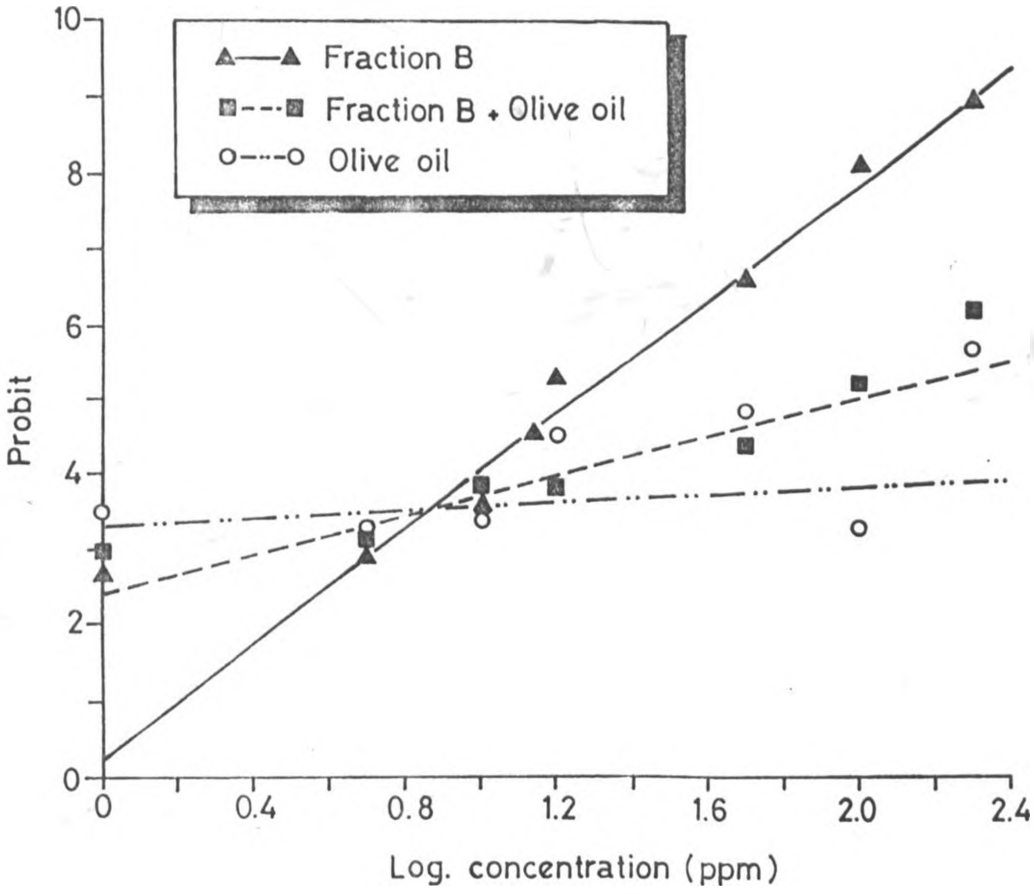


Figure 4: Log-concentration/probit mortality regression lines for *Ae. aegypti* second instar larvae treated with various concentrations of fraction B from *M. volkensii*, Olive oil, and fraction B + Olive oil (n=20).

= 2.630; d.f. = 3, 16; $p > 0.05$ and q = 0.341; d.f. = 3, 16; $p > 0.05$ respectively.

A mixture of fractions (4:6&1:1+B) had LC_{50} value of 11.2ppm (95% C. L. range 8.8-14.2ppm) which was almost equivalent to the mean LC_{50} (13.8ppm) of the two fractions when separate but slightly greater than that of fraction 4:6&1:1 alone and lower than that of fraction B. The LC_{95} for the mixture of fractions (4:6&1:1+B) was 44.9ppm. A statistical test which was performed to find out if there was any difference between the slopes of *M. volkensii* test substances and azadirachtin A (Figure 5) revealed that there was no significant difference between the slopes, F = 2.551; d.f. = 4, 30; $p > 0.05$. The same relationship was also observed in the mixtures where it was found that there was no significant difference between the slopes of fractions A, B, and a mixture of fractions (A+B) (Figure 6), F = 1.463; d.f. = 2, 18; $p > 0.05$. Similarly there was also no significant difference between the slopes of fractions 4:6&1:1, B and a mixture of fractions (4:6&1:1+B), F = 0.307; d.f. = 2, 18; $p > 0.05$ (Figure 7).

3.1.1.2 Total mortality.

Results presented in Tables 3 and 4, and shown graphically in Figure 8 indicate the percentage total mortalities. The percentage total mortality of various developmental stages are shown in Tables 4, 5 and 6, and

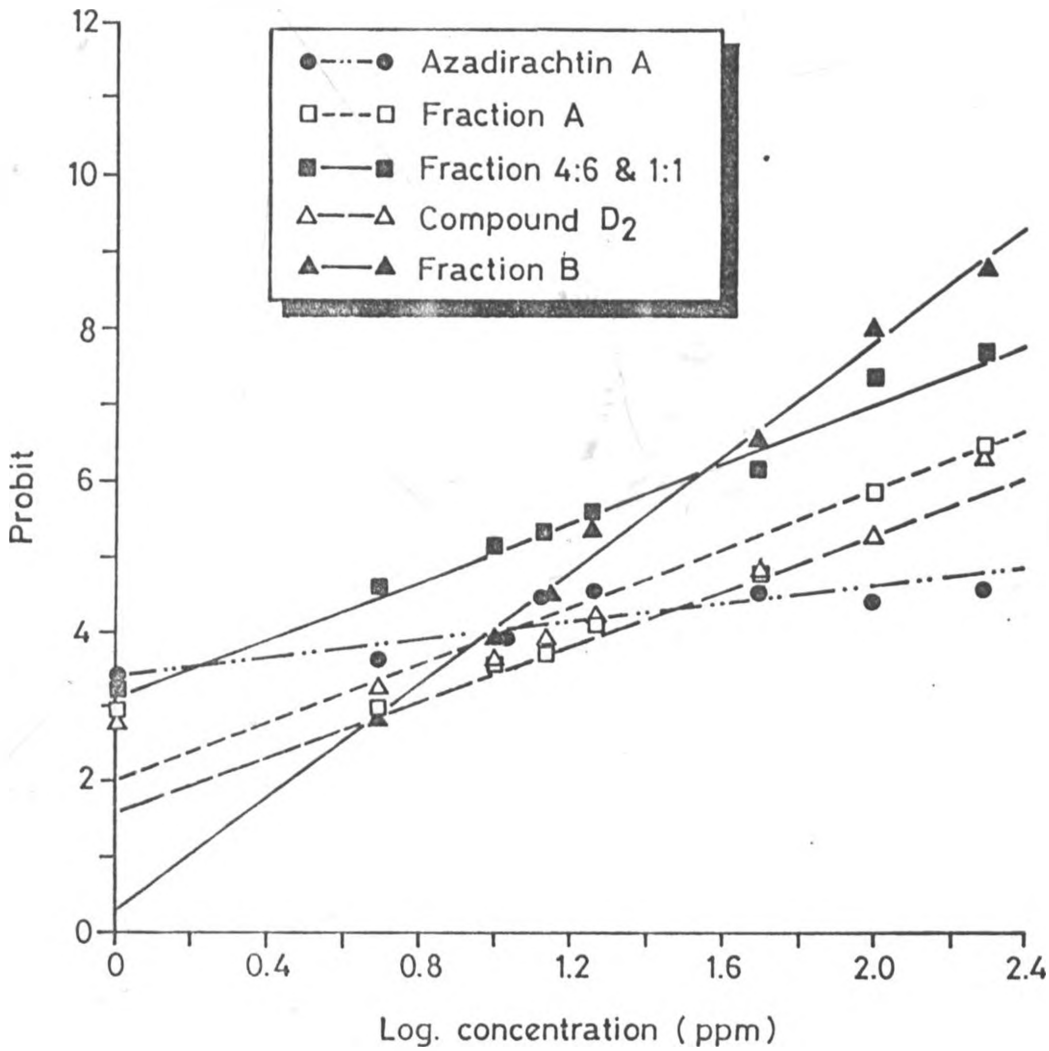


Figure 5: Log-concentration/probit mortality regression lines for *Ae. aegypti* second instar larvae treated with various concentrations of fractions A, B, and 4:6&1:1 and compound D_2 from *M. volkensii*, and azadirachtin A (n=20).

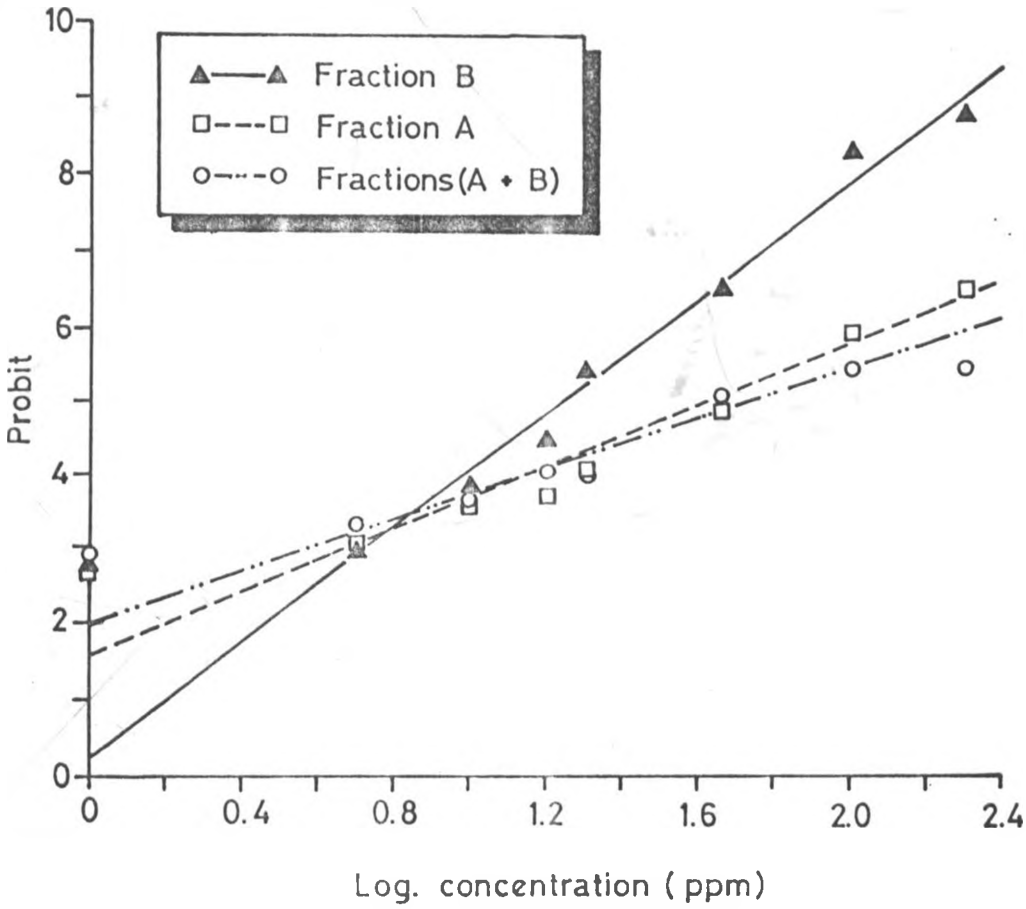


Figure 6: Log-concentration/probit mortality regression lines for *Ae. aegypti* second instar larvae treated with various concentrations of fractions A and B, and a mixture of fractions (A+B) from *M. volkensii* (n=20).

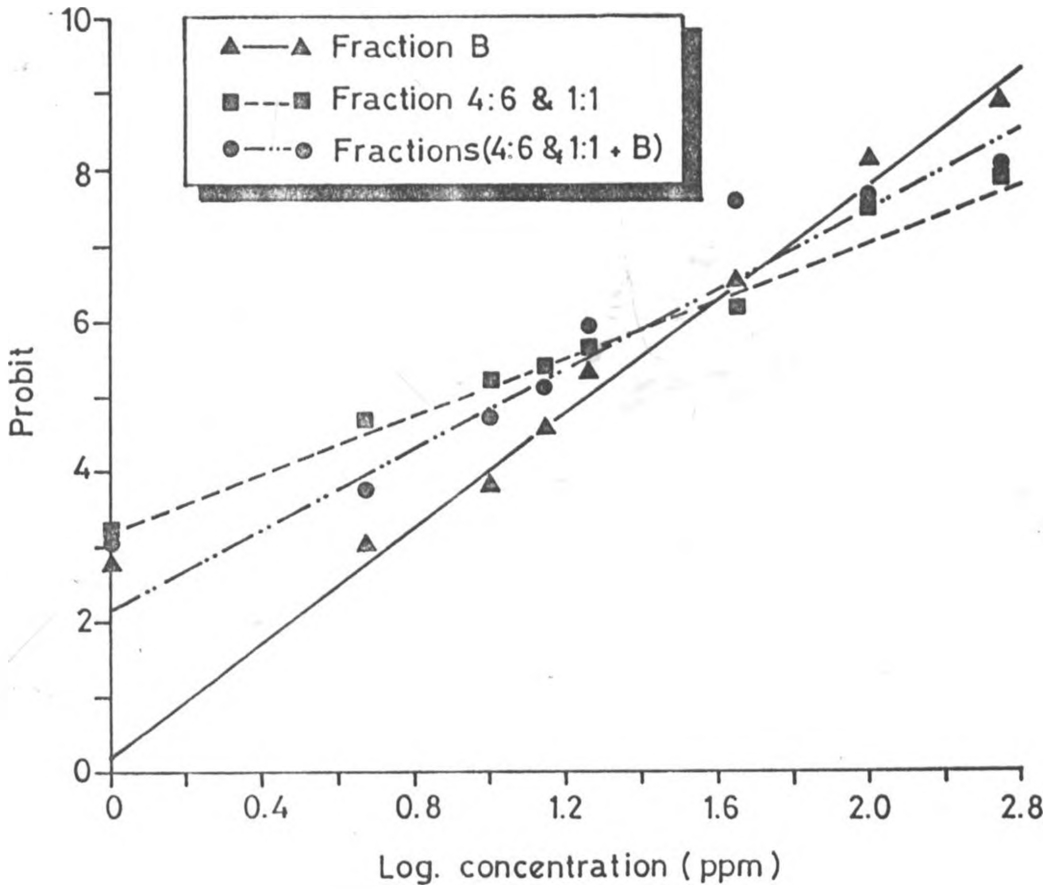


Figure 7: Log-concentration/probit mortality regression lines for *Ae. aegypti* second instar larvae treated with various concentrations of fractions B and 4:6&1:1, and a mixture of fractions (4:6&1:1 + B) from *M. volkensii* (n=20).

graphically represented in Figures 9 and 10.

When total mortality was used as a criterion for the effects of the test substances on the larvae over a period of time (Table 3 and Figure 8), it was found that in the test solutions of azadirachtin A at concentrations 5-200ppm, total mortality was high (100%) and occurred mostly in the larval stages. At 2.5ppm, azadirachtin A caused a total mortality of 60% with the larval stage comprising only 25% (Table 4), while at 1ppm the total mortality was 53% with the total mortality at larval stage being 38% (Table 5 and Figure 9). All the *M. volkensii* test substances caused total mortality rates of 100% at concentrations 50-200ppm (Table 3 and Figure 8) which occurred exclusively in the larval stage (Table 5 and Figure 9). At 20ppm, almost all the *M. volkensii* test substances still caused total mortality rates of 100% apart from fraction A which caused a total mortality rate of 47%. At concentrations of 10 and 15ppm, with the exception of fraction A which caused again lower total mortality rates of 11% and 21% respectively, other *M. volkensii* test substances caused total mortality rates greater than 50%. But surprisingly, the total mortality rate of azadirachtin A test solutions at the lowest concentration tested, 1ppm, was greater than those of all the *M. volkensii* test substances at the next concentration of 5ppm (Table 3 and Figure 8).

Table 3: Percentage total mortality (after correction by Abbott's formula) of the various developmental stages of *Ae. aegypti* in different concentrations of the test substances (n=20).

Test substance	% total mortality of various stages							
	Concentration in ppm							
	1	5	10	15	20	50	100	200
Fraction A (23)	11	11	11	21	47	100	100	100
Fraction B (24)	0	11	58	79	100	100	100	100
Fractions (A + B) (32)	16	37	68	84	95	100	100	100
Fraction 4:6&1:1 (8)	0	32	89	95	100	100	100	100
Fractions (4:6&1:1 + B) (8)	0	26	79	100	100	100	100	100
Compound D_2 (20)	21	21	63	84	100	100	100	100
Azadirachtin A (30)	53	100	100	100	100	100	100	100

Values in parentheses indicate the time (in days) when percentage total mortality of the various developmental stages was determined (Cf. Tables 1 and 5).

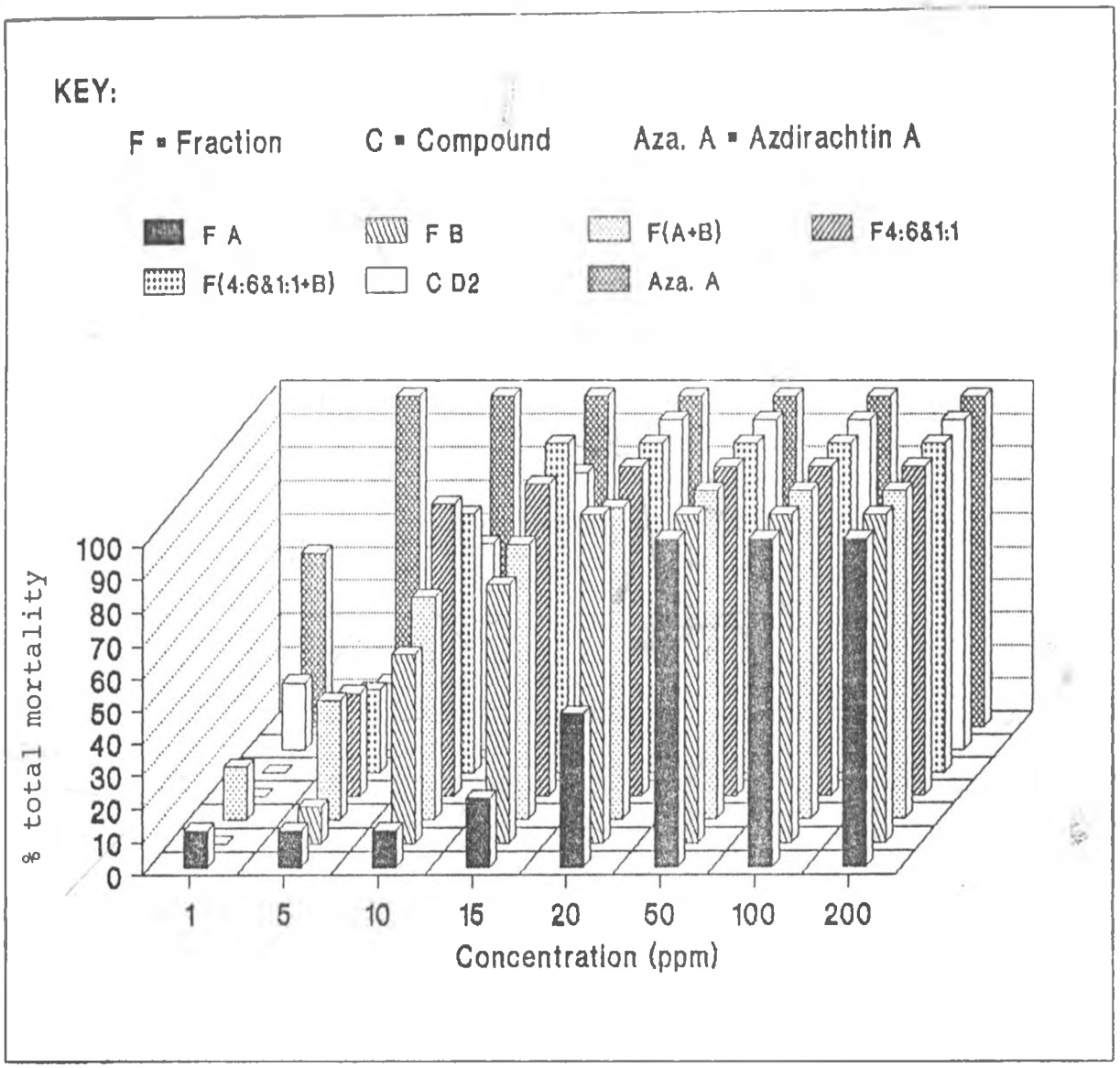


Figure 8: Percentage total mortality (after correction by Abbott's formula) of various developmental stages of *Ae. aegypti* in different concentrations of the test substances (n=20). Time span is indicated in Table 3.

Table 4: The effect of azadirachtin A (Aza. A) at 2.5ppm on various developmental stages of the second instar larvae of *Ae. aegypti* after 35 days (n = 20).

	Percentage total mortality				% total of emerged adults
			Intermediate stage		
	larval	Pupal	4th instar larvae-pupal	pupal-adult	
Control	10	0	0	0	90
Aza. A	25	20	10	5	40

n = number of second instar larvae used per replicate for each test.

Table 5: Percentage total mortality (after correction by Abbott's formula) of *Ae. aegypti* larval instars in different concentrations of the test substances (n=20).

Test substance	% total mortality of the larval instars							
	Concentration in ppm							
	1	5	10	15	20	50	100	200
Fraction A (23)	6	6	6	6	26	72	100	100
Fraction B (24)	0	6	11	32	79	100	100	100
Fractions (A + B) (32)	11	11	16	21	37	100	100	100
Fraction 4:6&1:1 (8)	0	26	79	89	100	100	100	100
Fractions (4:6&1:1 + B) (8)	0	6	32	63	100	100	100	100
Compound D_2 (20)	11	11	42	47	63	100	100	100
Azadirachtin A (30)	38	95	100	100	100	100	100	100

Values in parentheses indicate the time (in days) when percentage total mortality of the larval instars was determined (Cf. Tables 1 and 3).

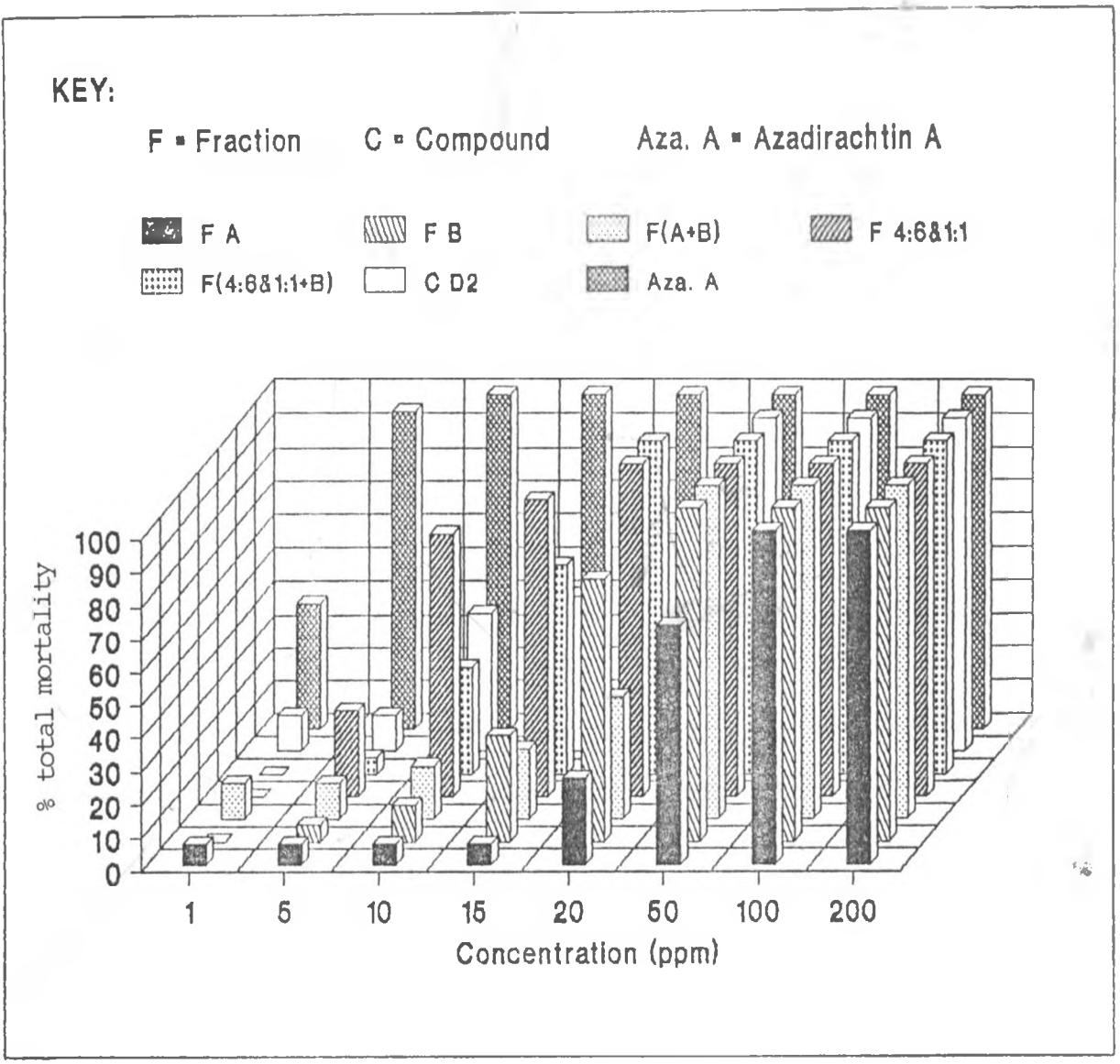
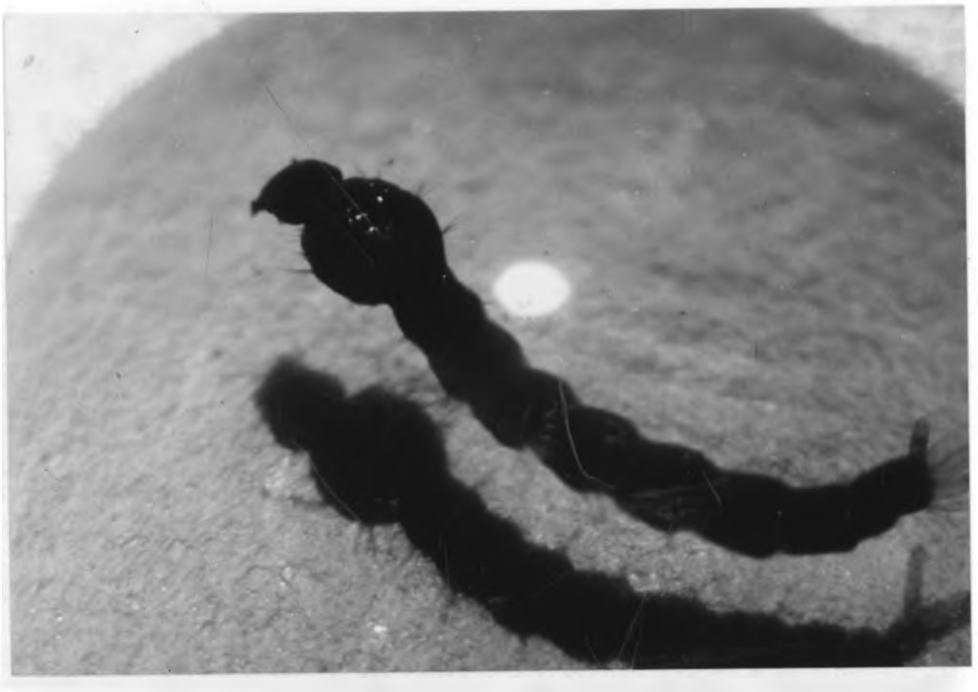


Figure 9: Percentage total mortality (after correction by Abbott's formula) of *Ae. aegypti* larval instars in different concentrations of the *M. volkensii* and azadirachtin A test substances (n=20). Time span is indicated in Table 5.

3.1.2 Morphogenetic effects of *M. volkensii* test substances and azadirachtin A on the second instar larvae of *Ae. aegypti* from the start of exposure in the test solutions until the emergence of adults.

3.1.2.1 Morphogenetic effects in the larval stages.

Most of the larvae which were dying in the test solutions of *M. volkensii* and azadirachtin A exhibited different types of features. In azadirachtin A test solutions at concentrations of 5-20ppm, majority of the dead larvae were moulting from second to third instar. Such larvae had the moulted exuviae pushed over the thorax region (Plates 1 (a)) and very few larvae had parts of the moulted head capsule remaining attached to the new one (Plate 1 (b)). However, the larvae which were dying in the later stages in these test substances of azadirachtin A were moulting from third to fourth instar and they had a gap between the head and thorax (Plate (1 (c)). The larvae which were dying in the solutions of *M. volkensii* test substances exhibited the three features above which were observed in the test solutions of azadirachtin A (Plates 1 (a), (b) and (c). In some cases the dead larvae in the solutions of *M. volkensii* test substances had the moulted skin from the head region floating on water while the lower part of it was still attached to the larval siphon (Plate 1(d)). Plate 1 (e) (larvae marked I) show normal live third and fourth instar larvae which did not exhibit any of the



a

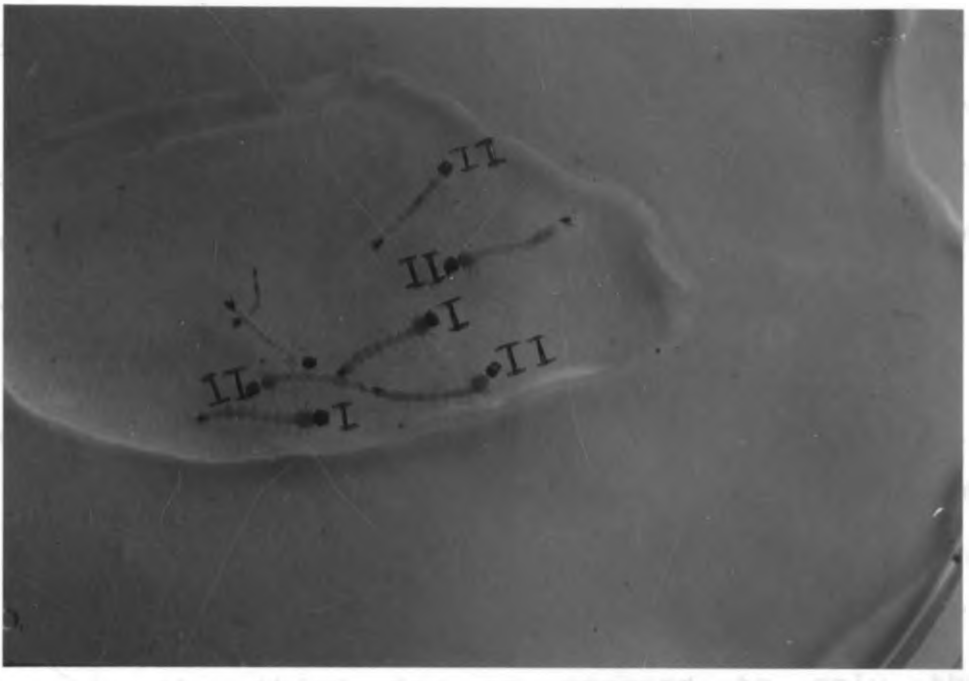
b

(Plate 1 cont.).

c

d

(Plate 1 cont.).



e

features mentioned above. The very few larvae which were dying in the controls had a very thin gap between the head-thorax region (Plate 1 (e) larvae marked II). This gap was not very conspicuous as it was in the larvae which were dying in the various concentrations of either azadirachtin A or *M. volkensii* test substances. These larvae which were dying in the controls, died mostly in the second instar, that is, 48 hours after the start of experiments.

18 unmelanised larvae were observed in the test solutions of azadirachtin A in concentrations of 5-15ppm on the third and fourth days after treatment, and in 20ppm on the third day as opposed to only three unmelanised larvae which were observed in the solutions of *M. volkensii* test substances (from the test solutions of compound D_2 only at 15ppm). These unmelanised larvae were able to survive for a short period of time before they died though the duration was not determined. Sluggishness and twisted S or U shaped resting postures were observed in the larvae in azadirachtin A test solutions at concentrations 10 and 15ppm on the ninth day after treatment while in 20ppm these characteristics were observed on the tenth day in the majority of larvae. In the solutions of *M. volkensii* test substances, these characteristics were eminent in a mixture of fractions (A+B) at 20ppm on the third day after treatment while in compound D_2 at 20ppm, they were observed on the second day and in 50ppm on the fourth

day. In fraction A at 50ppm, these characteristics were observed as early as after 24 hours only. There were no larvae showing such characteristics in fractions 4:6&1:1 and B.

3.1.2.2 Intermediate stages of development.

During the dosage mortality tests, various categories of intermediate stages were observed in the solutions of the test substances at lower concentrations only. The intermediate categories which were observed included partly escaped pupae with pupal abdomen, but head of a larva with the moulted larval skin still attached to it (Plate 2); dead unmelanised pupae (Plate 3); partly melanised pupae (Plates 4); dead melanised late pupae stage (Plate 5); adults partly escaped from the pupal exuviae (Plate 6, pupal-adults labelled I) and adults largely escaped but with some appendages still attached to the pupal exuviae (Plate 6, pupal-adults labelled II). In all the test substances, only three non viable adults were observed. Two of them were found in the test solution of fraction 4:6&1:1 at 5ppm while one was from the test solution of compound D_2 at the same concentration. Such adults had deformed wings and were found drowned in the test solutions since they could not expand their wings and fly away.

The mean percentage of the various categories of intermediate stages described above did not exceed 20% in any of the test substances. They were only observed in the lower concentrations (1-15ppm) of all the test

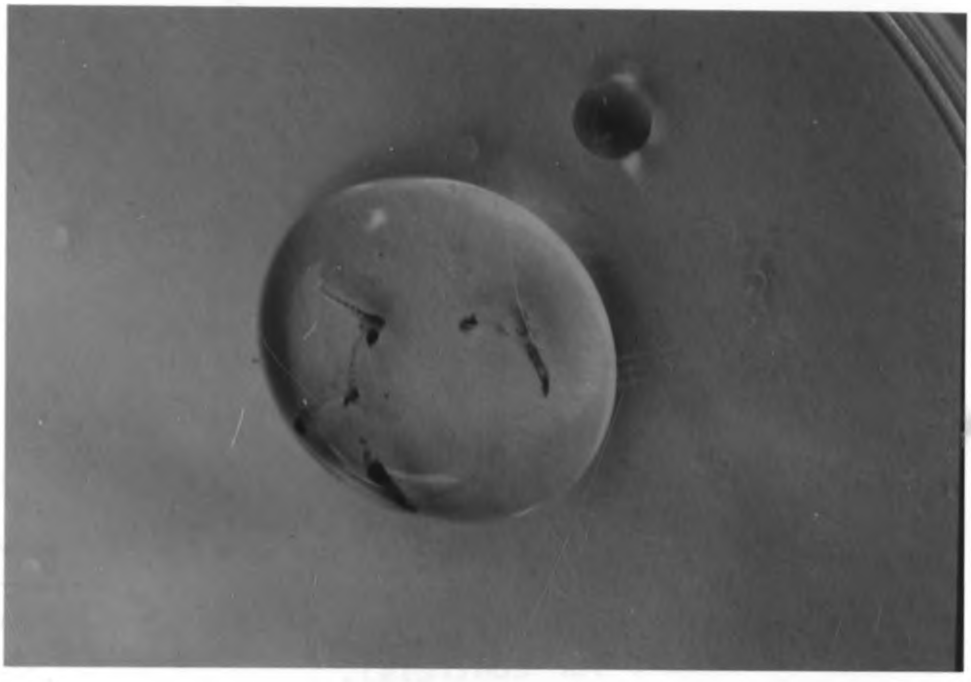


Plate 2: Dead partly escaped pupae with a pupal abdomen but head of a larva with the larval exuviae still attached (cf. Plates 1 (e) (i) and 7 for controls).

Plate 3: Dead unmelanised pupae in the early stage (cf. Plate 7 for controls).

Plate 4: Dead partly melanised pupae in the early stage Note an abnormally conspicuous wing bud in the larvae on the right (cf. Plate 7 for controls).

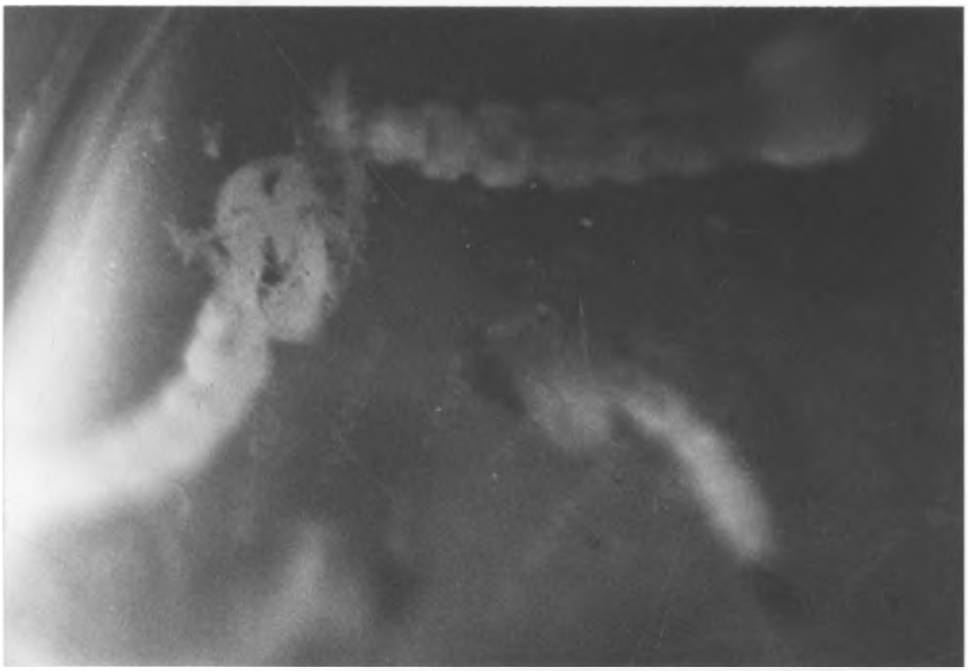


Plate 3

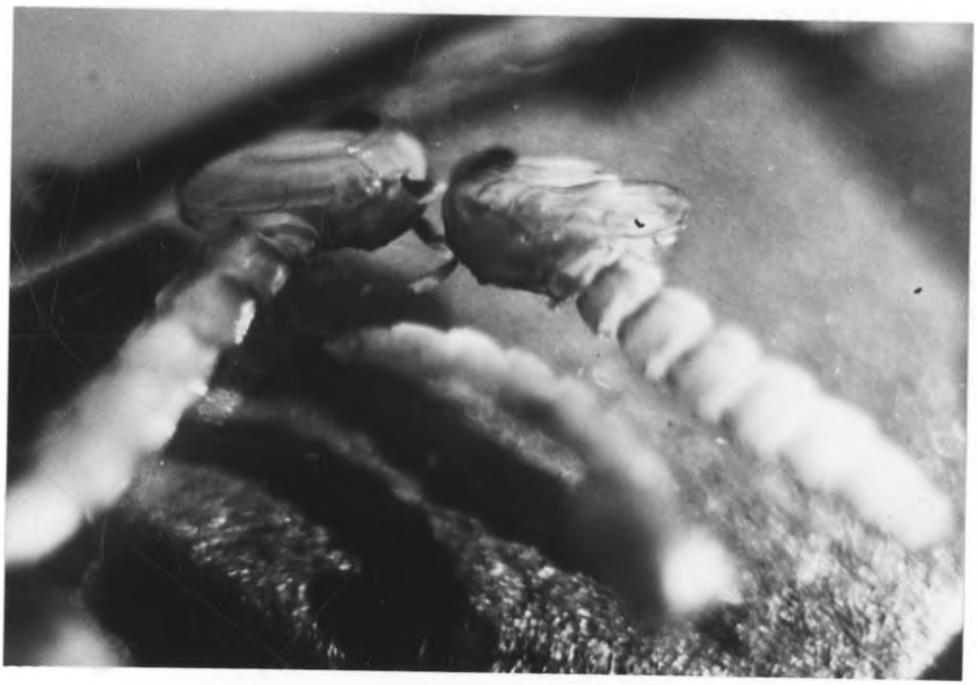


Plate 4

Plate 5: Dead pupae in the late stage (cf. Plate 7 for controls).

Plate 6: Pupal-adult intermediate stage marked:-

I Dead partly escaped adults from the pupal exuviae.

II Dead largely escaped adults but with some appendages still attached to the pupal exuviae.

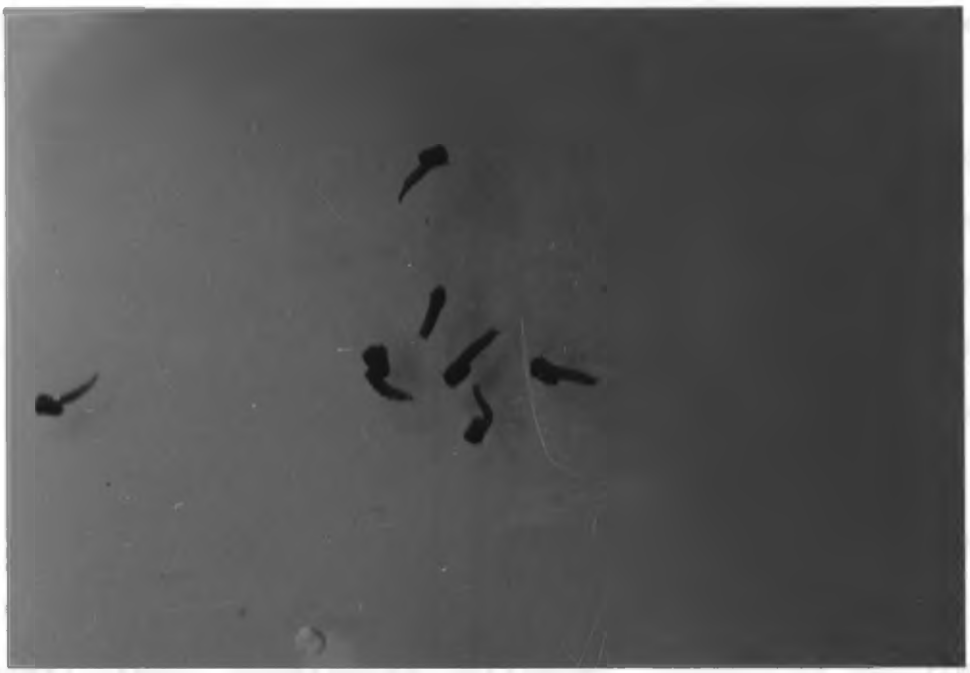


Plate 5

Plate 6

substances where the larvae were able to survive up to the later stages of development. Azadirachtin A test solutions produced the least mean percentage total of the various intermediate stages. Fourth instar larvae-pupal intermediate and the dead pupae categories were only 2% each (Table 6 and Figure 10). However, at 2.5ppm azadirachtin A produced mean percentage totals of 5% for the pupal-adult intermediate stage while those of fourth instar larvae-pupal intermediate and dead pupae stages were 10% and 20% respectively (Table 4).

The mean percentage total of the various intermediate stages resulting from the solutions of *M. volkensii* test substances differed depending on the the test substance under investigation. A mixture of fractions (A+B) produced the highest mean percentage total of the various intermediate stages of which the fourth instar larvae-pupal intermediate stage was 19%, dead pupae stage was 18% and the pupal-adult intermediate stage was 2%. Fraction B produced 15% fourth instar larvae-pupal intermediate stage, 6% dead pupae stage and 7% pupal-adult intermediate stage. Compound D_2 produced 6% fourth instar larvae-pupal intermediate stage, 12% dead pupae stage and 4% pupal-adult intermediate stage. A mixture of fractions (4:6&1:1+B) produced 9% fourth instar larvae-pupal intermediate stage, 7% dead pupae stage and 4% pupal-adult intermediate stage while fraction A gave 6% for the fourth instar larvae-pupal and dead pupae stages

Table 6: Mean percentage of dead intermediate stages and pupae resulting from various concentrations of the test substances (n=20).

Test substance	Mean percentage of dead intermediate stages		
	4th instar larvae-pupal	pupae	pupal-adult
Fraction A {23}	6	6	2
Fraction B {24}	15	7	2
Fractions (A + B) {32}	19	18	2
Fraction 4:6&1:1 {8}	0	3	1
Fractions (4:6&1:1 + B) {8}	9	7	4
Compound D_2 {20}	6	12	4
Azadirachtin A {30}	2	2	0

Values in parentheses indicate the time (in days) when mean percentage of dead intermediate stages and pupae were determined.

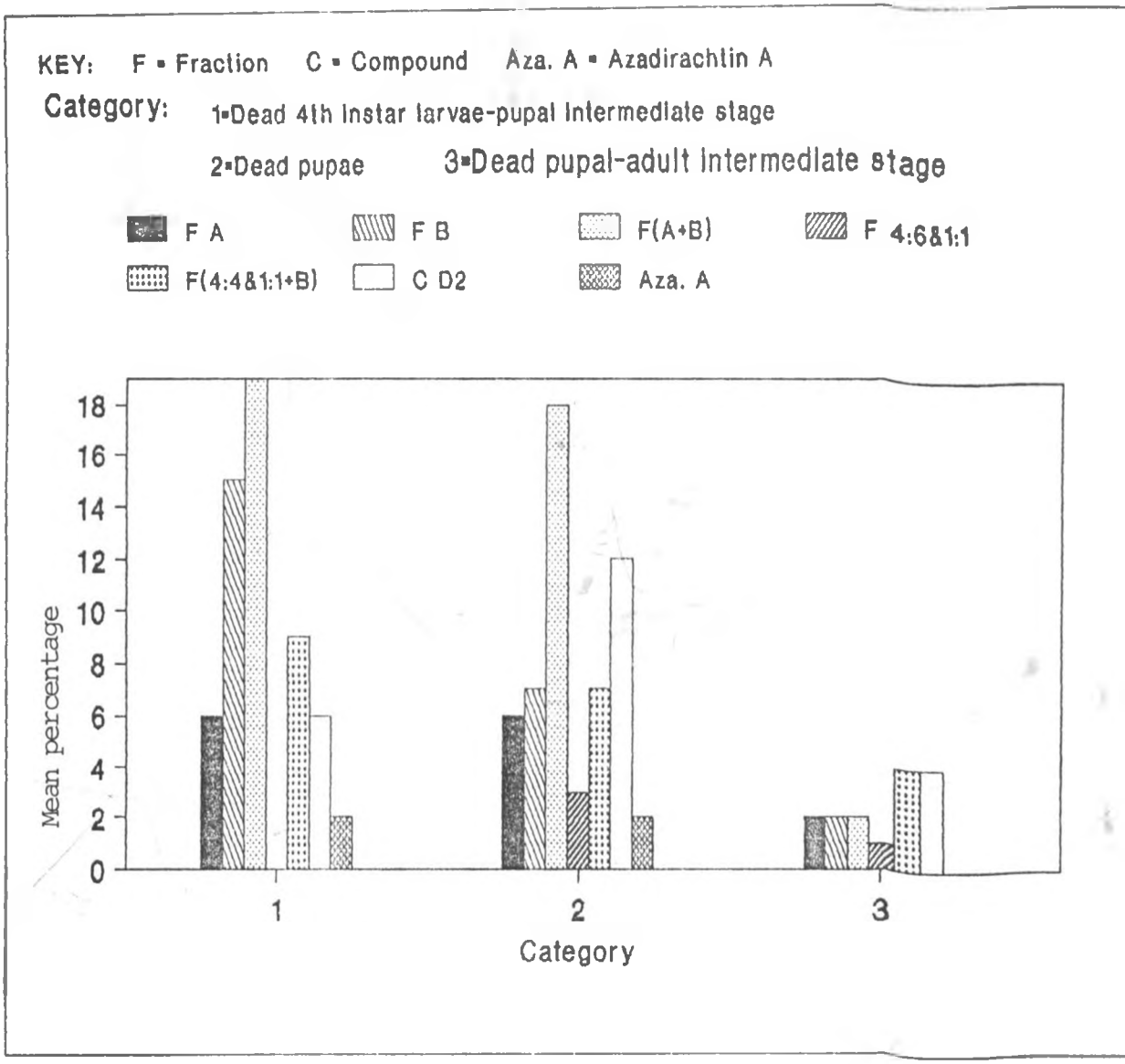


Figure 10: Mean percentage of dead intermediate stages and pupae resulting from various concentrations of the *M. volkensii* test substances and azadirachtin A (n=20). Time span is indicated in Table 6.

each, and 2% pupal-adult intermediate stage. Fraction 4:6&1:1 gave the least mean percentage total of the various categories of intermediate stages. It gave 0% fourth instar larvae-pupal intermediate stage, 3% dead pupae stage and 1% pupal-adult intermediate stage (Table 6 and Figure 10). From the above results it is clearly seen that the mean percentage total of the pupal-adult intermediate category was very low in all the test substances and did not exceed 5%. Plates 7 (a) and (b) show normal early and late pupal stages respectively which were in the control batch without any of the above mentioned features which were observed in their experimental counterparts (cf. Plates 2, 3, 4 and 5 for treated specimens). The pupae which were dying at later stages (when adults were just about to emerge) on close examination were found to lack properly developed adult features (Plate 8 (a)) as opposed to their counterparts in the control which had visible properly developed adult features (Plate 8 (b)).

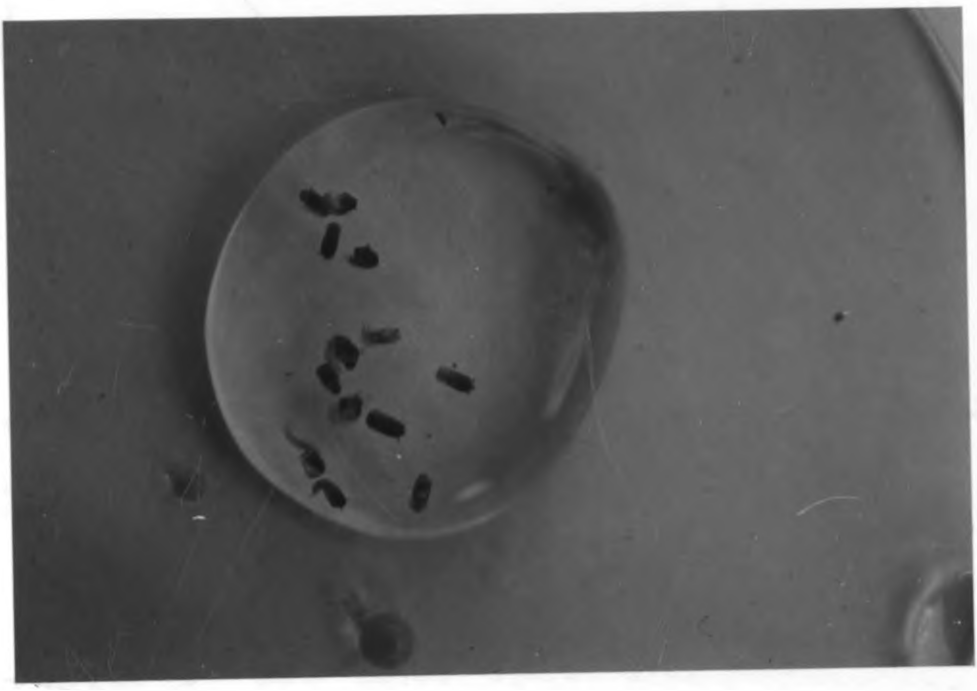
3.1.3 Emergence of adults from the solutions of the *M. volkensii* test substances and azadirachtin A.

From Table 7 and Figure 11, it is evident that in all the various concentrations of azadirachtin A test solutions, emergence of adults was very poor with only 43% emerging from 1ppm while in concentrations of 5ppm and above, no adult emerged. At a concentration of 2.5ppm of azadirachtin A it was only possible for 40% of

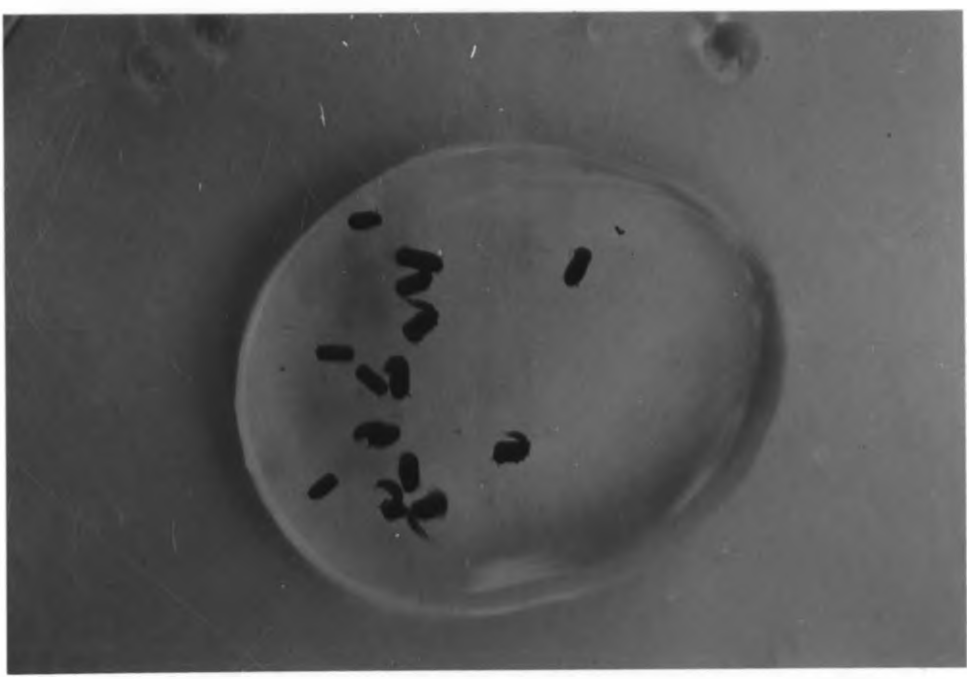
Plate 7: Normal live pupae in the early and late stages (cf. Plates 2, 3, 4 and 5 for treated specimens).

a: Normal live pupae in the early stage.

b: Normal live pupae in the late stage.



a



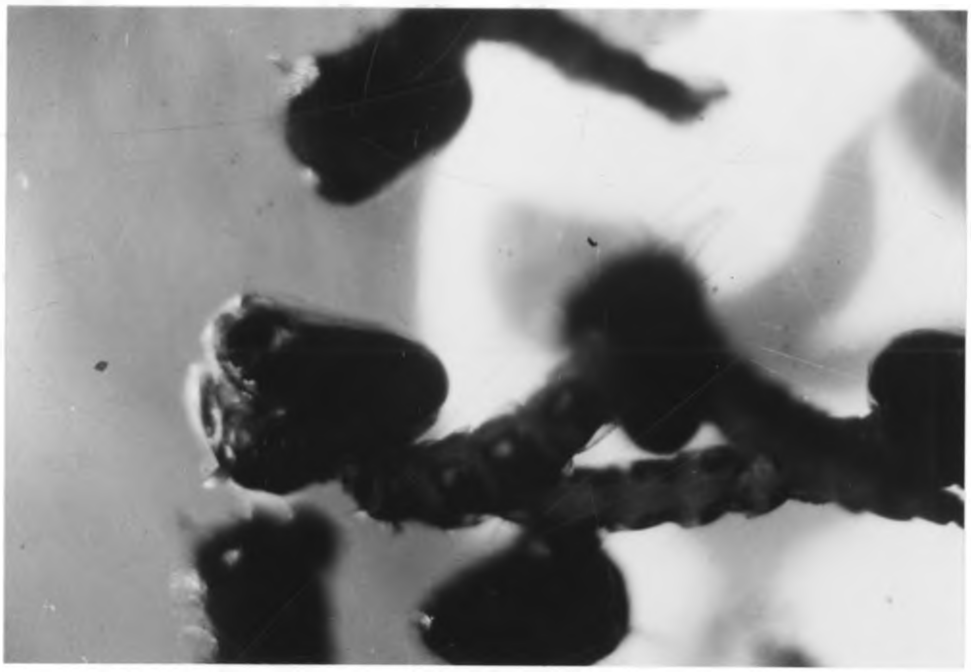
b

Plate 8: Dead late stage pupae from a mixture of fractions (A+B) and normal live late stage pupae from the control.

a: Dead late stage pupae from a mixture of fractions (A+B) lacking properly developed adult features.

b: Normal live late stage pupae from the control with properly developed and visible adult features.

a



b

Table 7: Percentage total emergence of adult *Ae. aegypti* from various concentrations of the test substances (n=20).

Test substance	% total of emerged adults							
	Concentration in ppm							
	1	5	10	15	20	50	100	200
Fraction A	85	85	85	75	50	-	-	-
Fraction B	95	85	40	20	-	-	-	-
Fractions (A + B)	80	60	30	15	5	-	-	-
Fraction 4:6&1:1	95	65	10	5	-	-	-	-
Fractions (4:6&1:1 + B)	95	70	20	-	-	-	-	-
Compound D_2	75	75	35	15	-	-	-	-
Azadirachtin A	45	-	-	-	-	-	-	-

Mean percentage of emerged adults in the control batch was 95%.

- (dash) indicates that no adult emerged.

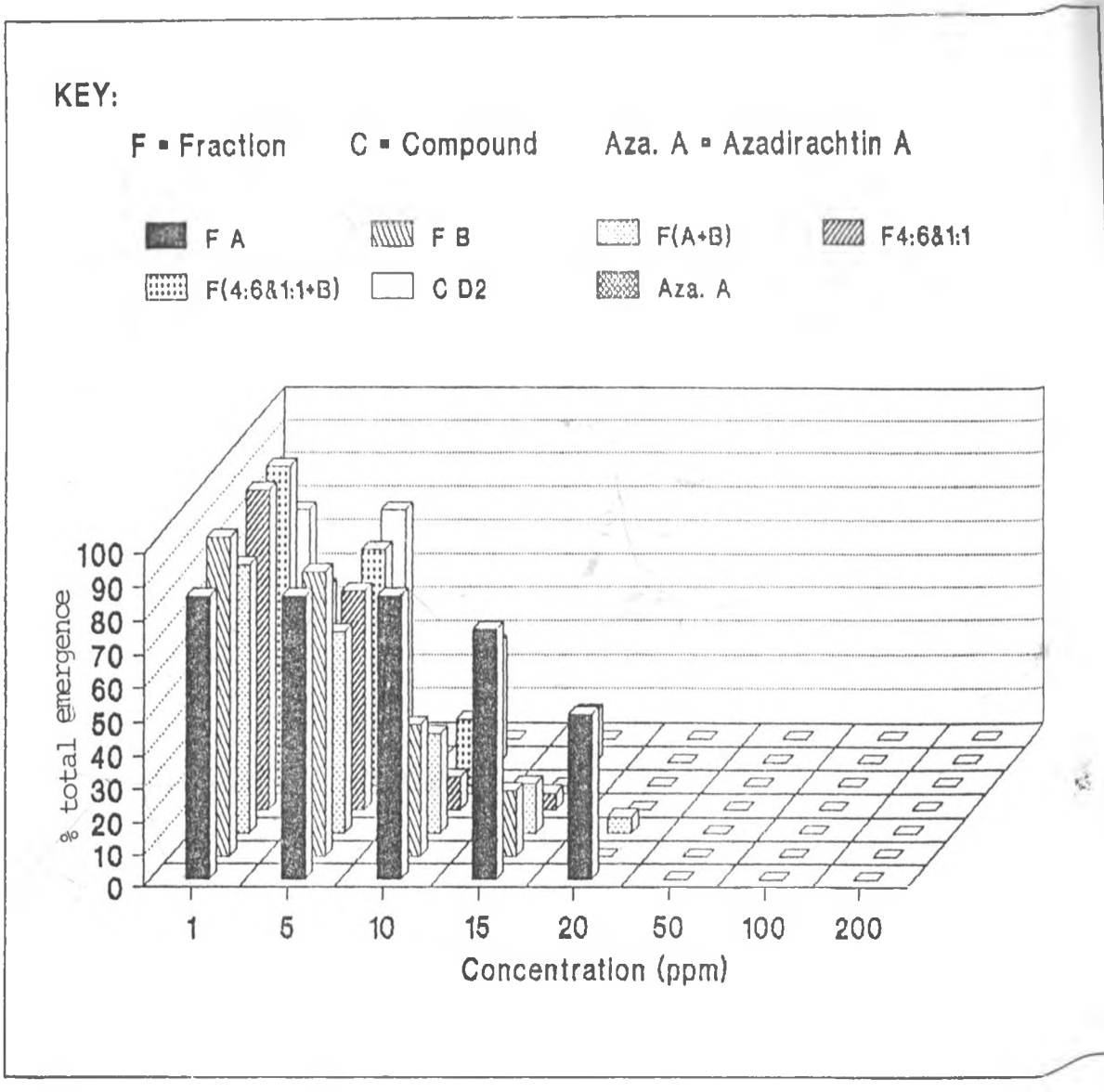


Figure 11: Percentage total emergence of adult *Ae. aegypti* from various concentrations of *M. volkensii* test substances and azadirachtin A (n=20).

adults to emerge (Table 4).

From the *M. volkensii* test substances solutions, with the exception of fraction A and a mixture of fractions (A+B) at 20ppm where 50% and 5% of adults emerged respectively, in concentrations of 20ppm and above no adults emerged in the other test substances (Table 7 and Figure 11). In a mixture of fractions (4:6&1:1+B) only 20% of adults emerged at 10ppm while there was no adult which emerged from 15ppm. In fraction 4:6&1:1 adult emergence rate was 10% and 15% from 10 and 15ppm test solutions respectively. In a mixture of fractions (A+B) and compound D_2 at concentrations, 10 and 15ppm, adult emergence was 30% and 15%, and 35% and 15% respectively. In fraction B at concentrations, 10 and 15ppm, adult emergence was 40% and 20% respectively while in fraction A at these concentrations, the emergence rate of adults was 85% and 75% respectively. Apart from fraction 4:6&1:1 and a mixture of fractions (A+B) where adult emergence at 5ppm was 65% and 60% respectively, the emergence of adults in the other *M. volkensii* test substances ranged between 70% and 95%. In the controls, the average emergence rate of adults was 95%. The adults which emerged from the highest concentrations of the *M. volkensii* and azadirachtin A test solutions were found to be normal just like the ones from the control. They laid eggs which hatched into normal larvae. These larvae were able to pupate normally. From the pupae, normal adults emerged though

their life span was not determined. No larval mortality or any category of the intermediate stages was observed among the larvae which hatched from the eggs laid by the adults which emerged from the solutions with the highest concentrations of all the test substances.

3.2 The effects of *M. volkensii* test substances and azadirachtin A on growth and development on the second instar larvae of *Ae. aegypti.*

In a mixture of fractions (A+B) at 20ppm and azadirachtin A at 2.5ppm, the exposed second instar larvae took a period of 35 and 28 days respectively before pupating (Table 8). Their growth rate was delayed for a longer period of time than the larvae which were in the control which took only 11 days. The larvae which were in the other *M. volkensii* test substances took a total period of about 19-24 days before pupating depending on the particular substance under test. The third and fourth instars (relative developmental stage, rDS) were the stages where prolongation of growth rates took place mostly. In a mixture of fractions (A+B) test solutions at 20ppm, the third rDS was the majority in any day of observation for 20 days as compared to the control where this stage took only three days. In the test solution of azadirachtin A at 2.5ppm, and fraction A at 50ppm, this stage was the majority for 12 days. The same stage was the majority for nine days in a mixture of fractions (4:6+1:1+B) test solution at 15ppm, and eight days in

Table 8: The effect of fractions and a compound from *M. volkensii*, and azadirachtin A on growth and development of *Ae. aegypti* second instar larvae (n=20).

Test substance	Duration taken in each rDS (days)				Total no. of days taken upto the end of pupation
	Instar II	Instar III	Instar IV	pupa	
Control 0ppm	1	3	4	3	11
*Fraction B 15ppm	2	8	13	-	23
Compound D_2 20ppm	4	8	11	-	23
*Fraction 4:6&1:1 10ppm	5	6	8	1	20
Fraction A 50ppm	2	12	10	-	24
*Fractions (4:6&1:1+B) 15ppm	6	9	4	-	19
!Fractions (A+B) 20ppm	6	20	9	-	35
!Azadirachtin A 2.5ppm	5	12	11	-	28

! indicates that fourth instars were very small just like the third instars in their late stage.

* indicates that the relative developmental stage in any particular day was difficult to determine due to high toxicity of the test substances.

- (dash) means there was no relative developmental stage (rDS) present.

each of the test solutions of compound D_2 at 20ppm and fraction B at 15ppm while in fraction 4:6&1:1 at 15ppm this stage took six days.

The fourth rDS was the majority in any day of observation in the test solutions of fraction B at 15ppm for 13 days, azadirachtin A at 2.5ppm and compound D_2 at 20ppm for 11 days each, fraction A at 50ppm for ten days, a mixture of fractions (A+B) at 20ppm for nine days, fraction 4:6&1:1 at 10ppm for eight days and in a mixture of fractions (4:6&1:1+B) test solutions at 15ppm for only four days. In the control this stage lasted for only four days. With the exception of test solutions of fractions A at 50ppm and B at 15ppm, where the second rDS was the majority in any day of observation for only two days, in the test solutions of other substances, the second rDS was the majority for about five days while in the control it took only one day. This indicates that these test substance had very little growth inhibition effect on the second rDS as compared to the third and fourth relative developmental stages. There was no day when the pupal stage was the majority relative developmental stage present in all the solutions of the test substances as was observed in the control where it took three days. It was only in the test solution of fraction 4:6&1:1 where this stage was the majority for only one day. This shows that there was no growth inhibition effect in the pupal stage since the few pupae which emerged into normal adults from the solutions of

some of the test substances took almost the same period of time as the ones which were in the control. In almost all the solutions of *M. volkensii* test substances, the emergence period of adults took 11 days longer than in the control where it lasted only three days. In the test solution of fraction 4:6&1:1 this period took only five days. In the test solutions of azadirachtin A at 1 and 2.5ppm, the adult emergence period took 14 and 19 days respectively longer than in the control where it lasted for four days. In most solutions of the test substances, the emerging period of adults was not uniform since there were some days with no adults emerging.

3.3 The effects of pellets formulated from the acetone extract fraction of *M. volkensii* on the third and fourth instar larvae of *Ae. aegypti*.

One hour after the larvae had been given the pellets formulated from the acetone extract fraction of *M. volkensii* as food, 50 out of 100 larvae were seen resting in either twisted S or U shaped postures and stretching very frequently. Only nine out of 100 larvae which were given the control pellets were seen in such postures though the S or U shaped postures were wider in size than the ones observed in the experimental larvae. The larvae resting in these postures in the control batch were not stretching frequently as was observed in the experimental larvae (Table 9). After 24 hours, only

Table 9: Mean number (± S.E.) of third and fourth instar larvae of *Ae. aegypti* resting in S or U postures after feeding on the newly formulated acetone extract and control pellets (n=100).

Duration (after giving the pellets)	Mean no. (± S.E.) of larvae resting in S or U postures (with ranks in parentheses)	
	Acetone extract pellets	Control pellets
1 hour	50 ± 4.7 (17)	9 ± 1.2 (13)
Day 1	20 ± 2.5 (16)	5 ± 0.9 (5.5)
Day 2	8 ± 0.7 (11)	9 ± 1.5 (13)
Day 3	7 ± 1.4 (9.5)	11 ± 0.8 (15)
Day 4	7 ± 1.6 (9.5)	9 ± 0.8 (13)
Day 5	5 ± 0.9 (5.5)	5 ± 0.6 (5.5)
Day 6	5 ± 1.4 (5.5)	6 ± 0.3 (8)
Day 7	3 ± 0.7 (3)	
Day 8	2 ± 0.6 (2)	
Day 9	1 ± 0.1 (1)	

$n_1=10$ $n_2=7$

$R_1=80$ $R_2=73$

Calculated Mann-Whitney statistics,

$$U' = n_2 n_1 + \frac{n_2 (n_2+1)}{2} - R_2 = 25$$

$U_{0.05\ (1),\ 10,\ 7} = U_{0.05\ (1),\ 7,\ 10} = 53$

$p > 0.05$; d.f. = 10, 7

20 larvae in the experimental group were resting in twisted S or U shaped postures and stretching frequently while in the control group only five larvae displayed such characters. A statistical test, the Mann-Whitney statistics, which was performed to find out if there was any significant difference in resting postures between the larvae which were given the newly formulated acetone extract pellets and the control ones revealed no significant difference, $U' = 25$; d. f. = 10, 7; $p > 0.05$ (Table 9).

The newly formulated acetone extract pellets were very effective on the third and fourth larval instar stages where they caused a mortality rate of 86% with only 13% larvae pupating into normal pupae. No mortality occurred on the larvae which were given the control pellets since 99% of the larvae pupated into normal pupae (Table 10 and Figure 12). The pupae arising from the larvae which were given the newly formulated pellets were slight affected since a mortality rate of 6% occurred while in the pupae given the control pellets, a mortality of only 1% occurred indicating no effect on the pupal stage. Only 3% fourth instar larvae-pupal intermediate category was observed from the larvae which were given the acetone extract pellets. From the control batch only 1% of such category was observed. 97% of the adults emerged from the larvae which pupated in the control while only 5% of adults emerged from the experimental group larvae which pupated. No growth

Table 10: The effect of pellets formulated from acetone extract fraction on the third and fourth instars of *Ae. aegypti* larvae (n = 100).

Category	Percentage	
	Control pellets	Acetone extract pellet
Dead 3rd & 4th instar larvae.	0	86
Dead 4th instar larvae -pupal intermediate stage.	1	3
Normal pupae formed.	99	13
Dead pupae.	1	6
Normal emerged adults.	*97	5

n = number of third and fourth instar larvae used per replicate for each test.

* indicates that one dead pupal-adult intermediate category was not included.

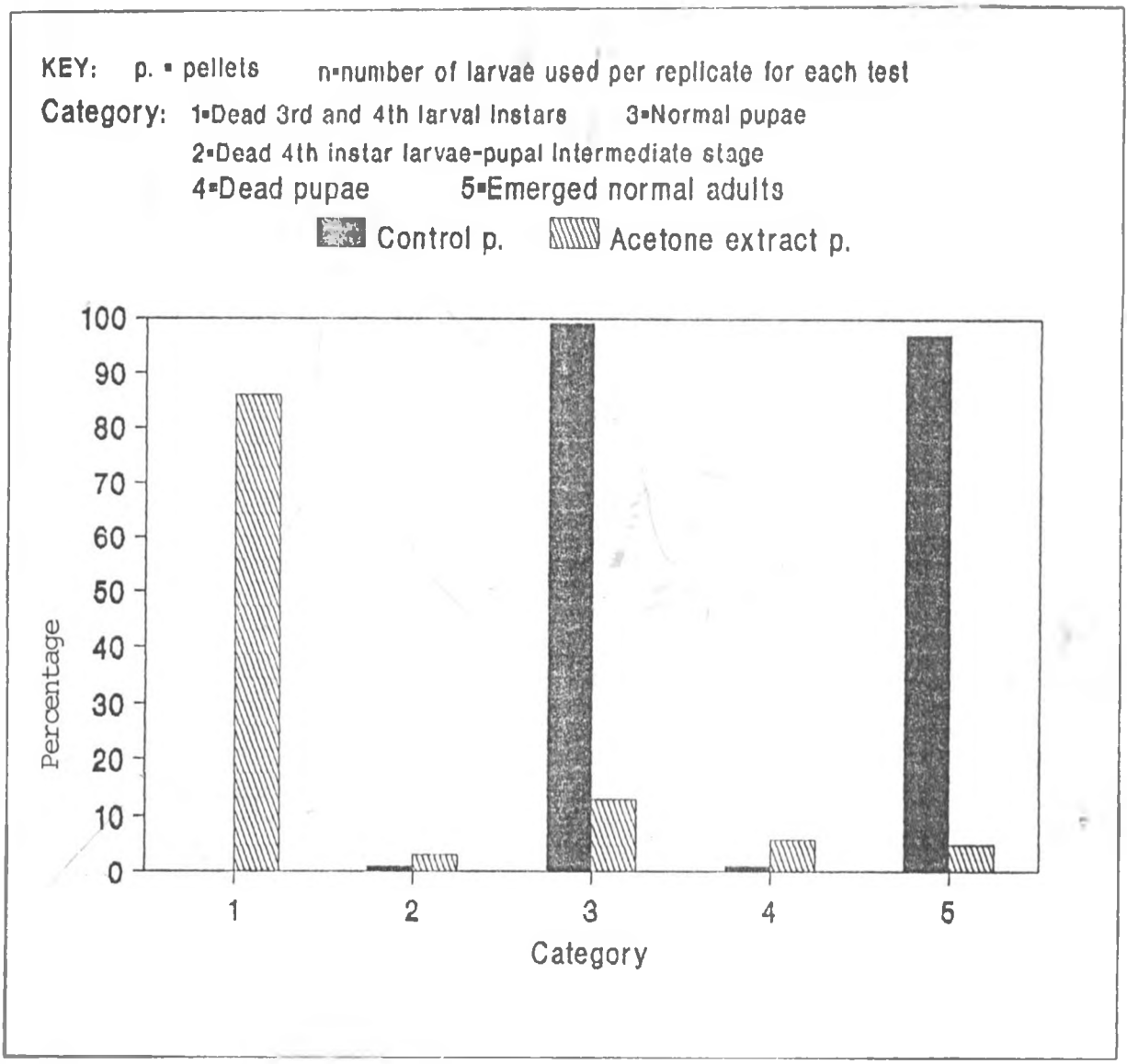


Figure 12: The effect of pellets formulated from the acetone extract fraction of *M. volkensii* on the third and fourth instars of *Ae. aegypti* larvae (n=100).

inhibition effect was observed between the larvae which were given the experimental and control pellets since their developmental period in both cases up to the end of adult emergence took the same time of 11 days.

3.4 The effect of different concentrations of *M. volkensii* and azadirachtin A test solutions on hatchability of *Ae. aegypti* eggs.

Hatchability of *Ae. aegypti* eggs increased with the increasing concentrations of the test substances differently depending on the test substance under investigation until optimum concentrations in some were reached. Fraction A, compound D_2 and azadirachtin A caused greater increase of hatchability with increasing concentrations resulting in over 75% of the larvae hatching at concentrations of 50, 20 and 10ppm respectively. Fractions B and 4:6&1:1 slightly increased hatchability with 55% and 65% of the larvae hatching respectively at the optimum concentrations of 10ppm and then inhibited hatching drastically at higher concentrations (above 50ppm) with only 3% and 26% of the larvae hatching respectively at 200ppm. There was no noticeable inhibition on hatchability of the eggs which were in the test solutions of fraction A, compound D_2 and azadirachtin A at higher concentrations since 75% of the larvae hatched in all the concentrations though some slight fluctuations were observed (Table 11 and Figure 13).

Hatchability of the eggs of *Ae. aegypti* in

Table 11: The effect of different concentrations of fractions and a compound from *M. volkensii* and azadirachtin A on the hatchability of *Ae. aegypti* eggs after 3 days (n=200).

Concentration (ppm)	Test substance	Percentage of hatched larvae
0	Fraction A	42
	Fraction B	59
	Fraction 4:6&1:1	54
	Compound D_2	48
	Azadirachtin A	67
5	Fraction A	40
	Fraction B	52
	Fraction 4:6&1:1	22
	Compound D_2	71
	Azadirachtin A	84
10	Fraction A	68
	Fraction B	64
	Fraction 4:6&1:1	56
	Compound D_2	89
	Azadirachtin A	100
20	Fraction A	52
	Fraction B	25
	Fraction 4:6&1:1	31
	Compound D_2	90
	Azadirachtin A	94
50	Fraction A	79
	Fraction B	46
	Fraction 4:6&1:1	83
	Compound D_2	76
	Azadirachtin A	89
100	Fraction A	72
	Fraction B	25
	Fraction 4:6&1:1	46
	Compound D_2	70
	Azadirachtin A	75
200	Fraction A	67
	Fraction B	03
	Fraction 4:6&1:1	26
	Compound D_2	80
	Azadirachtin A	97

n=number of eggs used per replicate for each test substance.

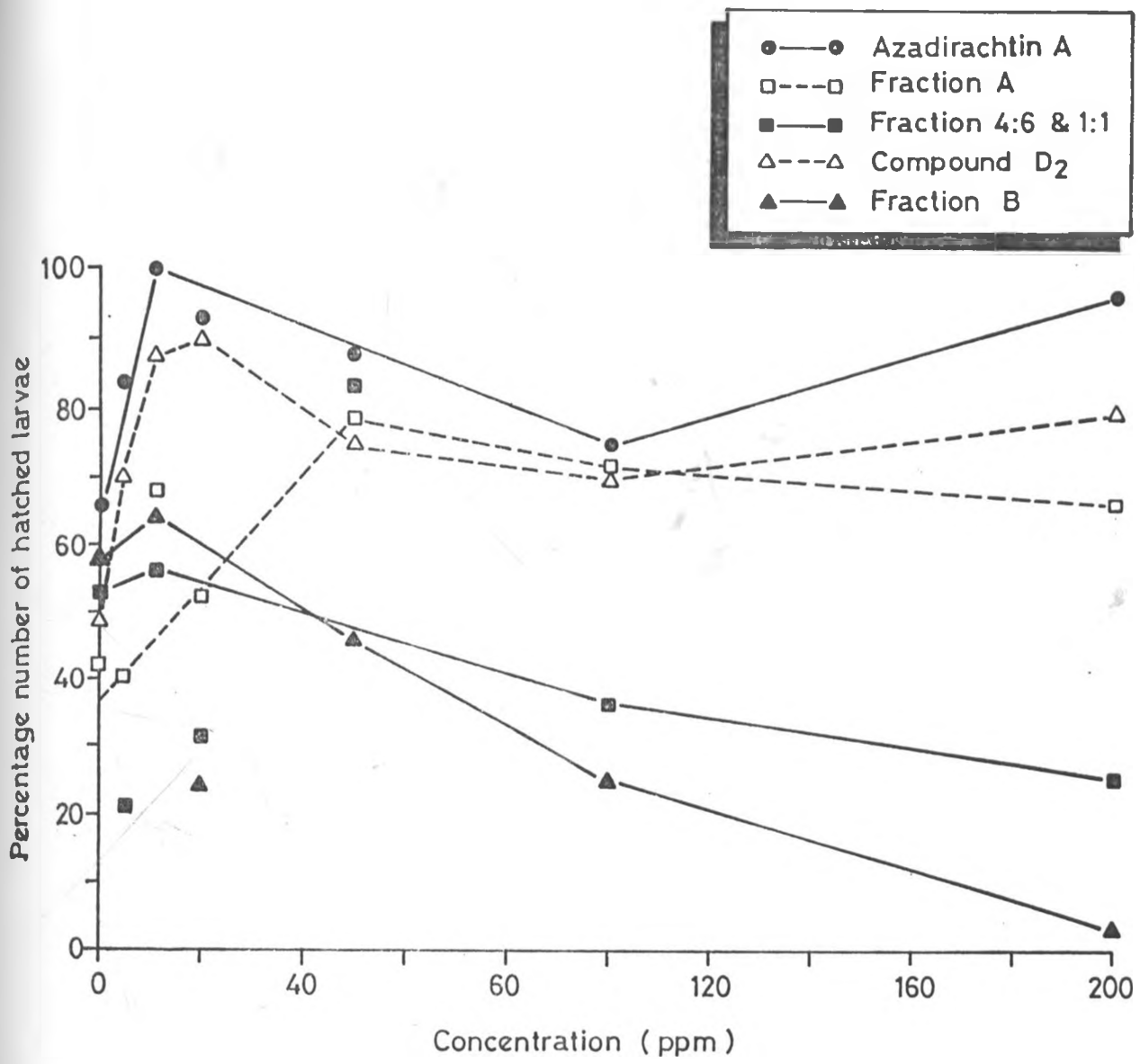


Figure 13: The effect of different concentrations of *M. volkensii* test substances and azadirachtin A on the hatchability of *Ae. aegypti* eggs after 3 days (n=200).

different concentrations of the test substances was also found to be time related. Most of the eggs which were in the test solutions of azadirachtin A and compound D_2 at 10ppm hatched earlier than those which were in the solutions of other test substances. In fact after 10 hours, 148 larvae had hatched from the test solution of compound D_2 and 176 from that of azadirachtin A while from the control only 69 larvae had hatched within the same time (Table 12 and Figure 14). Majority of the eggs which were in the test solutions of fractions A and 4:6&1:1 at 50ppm hatched after 24 and 32 hours respectively. In the control, and fraction B at 10ppm, majority of the larvae hatched also after 32 hours but they were fewer (127) than those which hatched from fraction 4:6&1:1 (165) after the same period of time. The larvae which hatched from azadirachtin A test solutions at 100ppm were not motile as was the case with the ones from the other concentrations. Prematurely hatched larvae with the egg cases still attached were observed in the test solutions of azadirachtin A at 200ppm and they were also not motile.

When the larvae which were hatched in different concentrations of the test substances were reared so that their survival rate could be determined, it was found that the larvae which hatched in the higher concentrations had very poor survival rate. With exception of the test solutions of fraction A where 90% of the larval mortality occurred on the larvae hatched

Table 12: The effect fractions and a compound from *M. volkensii* and azadirachtin A on the hatchability rate of *Ae. aegypti* eggs (n=200). Values in parentheses are concentrations in ppm.

Time (hours)	Mean no. of hatched larvae ± S.E.					
	Cntr. (0*)	F_A (50)	F_B (10)	$F_{4:6\&1:1}$ (50)	C D_2 (10)	Aza. A (10)
0	0	0	0	0	0	0
6	35 ±5	7 ±1	45 ±6	51 ±7	101 ±14	120 ±15
10	69 ±10	44 ±6	94 ±13	85 ±10	148 ±17	176 ±20
24	81 ±11	143 ±20	99 ±11	119 ±12	163 ±18	183 ±16
32	127 ±18	157 ±22	127 ±15	165 ±17	176 ±18	197 ±18
48	131 ±18	158 ±18	128 ±10	166 ±20	176 ±18	198 ±20
72	133 ±17	158 ±18	128 ±10	166 ±20	179 ±16	198 ±20

n = number of eggs used per replicate for each test substance.

Cntr. = Control F_A = Fraction A

F_B = Fraction B $F_{4:6\&1:1}$ = Fraction 4:6&1:1

C D_2 = Compound D_2 Aza. A = Azadirachtin A

* The concentrations used are the ones in which the highest number of larvae hatched in the controls and in each of the test substances.

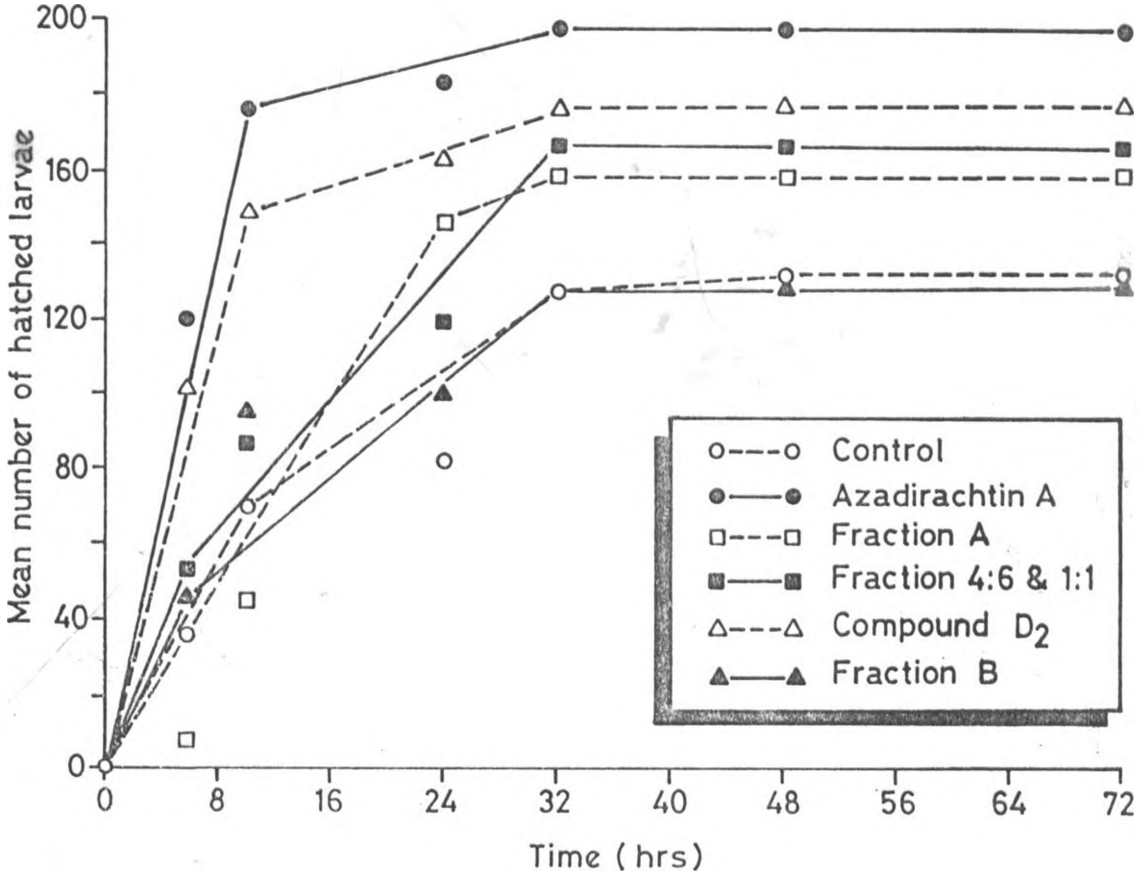


Figure 14: The effect of *M. volkensii* test substances and azadirachtin A on the hatching rate of *Ae. aegypti* eggs (n = 200).

in 200ppm after one week, in the other test substances all the larvae, 100%, which hatched in 100-200ppm were dead after one week (Table 13). However, in the test solutions of compound D_2, azadirachtin A and fraction B, mortality of 100%, 72% and 71% were observed in the larvae which were hatched in 50ppm test solutions after one week. In the test solutions of fractions A and 4:6&1:1 at 50ppm, there were no high mortality rates after one week since only 30% and 42% of the larvae were dead respectively. Majority of the larvae which were dying in the test solutions of compound D_2 at 100-200ppm were moulting from second to third instar.

Percentage total mortality rates (after correction by Abbott's formula) of the various larval instars of *Ae. aegypti* resulting from the larvae hatched in the solutions of the test substances at different concentrations are presented in Table 14 and depicted graphically in Figure 15. In all the test substances apart from fraction A at 100 and 200ppm where total mortality of the hatched larvae was 53% and 90% respectively, total mortality of 100% was recorded from the larvae which were hatched in the other test substances at 100 and 200ppm. Least total mortality rates were recorded from the larvae which hatched in the test solutions of azadirachtin A, fractions A and 4:6&1:1 at lower concentrations but mortality rates of 53% and 91% occurred on the larvae which hatched in 50ppm test solutions of fraction 4:6&1:1 and

Table 13: Percentage mortality (after correction by Abbott's formular) of the larvae of *Ae. aegypti* hatched in the *M. volkensii* and azadirachtin A test solutions at 50, 100 and 200ppm after one week (n=200).

Test substance	% mortality of the hatched larvae		
	Concentration in ppm		
	50	100	200
Fraction A (10)	30	46	90
Fraction B (10)	71	96	100
Fraction 4:6&1:1 (10)	42	97	100
Compound D_2 (8)	97	100	100
Azadirachtin A (10)	97	100	100

Values in parentheses indicate the time (in days) when percentage mortality of the larvae was determined.

Table 14: Percentage total mortality (after correction by Abbott's formula) of various larval instars of *Ae. aegypti* resulting from eggs hatched in test solutions of different concentrations of the test substances (n=200).

Test substance	% total mortality of the larval instars					
	Concentration in ppm					
	5	10	20	50	100	200
Fraction A (10)	9	7	14	33	53	90
Fraction B (18)	22	34	41	79	100	100
Fraction (17) 4:6&1:1	17	14	16	53	100	100
Compound D_2 (23)	37	5	46	97	100	100
Azadirachtin A (20)	9	6	16	91	98	100

Values in parentheses indicate the time (in days) when percentage total mortality of various larval instars was determined.

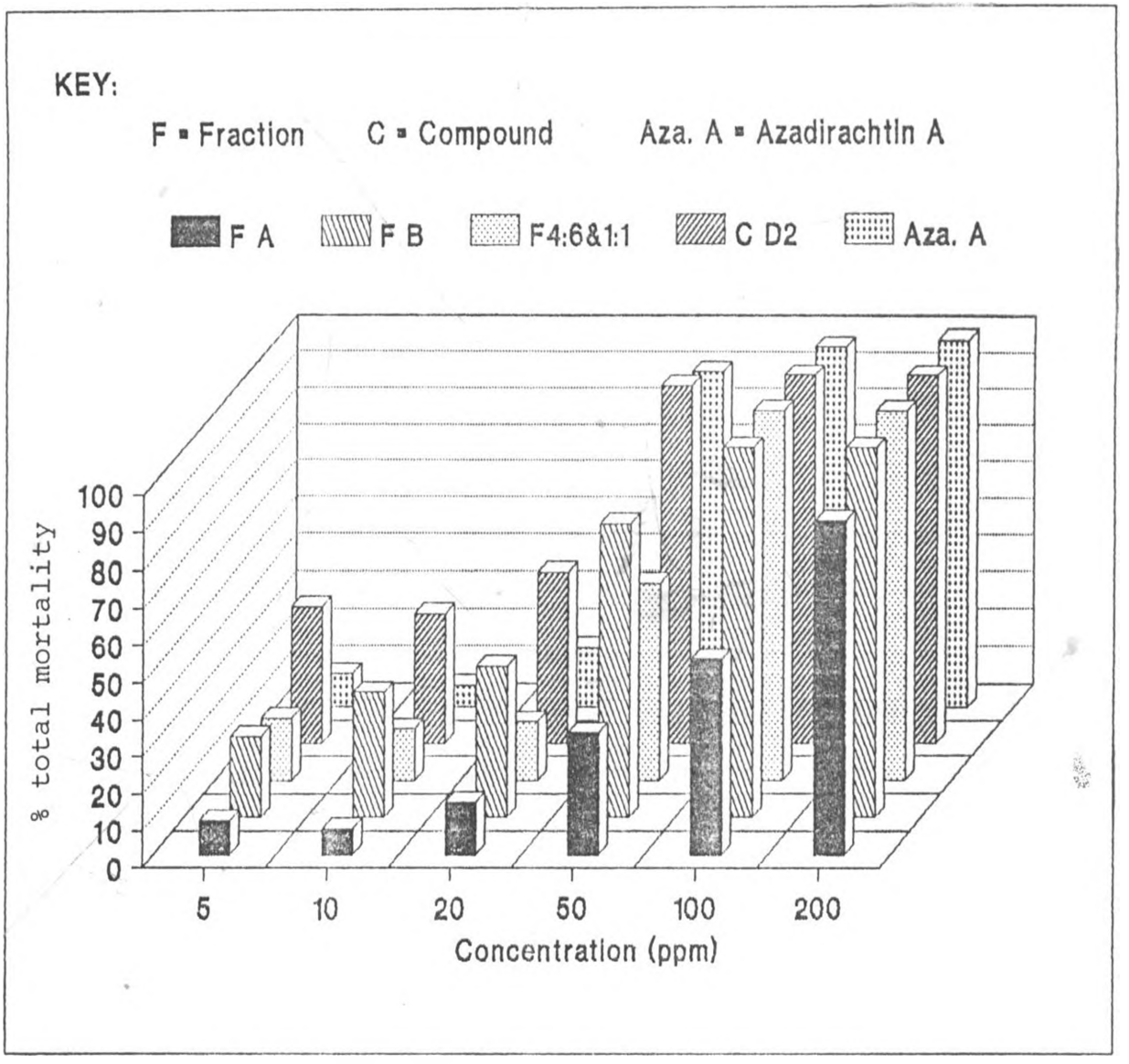


Figure 15: Percentage total mortality (after correction by Abbott's formula) of various larval instars of *Ae. aegypti* resulting from eggs hatched in solutions of different concentrations of the *M. volkensii* test substances and azadirachtin A (n=200). Time span is indicated in Table 14.

azadirachtin A respectively. Fraction B and compound D_2 at lower concentrations (5-20ppm) caused total mortality rates ranging between 22%-46%.

There were very few intermediate categories resulting from the larvae which were hatched in different concentrations of the test substances. No fourth instar larvae-pupal intermediate stage was observed from the larvae which hatched in the test solutions of compound D_2 while only 1% was observed from the solutions of the remaining test substances. 1% dead pupal-adult intermediate stage was observed from the larvae which hatched in the test solution of fraction B, compound D_2 and azadirachtin A while there was none from the larvae which were hatched in the test solutions of fractions A and 4:6&1:1. Dead pupal stage was the mostly observed stage with 4% occurring in the larvae which were hatched from the test solutions of fractions A and B, 2% from the larvae which hatched in the test solutions of fraction 4:6&1:1 and 1% from the larvae which hatched from the test solutions of compound D_2 and azadirachtin A (Table 15 and Figure 16). From the results presented in Table 16 and Figure 17, it is evident that no adults emerged from the larvae which hatched in the solutions of almost all the test substances at 100 and 200ppm except in fraction A where 44% and 10% of adults emerged from the larvae which were hatched in 100 and 200ppm respectively, and 2% from azadirachtin A at 100ppm. Emergence rate of above 50%

Table 15: Percentage of dead intermediate stages and pupae resulting from eggs hatched in test solutions of different concentrations of the test substances (n=200).

Test substance	Percentage Dead intermediate stages		
	4th instar larvae-pupal	pupal	pupal-adult
Fraction A (10)	1	4	0
Fraction B (18)	1	4	1
Fraction (17) 4:6&1:1	1	2	0
Compound D_2 (23)	0	1	1
Azadirachtin A (20)	1	1	1

Values in parentheses indicate the time (in days) when percentage of dead intermediate stages and pupae were determined.

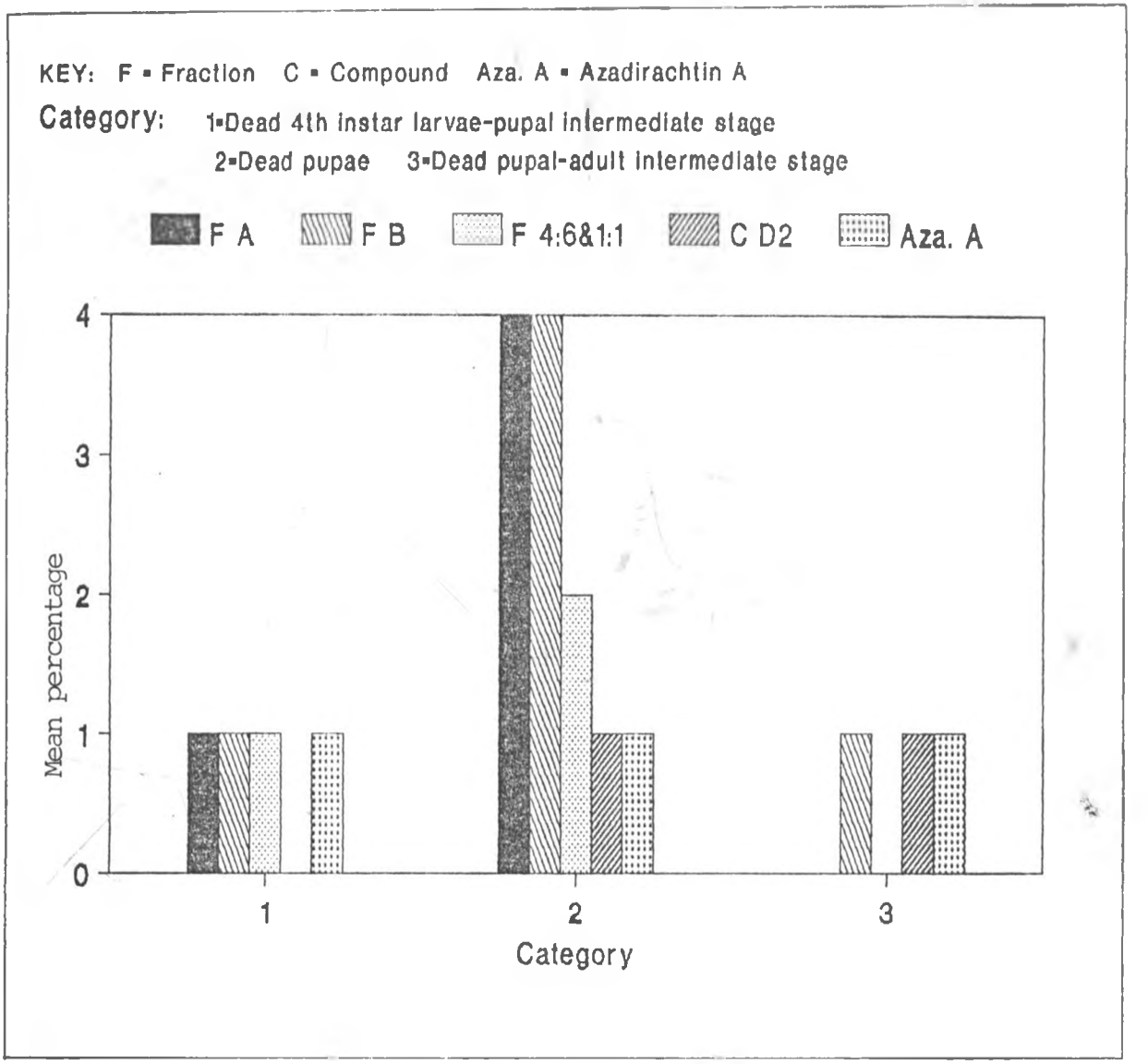


Figure 16: Mean percentage of dead intermediate stages and pupae resulting from eggs hatched in solutions of different concentrations of the *M. volkensii* test substances and azadirachtin A (n=200). Time span is indicated in Table 15.

Table 16: Percentage total emergence of adult *Ae. aegypti* from eggs hatched in solutions of different concentrations of the test substances (n=200).

Test substance	% total of emerged adults Concentration in ppm						
	0.0	5.0	10.0	20.0	50.0	100.0	200.0
Fraction A	87	86	89	86	67	44	10
Fraction B	75	77	61	57	21	0	0
Fraction 4:6&1:1	92	81	85	79	46	0	0
Compound D_2	73	63	64	54	3	0	0
Azadirachtin A	91	89	93	83	9	2	0

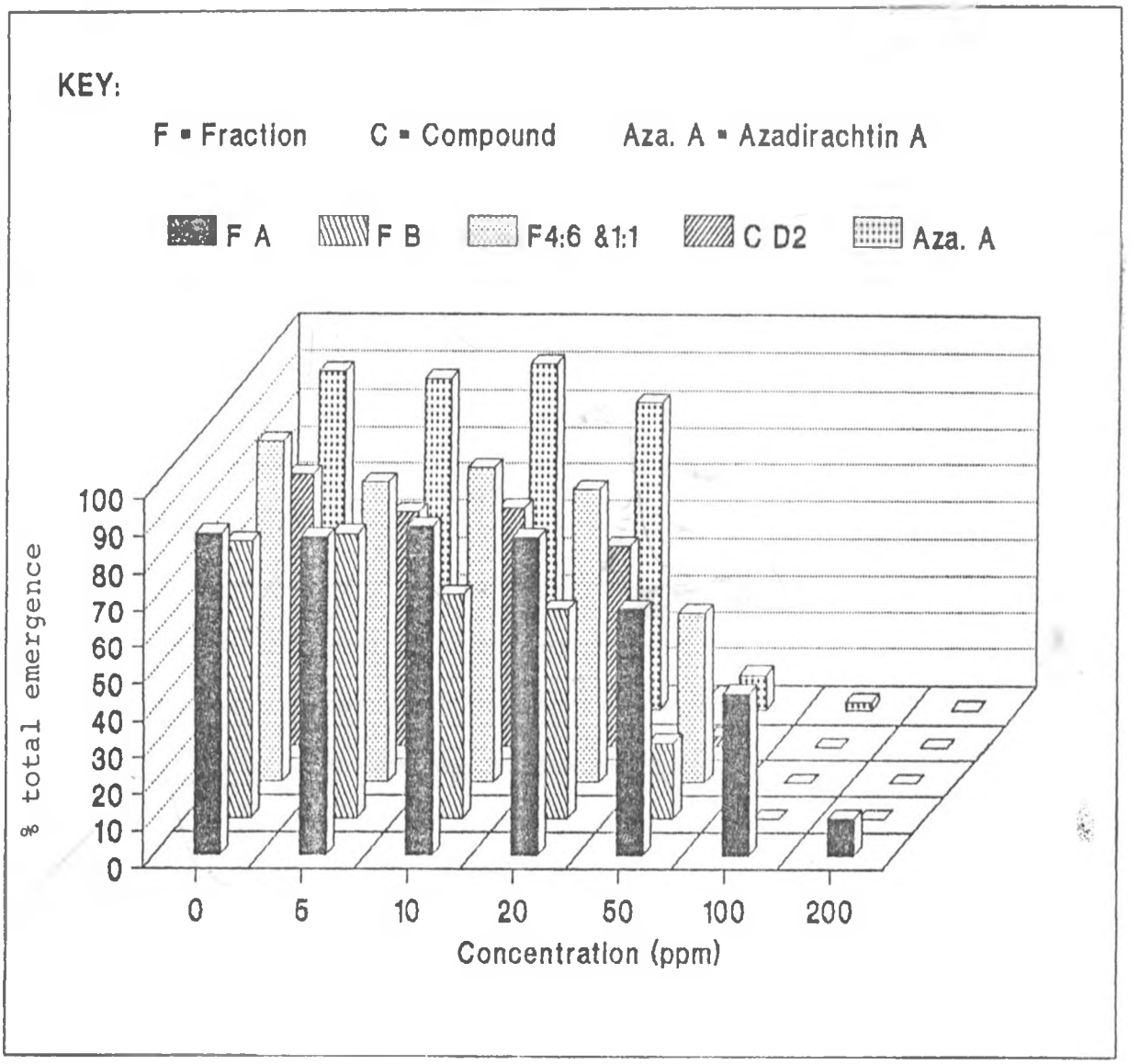


Figure 17: Percentage total emergence of adult *Ae. aegypti* from eggs hatched in various concentrations of the *M. volkensii* test substances and azadirachtin A (n=200).

was only recorded from the larvae which were hatched in the test solution of fraction A at 50ppm. At the same concentration in the test solutions of fraction 4:6&1:1, the emergence rate of the adults was 47%, in fraction B it was 21% and from the test solution of azadirachtin A and compound D_2, it was as low as less than 10%. Apart from the test solutions of fraction B and compound D_2 where the rate of adult emergence was 57% and 54% respectively at 20ppm, in the solutions of the other test substances the adult emergence rates were higher and ranged between 63%-86% at concentrations 5-20ppm and did not vary from the controls. The emergence period of adults from the larvae which were hatched in the test solutions of fractions A, B and 4:6&1:1 at different concentrations took a period of six days as was in the controls but in the test solutions of azadirachtin A at 20 and 50ppm and in compound D_2 at 10, 20 and 50ppm, the emergence period of the adults took three and four days respectively longer than in the controls.

3.5 The effect of solutions of the *M. volkensii* test substances on the oviposition behaviour of adult *Ae. aegypti* females.

At 50ppm, it was only in the test solution of fraction 4:6&1:1 where the percentage of eggs laid was low, i. e., 59%. In the test solutions of fractions A and B, and compound D_2 there were higher percentages of

eggs laid namely 93%, 85% and 95% respectively (Table 17). At 100ppm, the percentage of eggs laid in the test solution of fraction B reduced to 55% while in the test solution of fraction 4:6&1:1, 53% of the eggs were laid which was only a very slight reduction from the previous value of 59% at 50ppm. There was no noticeable reduction in the percentage of eggs laid in the test solution of fraction A and compound D_2 at 100ppm since 98% and 92% of the eggs were laid in each case respectively. At 200ppm all the solutions of *M. volkensii* test substances deterred oviposition of the adult female mosquitoes drastically with only 33%, 22%, 15% and 17% of the eggs being laid in the test solutions of fractions A, B and 4:6&1:1, and compound D_2 respectively. Experiments were not performed using the test solutions of azadirachtin A with regard to this aspect due to limitation of the test material.

From the choice tests which were carried out using the test solutions of *M. volkensii* at the concentration (200ppm) which caused a drastic reduction in oviposition by less than 50%, all the solutions of *M. volkensii* test substances deterred oviposition significantly as compared to the controls. In all cases $p < 0.05$ and X^2 values were 25.0, 10.2, 10.2 and 6.8 (d.f. = 1 in each case) for the test solutions of fractions A, B and 4:6&1:1, and compound D_2 respectively (Table 18).

Table 17: Mean number and percentage of eggs laid by *Ae. aegypti* females in solutions of various concentrations of the *M. volkensii* test substances and control (n=20).

Test Substance	Concentration in ppm 50 Mean no. ± S.E.	%	100 Mean no. ± S.E.	%	200 Mean no. ± S.E.	%
Control*	1399 ±107	100[1]				
Fraction A	1292 ±155	93	1378 ±153	98	466 ±53	33
Fraction B	1184 ±79	85	782 ±86	55	305 ±35	22
Fraction 4:&1:1	852 ±103	59	755 ±88	53	212 ±23	15
Compound D_2	1335 ±150	95	1287 ±142	92	233 ±24	17

* In order to work out the percentage of eggs laid in the control, the mean number of eggs laid in that control (1399) was taken to be equivalent to 100%. It has been reported that the batch size of eggs laid by a female of *Ae. aegypti* is normally below 100 (Woke *et al.*, 1956; Ingram, 1954) but the average maximum is 76 eggs per female (Roy, 1936) which is almost equal to the size of eggs laid by each female in the control.

[1] Only one control batch was done with the three different concentrations of the test substances.

Table 18: Mean number and percentage of eggs laid by *Ae. aegypti* females in solutions of the *M.* test substances substances at 200ppm, and in control. Chi-square comparison results are shown as well (n=20).

Test substance	Mean no. ± S.E.	%	X^2 value
Fraction A	137 ± 15	25	25.0*
Control	409 ± 49	75	
Fraction B	290 ± 31	34	10.2*
Control	557 ± 61	66	
Fraction 4:6&1:1	190 ± 22	34	10.2*
Control	370 ± 43	66	
Compound D_2	301 ± 33	37	6.8*
Control	509 ± 83	63	

* significant at probability, p less than 0.05.

Degrees of freedom (d.f.) = 1.

Chi-square (X^2) comparison was performed by assuming that if the test solutions of *M. volkensii* did not influence the site selection behaviour of the ovipositing females then each petri-dish should have contained 50% of the total number of the eggs laid.

3.6 Hatchability of the eggs of *Ae. aegypti* laid in the oviposition deterring solutions (200ppm) of *M. volkensii* test substances as a measure of any ovicidal and post-embryonic effects.

Low numbers of larvae, 152, hatched from the eggs which were laid in the test solution of compound D_2 while 193 larvae hatched from the eggs which were laid in the test solution of fraction B thus showing some ovicidal effects. The number of larvae which hatched from the eggs which were laid in the test solutions of fraction 4:6&1:1 was 258. Almost all the eggs which were laid in the test solutions of fraction A hatched (384 larvae) just like in the control where 397 eggs hatched into larvae (Table 19 and Figure 18).

When the larvae which hatched from the eggs laid in the oviposition deterring solutions of *M. volkensii* were reared up to the emergence of adults so that their survival rate could be determined, only 28 larvae survived upto adults from the larvae which hatched from the eggs laid in the test solution of fraction 4;6&1:1. Less than half of the larvae (78) which hatched from the eggs laid in the test solution of fraction B survived upto adults. Almost half of the larvae (70) which hatched from the eggs laid in the test solution of compound D_2 survived upto adults while more than half of the larvae (249) which hatched from the eggs laid in the test solution of fraction A survived upto adults. In

Table 19: Mean number (± S.E.) of hatched larvae and adults arising from eggs of *Ae. aegypti* laid in the highest concentration (200ppm) of *M. volkensii* fractions and a compound tested (n = 400).

Develop. stage	Mean number (± S. E.)				
	Cntr.	F_A	F_B	$F_{4:6\&1:1}$	C D_2
Hatched larvae	397 ± 31	384 ± 43	193 ± 22	258 ± 23	152 ± 20
Emerged adults	364 ± 11	249 ± 8	*78 ± 4	28 ± 2	70 ± 6

n = number of eggs used per replicate for each test.

Develop. stage = Developmental stage.

Cntr. = Control F_A = Fraction A F_B = Fraction B

$F_{4:6\&1:1}$ = Fraction 4:6&1:1 C D_2 = Compound D_2

* indicates that 4 ± 1 dead pupal-adult intermediates were observed.

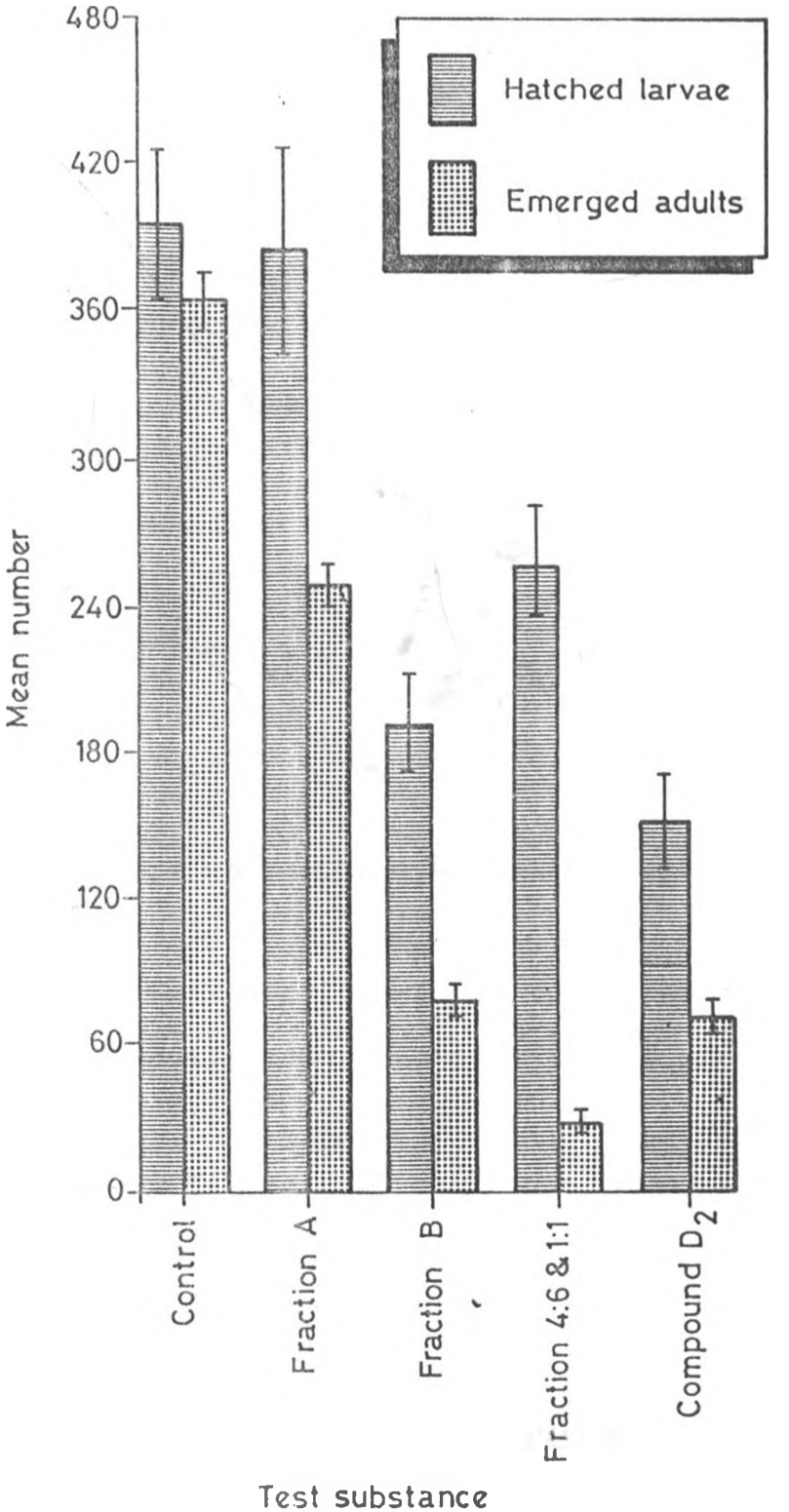


Figure 18: Mean number of larvae hatched and emerged adults from eggs of *Ae. aegypti* laid in the oviposition deterring concentration (200ppm) of the *M. volkensii* test substances (n = 400). The bars indicate S. E.

the control majority of the larvae (364) which hatched survived until the adults emerged (Table 19 and Figure 18).

Only four dead pupal-adult intermediates were observed from the larvae which hatched from the eggs which were laid in the oviposition deterring test solutions of fraction B. Other intermediate categories of development were not observed. Growth inhibition effect was also not observed on the larvae which hatched from the eggs which were laid in the oviposition deterring test solutions of the *M. volkensii* test substances.

3.7 The effect of fraction B from *M. volkensii* on three culicine mosquitoes in Chiromo campus during the limited field trial studies.

In this investigation environmental factors such as temperature (heat), light and rainfall (relative humidity) which were likely to have some effects on the activity of fraction B by either increasing or decreasing the concentrations under trial were not taken into consideration. Such environmental factors were assumed to represent the actual conditions in the field. Rates of evaporation of the test solutions from both the control and experimental tins were also assumed to be equivalent to the rates at which more water was getting into the tins hence, fluctuation in volume was expected to be negligible.

Three species of culicine mosquitoes; *Ae. aegypti*

and two *Culex* species of which one species was the majority were identified in the study area within Chiromo campus. On the ninth day after the start of the experiment there were very many first instar larvae of which 1425 were in the control tins and 1595 were in the experimental tins which had 25ppm of the test solutions (Table 20). No first instar larvae were present in the tins which had 50 and 100ppm test solutions after nine days. Only 61 dead first instar larvae were removed from the tins with 50ppm test solutions at that time. On the 14th day, the latest instar of development present in the control tins and the ones with 25ppm test solutions was the second instar of which 102 were in the control tins and 123 were in the test solutions having 25ppm. In the tins which had 50ppm test solutions there were 512 first instar larvae only while at the same time there were still no first instar larvae present in the tins which had 100ppm test solutions though a few dead ones (27) were found and removed.

There were 97, 367 and 9 third larval instar present in the control tins and the ones which had 25 and 50ppm test solutions respectively on the 18th day as the latest stage of development but in the tins which had 100ppm test solutions there were still no larval instars present. On the 22nd day the latest stage of development present in the control tins and the ones with 25ppm test solutions was still the third instar. In the tins which had 50 and 100ppm test solutions there were only 448 and 70 first instar larvae respectively.

Table 20: The effect of fraction B from *M. volkensii* on three species of culicine mosquitoes available in Chiromo campus during the limited field trial studies.

Duration (day)	Conc. (ppm)	Developmental stage present: Instar I	Instar II	Instar III	Instar IV	pupa	Adult	Total
9	0	1423	-	-	-	-	-	1423
	25	1595	-	-	-	-	-	1595
	50	0[a]	-	-	-	-	-	0
	100	0	-	-	-	-	-	0
14	0	1603	102	-	-	-	-	1704
*	25	1436	123	-	-	-	-	1559
	50	512	0	-	-	-	-	512
	100	0[a]	0	-	-	-	-	0
18	0	711	209	97	-	-	-	1017
*	25	174	394	267	-	-	-	835
**	50	522	94	9	-	-	-	625
	100	0	0	0	-	-	-	0
22	0	589	102	251	-	-	-	942
	25	210	168	448	-	-	-	826
	50	448	0[b]	0	-	-	-	448
	100	70	0	0	-	-	-	70
26	0	664	213	302	0	-	-	1179
	25	490	399	396	14	-	-	1299
*	50	803	318	0	0	-	-	1121
	100	252	0[b]	0	0	-	-	252
30	0	807	584	223	18	-	-	1632
	25	686	407	435	21	-	-	1549
	50	537	373	19	0	-	-	929
	100	168	140	0	0	-	-	308
34	0	497	592	256	40	0	-	1385
	25	655	331	265	23	1	-	1275
	50	443	448	89	0	0	-	980
	100	159	135	0	0	0	-	294
38	0	370	302	314	15	23	-	1024
	25	636	327	233	8	11	-	1215
	50	200	544	47	5[c]	0	-	796
**	100	488	75	5	0	0	-	568

(table 20 cont.).

42	0	388	279	295	13	21	0	996
	25	638	259	216	8	13	11	1145
	50	359	407	30	7	0	0	803
	100	426	103	1	0	0	0	530
46	0	394	257	344	14	18	1	1028
	25	579	349	212	12	6	13	1171
	50	303	464	81	5	4	0	857
	100	378	156	197	0	0	0	731
50	0	418	289	345	11	10	5	1078
	25	523	370	238	30	9	6	1176
	50	339	274	87	13	7	3	723
	100	496	211	105	0[c]	0	0	812
54	0	520	307	314	19	16	10	1186
	25	511	342	292	24	18	7	1192
	50	379	292	92	18	1	0	782
	100	451	241	93	2	0	0	787
58	0	481	336	299	21	13	11	1161
	25	534	335	289	20	17	6	1201
	50	341	272	83	15	0[e]	0	711
	100	405	269	81	9	0[d]	0	764
62	0	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-

Conc. = Concentration

- (dash) means there was no any developmental stage present.

[a] Dead first instar larvae were seen.

[b] Dead second instar larvae were seen.

[c] Dead moulting third larval stage were seen.

[d] Dead 4th instar larvae-pupal intermediate stage were seen.

[e] Dead pupae were seen.

! All the test solutions had evaporated and there was no data collected.

* and ** Indicate patricular days when the activity of 50ppm was almost similar to that of 25ppm, and that of 100ppm was also almost similar to that of 50ppm respectively.

100 dead second instar larvae were observed in the tins which had 50ppm test solutions on the 22nd day.

There were very few fourth instar (14) present as the latest stage of development in the tins which had 25ppm test solutions on the 26th day while in the control tins there were no fourth instars yet. It was on the 30th day when fourth instar larvae were present in the control tins as the latest stage of development. The tins which had 50ppm test solutions had 318 second instar larvae only as the latest stage of development at the same time (26th day) while in the 100ppm test solution tins there still were first instar larvae (252) but few dead second instar larvae (8) were observed. At the same time (on day 30) there were 19 third and 140 second instars present in the 50ppm and 100ppm test solution tins respectively as the latest developmental stages. There were no new developmental stages present in the control tins and the experimental ones on the 34th day except for one pupa which was present in the 25ppm test solution tins. It was on the 38th day when 23 and 11 pupae were present in the control tins and the experimental tins with 25ppm test solutions respectively as the latest stage of development. By this time, only five fourth instar larvae were present in the tins which had 50ppm test solutions as the latest stage of development while in the 100ppm test solution tins, the latest stage of development was the third instar which were only five.

The first few adults (11) emerged from the 25ppm

test solution tins on day 42 while in the control and the other experimental tins, the only noticeable change was on day 46 in the 50ppm test solution tins where four pupae were present as the latest stage of development. On day 50, some adults emerged from the control tins and 50ppm test solution ones as the latest stage of development while in the 100ppm test solution tins, dead moulting third/fourth instar larvae were seen. On day 58, three dead fourth instar larvae-pupal intermediate stage were observed from 100ppm test solution tins. Close examination of the samples of larvae taken from treated and the control tins revealed that first, second and third instar larvae died during moulting to the next larval stages. Most of the fourth instar larvae in 50ppm test solution tins died as pupae. By the time the experiment ended on the 62nd day, only three adults had emerged from the 50ppm test solution tins while in 100ppm test solution tins, the pupal developmental stage was never attained.

When determining the biodegradation rate of fraction B during the field trial test, it was found that after a period of about 10 days the activity of the 50ppm test solutions (on day 26) had been reduced and was almost similar to the one of 25ppm test solutions (on days 14-18) while the activity of 100ppm test solutions (on day 38) approached the one of 50ppm test solutions (on day 18) after a period of 20 days (Table 20).

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION

It has been shown that an individual's susceptibility to a poison is influenced by different parameters such as weight, sex, age, environmental temperature and humidity, time of contact with poison, and genetic heterogeneity or variability of the insect population under test (Brown and Pal, 1971). An attempt was made to standardise all these parameters during the experimentation whereby the test larvae (second instar), adults, and the eggs were picked randomly from the population which was assumed to be homogeneous with respect to the genetic composition. This homogeneity was tested by Chi-square statistics at degrees of freedom less two the number of responding concentrations when evaluating toxicity of the various test substances with the second instar larvae of *Ae. aegypti*. Homogeneity was found to hold true in all cases at 0.05 probability level (Appendix 1).

When comparing the effect(s) of the active principles in *M. volkensii* test substances and azadirachtin A, a stage-specific difference in biological activity becomes visible depending on the test substance under investigation. It is clear from the results presented on toxicity that at a higher concentration of 200ppm, all the *M. volkensii* test

substances had acute toxicity within 48 hours but there were two test substances (fractions 4:6&1:1 and B) which exhibited acute toxicity at even moderate concentrations of 50 and 100ppm. Fraction 4:6&1:1 had acute toxicity at even a lower concentration of 20ppm. No acute toxicity was observed in any concentration of azadirachtin A within 48 hours. When the LC_{50} values were taken into consideration, fraction 4:6&1:1 had the most acute LC_{50} value of 9.4ppm followed by fraction B which had LC_{50} value of 18.2ppm. Fraction A and compound D_2 had low LC_{50} values of 44.5 and 55.3ppm respectively. Azadirachtin A did not show any lethal effect since its LC_{50} value was greater than 200ppm. These results are in agreement with similar studies by Mwangi and Mukiama (1988) who found that compounds from *M. volkensii* cause lethal effects which occur within 48 hours on the second instar larvae of *An. arabiensis*. On the other hand, Zebitz (1984) reported that even after exposure of the second instar larvae of *Ae. aegypti* to excessive concentrations of neem seed kernel extracts and azadirachtin, there was no acute toxicity.

Data presented on the activity of some mixtures of the test substances of *M. volkensii* revealed that there was no noticeable synergism effect when the two toxic fractions 4:6&1:1 and B or one very toxic fraction , B, and a less toxic one A, were mixed. So far no data are yet available on the synergistic effects of the extracts of either the neem tree or the Chinaberry tree.

The fact that the oily nature of fraction A lowers the acute toxicity of fraction B, which was further confirmed when fraction B was mixed with a neutral oil, olive oil, has some significance on the practical application of these toxic fractions from *M. volkensii* for mosquito larvae control programmes on open water surfaces. In the field application of the toxic fractions of *M. volkensii* for the control of mosquito larvae, oily water surfaces should be avoided since they are likely to lower the acute toxicity of these fractions though it is known that during formulation of the majority of inorganic and synthetic pesticides, different types of oils are added to enhance their biological activities. In such cases different types of oils act as inhibitors to evaporation, wash off by rain-water, and resistance mechanisms of insect pests or vectors. This is the first report on the effect of oil in lowering the acute toxicity of the extracts from *M. volkensii*.

It is very interesting that azadirachtin A did not show any acute toxicity after 48 hours in the highest concentration tested but it caused the highest percentage total mortalities (100%) over a longer duration at concentrations of 5-15ppm which were tested than almost all the test substances of *M. volkensii* at the same concentrations. Even at the lowest concentration tested, 1ppm, azadirachtin A still caused the highest percentage total mortality rate (53%) than

all the *M. volkensii* test substances at the same concentration. It is therefore possible to conclude that the physiological effects of azadirachtin A occur at later stages during the mosquito larval development after treatment and they cause greater effects at even very low doses than those of *M. volkensii* test substances thus, mimicking some insect growth hormones or their analogues.

Morphogenetic effects were evident when the second instar larvae of *Ae. aegypti* were exposed to the test solutions of *M. volkensii* and azadirachtin A until the emergence of adults. Higher percentage total mortalities which occurred during the various larval stages in the solutions of test substances showed a stronger larvicidal effect of the test substances, however, some pupicidal effect was also observed. Al-Sharook *et al.*, (1991) also observed some larvicidal and pupicidal effects on the larvae of *Cx. pipiens molestus* when using the acetone extract fraction from *M. volkensii* and azadirachtin A. Unfortunately, Mwangi and Mukiama (1988) did not record any death during the pupal stage. Majority of the deaths observed in the pupal stage were as a result of the fourth instar larvae failing to ecdyse into perfect pupae thus producing deformed pupae which were short lived with some dying within 24 hours after ecdysis. Nevertheless, the pupae which were dying at later stages (when adults were just about to emerge) on close examination were found to

lack properly developed adult features as opposed to their counterparts in the control which had visible properly developed adult features.

The occurrence of greater numbers of different intermediate categories and higher larval percentage total mortality rates in the test solutions of *M. volkensii* shows a wide range of activity of the active principles in *M. volkensii* than azadirachtin A. The least number of different intermediate stages resulting from fraction 4:6&1:1 of *M. volkensii* and azadirachtin A can be explained by the fact that these two test substances eliminate most of the larvae during their early larval developmental stages before reaching the stages where intermediate stages can be observed.

The occurrence of different features in the dead moulting larvae together with the presence of various intermediate stages of development, unmelanised larvae, sluggishness and twisted S or U shaped resting postures in the solutions of different test substances, probably indicates some physiological interference these test substances exert on the moulting and growth processes thus acting as insect growth inhibitors. Surprisingly, the transformation from pupal to adult stage was least affected since there were only three non-viable adults and very few pupal-adult intermediate stages arising from the solutions of all the test substances as indicated in the results that the mean percentage total of the pupal-adult intermediate category was very

low in all the test substances and did not exceed 5%.

According to Mwangi and Mukiama (1988) the sluggish movement and peculiar coiling of treated larvae suggest some neural or muscular disturbance by the active principal(s) which may be causing acute lethal effect. This type of effect was not observed on the larvae treated with superlethal doses of neem seed kernel (Zebitz, 1984). Mwangi and Mukiama (1988) further stated that the delayed lethal effect of the extract however, was more likely to be caused by a disturbance of the endocrine mechanisms that regulate moulting and metamorphosis. This mechanism of action has been postulated previously for neem seed kernel extracts (Rembold, 1984; Zebitz, 1986). Failure for some experimental larvae to ecdyse, melanise, and the production of fourth instar larvae-pupal and pupal-adult intermediates, and some adults with malformed wings, would seem to support a similar postulation for *M. volkensii* test substances (Mwangi and Mukiama, 1988).

High percentage total emergence of adults was only observed from the lower concentrations, i.e. less than 20ppm, of the solutions of different *M. volkensii* test substances which still varied depending on the particular test substance under investigation. These adults were found to be normal and no lethal effect was observed in the next generation. These results suggest that the active compounds in *M. volkensii* and azadirachtin A might be similar in their activity and

for physiological response to occur, a threshold must be reached. This therefore explains why it was possible for normal adults to emerge from lower concentrations. But, Mwangi and Mukiama (1988) found that it was not possible to produce healthy adults of *An. arabiensis* when larvae were reared in water containing as little as 1 µg/ml of certain fractions of *M. volkensii* extracts. The active compounds from *M. volkensii* extracts (Mwangi and Mukiama, 1988, 1989; Mwangi, 1989; Al-Sharook *et al.*, 1991) and the neem tree (Zebitz, 1986) have been found to exhibit various degrees of toxicity to different mosquito species with *Ae. aegypti* responding to higher doses. The lethal effects caused by the active compounds from *M. volkensii* extracts on the mosquito larvae occur earlier than the ones caused by the extracts from the neem tree (Mwangi and Rembold, 1988). The results of the present study therefore tend to suggest that the extracts from *M. volkensii* can be effectively used in the control of different mosquito species but further bioassays on various species of mosquitoes is still required.

Exposure from the second instar larvae to adult emergence resulted in an obvious delay of development though it differed with the stage of development and the test substance. All the test substances of *M. volkensii* and azadirachtin A showed good growth inhibition activity mostly on the third and fourth instars with the second instar being only slightly affected. Most of the

larvae with extended longevity were smaller in comparison to the controls in the corresponding instar especially those treated with azadirachtin A at 5ppm where none of them attained adulthood. Majority of the larvae with extended longevity died mostly during ecdysis at various intermediate stages. In some solutions of the test substances such larvae resulted in very small adults as compared to the adults emerging from the controls. No growth inhibition effect occurred on the pupal stage since the few pupae which emerged into normal adults from the solutions of some of the *M. volkensii* test substances took almost the same period of time as the ones which were in the control. Overall, the test solutions of azadirachtin A, and a mixture of fractions (A+B) showed good growth inhibition effects although azadirachtin A produced the best growth inhibition effect at a lower concentration of 2.5ppm than all the *M. volkensii* test substances.

Mwangi and Rembold (1988) concluded that the active compound in *M. volkensii* has acute toxicity at higher doses, and growth inhibiting activity at lower doses, to some extent this does not seem to be true. It is apparent that there are two types of compounds in *M. volkensii* fruit extract: one group of compounds is acutely toxic to mosquito larvae. According to this investigation it is present in fractions 4:6&1:1 and B; the second group is growth inhibiting and is present in almost all the test substances of *M. volkensii*, namely,

fractions 4:6&1:1, A and B, and compound D_2. These results therefore support the later findings of Mwangi and Mukiama (1988) who also made similar conclusions. Azadirachtin A seems to be exclusively a growth inhibiting compound. The growth inhibitory mode of action of the bioactive *M. volkensii* compounds may be like that of azadirachtin A as was proposed by Al-Sharook *et al.*, (1991). It is noteworthy that even at a higher concentration of 50ppm, fraction A still shows good growth inhibition effect and despite the fact that it lowers the acute toxicity of fraction B when they are mixed together, the mixture had the highest growth inhibition effect than all the *M. volkensii* test substances. This might suggest that some growth inhibiting activity is lost during further purification of *M. volkensii* extracts. Mwangi and Rembold (1988) found that some growth inhibiting activity is actually lost during the concentration of the active material. Acute toxicity might also be lost during further purification processes since a very pure compound like D_2 lacks acute toxicity as compared to Fraction B which is a mixture of a number of compounds. Also the toxicity of fraction 4:6&1:1 which has been purified further is only about twice that one of fraction B. Mwangi and Rembold (1988) also reported that the acetone soluble fraction of *M. volkensii* was only 5-6 times more active than the crude extract. This further points out that at certain stages of purification, the active compounds

in the fruit extracts of *M. volkensii* are lost.

The structure of the bioactive compounds in *M. volkensii* however still remain to be determined (Mwangi, 1989). Although limonoids such as volkensin with demonstrable antifeedant activity against armyworm *Spodoptera frugipenda*, and salannin (Rajab *et al.*, 1988) have been isolated, they may not be contributing to the bulk of the activity in the crude exract since they are inactive in *Aedes* bioassay (Mwangi and Bentley unpublished observation). These active compounds are likely to be limonoid tetranortriterpenoids similar to those in the neem tree, although azadirachtin is not present in detectable quantities in the fruit extracts of *M. volkensii* (Mwangi, 1989; Al-Sharook *et al.*, 1991). According to Al-Sharook *et al.*, (1991), the good water solubility of the active compounds in the acetone extract fraction of *M. volkensii* indicates the presence of saponins as toxic principles, and hence make the acetone extract from *M. volkensii* an interesting candidate for its application against mosquito larvae and other aquatic vectors of diseases.

The greater number of larvae resting in the S or U shaped postures and stretching frequently after feeding on the newly formulated acetone extract pellets within 24 hours show that the newly formulated acetone extract pellets had some acute physiological effects on the larvae within that time. Since Mwangi and Mukiama (1988) postulated that the sluggish movement and

peculiar coiling of the larvae treated with *M. volkensii* extracts seem to suggest some neural or muscular disturbance by the active compounds in those extracts, the resulting S or U resting postures and frequent stretching observed from the larvae which were given the acetone extract pellets within the first 24 hours, can be explained in the same way. But the fact that later on there was no significant difference in resting postures between the larvae which were given the newly formulated acetone extract and the control pellets, can be as a result of the larvae which were given the acetone extract pellets developing some tolerance or excreting the active compounds in *M. volkensii*.

Failure of growth inhibition effects being observed on the larvae which were given the newly formulated acetone extract pellets is likely to support the theory which was put forward by Mwangi and Rembold (1988) that since the *M. volkensii* extracts contain antifeedant activity, it is likely that part of the poor growth is due to the antifeedant activity and not growth regulation as such. This seems to be true because in this investigation, during the process of formulation of the new acetone extract pellets, the acetone extract fraction was coated with the jelly crystals to prevent the antifeedant effect which could have repelled the larvae when they attempted to feed on the pellets. The larvae fed on the pellets and no growth inhibition activity was observed. Mwangi and Rembold (1988) and Al-

Sharook *et al.*, (1991) observed growth inhibition effects when the second instar larvae of *Ae. aegypti* and the third instar larvae of *Cx. pipiens molestus* respectively were exposed to the *M. volkensii* acetone extract fraction test solutions. However, more data is needed to determine the contribution of each of these two modes of action for the active compound in the extract.

The high larval mortality which occurred on the third and fourth larval instars of *Ae. aegypti* when they were given the pellets formulated from the acetone extract fraction of *M. volkensii* show that these pellets can be the most effective strategy for the control of mosquito larval stages when applied on water as larval food since *Ae. aegypti* breeds mostly in artificial containers and further more this extract so far has not been reported to have any toxic effects on mammals. Better results can be obtained if a method can be found of formulating pellets from the toxic fractions of *M. volkensii* like 4:6&1:1 and B.

The increase of hatchability with increasing concentrations and the cause of earlier hatching in the solutions of less toxic test substances (fraction A, compound D_2 and azadirachtin A), and the slight increase in hatchability with increasing concentrations and the cause of late hatching in the solutions of acute toxic (lethal) test substances (fractions 4:6&1:1 and B) on *Ae. aegypti* eggs seem to be a very interesting

phenomenon. This shows again how the active compounds in *M. volkensii* have a diverse range of activity and which again falls under two groups. The less toxic group of test substances from *M. volkensii* (fraction A and compound D_2) act as hatching stimulants while the acute toxic group (fraction 4:6&1:1 and B) act as hatching inhibitors. Azadirachtin A acts only as a hatching stimulant. This aspect is worth further investigating though a brief physiological explanation is given below.

In *Ae. aegypti* the first indication of hatching is the appearance of a slow-widening transverse crack in the dorsal surface of the egg shell towards anterior end caused by the pulsatile movements of the larva (Christopher, 1960). Therefore the solutions of the less toxic test substances may be causing some irritation effects on the embryo leading to wriggling and thus increasing the frequency of the pulsatile movements as the concentration increases. This resulted in increased pulsatile movements which caused earlier hatchability. But the rate at which irritation was being caused varied depending on the toxicity level of the test substance under investigation. On the other hand, the solutions of the acute toxic (lethal) test substances might be killing the embryo immediately they are in contact with it as their concentration increases thereby causing very little irritation effects on the embryo resulting into very few or no pulsatile movements which could increase hatchability. Omollo (1982) also

observed some inhibition effects on the hatchability of *Stomoxys* and *Locusta* eggs when using different concentrations of the aqueous extracts of *M. volkensii*, *A. indica* and *M. azedarach.* Dead larval stages, very few or no adults emerging from higher concentrations (100-200ppm), and very low mean percentage of intermediate stages arising from the eggs which hatched from the solutions of various test substances indicate at least the presence of some active compounds which interfere with the post-embryonic development in *Ae. aegypti.*

The effect of *M. volkensii* extracts on the oviposition response of mosquitoes has not been well investigated. It appears from the results of the present study that at a higher concentration (200ppm), the *M. volkensii* test substances are both antiovipositant and ovicidal. Indeed, Jiang' (unpublished paper) observed that aqueous extract from the fruit kernels of *M. volkensii* at a higher concentration of 100ppm had a strong repellant effect on the egg laying behaviour on adult females of *Cx. pipiens* for about one week. Similarly, (Yadav, 1985) reported that treating green gram (*V. radiata*) seeds with 30mg neem seed oil/10g seed was ovicidal and 50mg/10g seed was antiovipositional in *C. analis* and 40mg/10g was antiovipositantal in *C. chinensis* and *C. analis* while Singh and Srivastava (1983) found the ethanol extract of the petroleum ether-extracted kernel oil to be antiovipositional deterrent

at 2.5% or higher concentrations against *Dacus dorsalis* (Hendel). It appears that relatively higher concentrations are required to achieve antiovipositional effect than an ovicidal effect (Parmar, 1986). The *M. volkensii* test substances seem to act as contact ovipositional deterrents since most of them are soluble in water and it is known that the chief stimulus for oviposition in *Ae. aegypti* is the actual contact with water (Kennedy, 1941).

No data are yet available on the effects of pure azadirachtin A on the oviposition behaviour of adult mosquitoes although azadirachtin has been found not to repel or inhibit oviposition response of *Heliothis armigera* females (Saxena and Rembold, 1983). Neem oil has been found to cause a considerable ovipositional deterrent effects to the females of *Culex* species but not to *Ae. aegypti* and the percent larval hatch of the eggs laid in the neem seed kernel extracts has also been found not to differ from the control (Zebitz, 1986).

The least number of adults emerging after the rearing of the larvae which hatch from the eggs which were laid in the oviposition deterring concentration of some test substances from *M. volkensii* is a clear indication of disruption of the post-embryonic development in *Ae. aegypti*. The delayed post-embryonic effects seem to suggest some direct effects on the embryos' programming cells by the active compounds in *M. volkensii* rather than the persistence activity.

It is surprising that the active compounds in *M. volkensii* can exert effect on the embryonic development of *Ae. aegypti* and at the same time act as an ovicide. Such actions of the compounds would seem to be the most practical means for mosquito control since it will be possible to eliminate to be the potential harmful stages of the mosquito as well as the next generation. Even if the application is not early enough to prevent hatching, the delayed effects will prevent metamorphosis into a normal reproductive adult but still if the adults emerge , they will be deterred from oviposition.

Information on the physiological action of the active compounds from the Meliaceae family, especially *M. volkensii*, still remains fragmentary. The fact that azadirachtin interferes with post-embryonic development (Redfern *et al.*, 1981; Sieber and Rembold, 1983; Gaabouch and Hayes, 1984; Schluter and Schulz, 1984) and reproduction (Streets and Schmutterer, 1975; Rembold and Sieber, 1981; Schulz and Schluter, 1984) suggest an interference with hormonal mechanisms or insect endocrine system. Indeed, influence on the ecdysteroid titre in hemi- and holometabolous insects was found by Redfern *et al.*, (1982), and Sieber and Rembold (1983). Mwangi (1982) observed the same effects using *M. volkensii* extracts on the nymphs of *S. gregaria.* More recently, Rembold *et al.*, (1984) reported a complete inhibition of juvenile hormone production in adult *L. migratoria* as a consequence of single injection of

azadirachtin. The effect of azadirachtin on these two morphogenetic hormones could be interpreted as interference with the neuroendocrine systems which controls ecdysone and juvenile hormones. May be the release of the trophic hormones from corpus cardiacum into the haemolymph is reduced or even inhibited by azadirachtin and that is the first step in its mode of action, which ends with inhibition of growth/moulting (Rembold, 1984). Likewise the same explanation can be offered for the active compounds in *M. volkensii*. Azadirachtin is similar in structure to insect moulting hormones (Nakanishi, 1975) (Appendix II) and apparently act as an insect ecdysis inhibitor (Kubo and Klocke, 1982), perhaps by influencing the quantity or quality of the "pool" of moulting hormone (Rembold *et al.*, 1982). The structures of some *M. volkensii* compounds (salannin and volkensin) which have been determined also have some similarities to azadirachtin and the insect moulting hormones (Appendix II). Though the structure of the compound(s) contributing to most of the activity in *M. volkensii* extracts has not yet been determined, it might also be similar to the structures of the known tetranortriterpenoids from the Meliaceae family, and the insect moulting hormones. These structural similarities suggest some relationship in biological activity (Leuschner, 1972). According to Rembold *et al.*, (1986), the epoxide structure is of major importance for growth inhibiting activity of all the azadirachtin

derivatives. Since the epoxide structure is also present in volkensin, salannin and other compounds (Figure 2) from *M. volkensii* (Appendix II), it might be contributing to most of the growth inhibition activity observed in the *M. volkensii* test substances.

Williams (1967) suggested that hormone analogues act in very small quantities. This property may be exhibited by the active compounds in *M. volkensii* test substances and therefore can be used as "Third Generation Pesticides" if a well planned investigation is carried out and the active compounds are isolated. Unlike juvenile hormones or their analogues which must be applied to insects at abnormal times in their developmental programmes in order to exert some physiological effects (Schneiderman, 1971), the active compounds in *M. volkensii* test substances can be applied at any time on any stage of development of an insect for morphogenetic effects to occur (Mwangi, 1982; Mwangi and Mukiama, 1988; Mwangi and Rembold, 1988; Mwangi, 1989; Mwangi and Kabaru, 1991; Rajab *et al.*, 1988; Al-Sharook *et al.*, 1991). This therefore shows that the active compounds in *M. volkensii* extracts have a long term (prolonged) effect and wide range of activities on various insects hence are superior to juvenile hormones or their analogues in terms of activity and can be used effectively in the control of disease vectors and insect pests.

Toxicity of the active compounds in the Meliaceae

family has not been thoroughly investigated and systematically studied in mammals. The derivatives from the other two members of the family (*A. indica* and *M. azedarach*) have been reported to be non-toxic to man and other mammals (see literature review). Apart from being non-toxic to mammals, extracts from the Meliaceae family have also been reported to show very little adverse effects on beneficial insects at higher concentrations. Awala (1990) found that the lady bird beetle, *Cheilomenes* species show very little response to the crude extract of *M. volkensii* at 4% which was very toxic to other insect pests. The predatory spider, *Lycosa pseudoannulata* (Boesberger and Strand) has also been found not to be adversely affected by the highest dose tested, 50µg neem oil/spider (Saxena *et al.*, 1983). Similarly, Mansour *et al.*, (1986) studying the effect of neem seed kernel extracts from different solvents on the predacious mite, *Phytseilus persimilis* and the phytophagous mite, *Tetranychus cinnabarinus* reported that all the extracts were considerably much more toxic to the pests than the predators. Zebitz (1986) when studying the effect of neem seed kernel extracts on non-target organisms also found that only the highest concentration (100ppm) of the enriched extract (AZT-VR-K-E) was considerably toxic to the water lice, *Asellus aquaticus* while the Guppies, *Lebistes reticulatus* were not adversely affected by any of the three concentrations (1, 10 and 100ppm) tested. Because the

impact of beneficial insects on pest insects is well known, the lack of adverse effects of the Meliaceae family extracts on predators must be considered of greatest importance when their field application is contemplated. This is also another advantage the extracts from this family have over the other earlier botanical (organic) pesticides derived from plants such as nicotine, pyrethrum powder (pyrethrin) and rotenone, and the newly developed synthetic pesticides which had been reviewed earlier on in this study.

At present very little is known concerning field application of *M. volkensii* extracts on the control of agricultural pests (Mwangi, 1989; Mwangi and Kabaru, 1991) while nothing is yet known on the control of mosquitoes in the field. However, data from the limited field trial studies within Chiromo campus using fraction B from *M. volkensii* indicate some promising results. The absence of the first instar larvae in the 50 and 100ppm test solutions for about two and three weeks respectively, and the fact that in the 50ppm test solutions only three adults emerged and none from the 100ppm test solutions after a period of two months show that *M. volkensii* extracts can successfully be used in the field though the activity of this fraction, B, was found to be lower in the field. This lowered activity might have been due to the environmental factors (light, temperature, heat and relative humidity) which have been found to affect the activity of the active compounds

like azadirachtin (Jacobson *et al.*, 1984). The slow growth rate which was observed from the larvae which were in both the control and experimental test solutions could not allow for any growth inhibition effect in the field to be assessed. The slower growth rate was likely to be due to lower temperatures (19 $\pm$ 2^{o}C) which were experienced during the limited field trial study period which commenced on 3rd May, 1992 and ended on 30th July, 1992.

The little information available on the field application of *M. volkensii* extracts on crops revealed that the extract can protect the crops for a period of between three to four weeks and the level of protection compared favourably with the commercially available insecticides, and that the biodegradation rate of the active limonoid in *M. volkensii* extracts take long enough for insect pests to be eliminated or repelled to alternative host plants (Mwangi, 1989; Mwangi and Kabaru, 1991). Similar results were also observed in the limited field trial study although the biodegradation rate of the active compound(s) in fraction B from *M. volkensii* was only about two to three weeks. Successful field trial experiments have been conducted using the extracts from the other members of the Meliaceae family (in reviews by Schmutterer *et al.*, 1981, 1984, 1986) and if the active compound(s) in *M. volkensii* is similar to the ones found in the exracts of the other Meliaceae species, then they also can be used in the field

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successfully for the control of crop insect pests and disease vectors.

From the current study, it can be concluded that the extracts of *M. volkensii* contain active compounds which act as insect growth regulators (IGRs), feeding inhibitors, antiovipositants and ovicides just like the extracts from the other members of the family. This knowledge has, however, not been commercially exploited, and a thorough understanding of the physiological basis for the action and effects of the active compounds in *M. volkensii* can help to develop this concept to a stage it could be employed in third world countries to reduce insect pest/vector damages as well as multiple ecological, health and social problems caused by improper application of inorganic and synthetic pesticides. Further more since *M. volkensii* flourishes in semi-arid areas, it could serve the purpose of afforestation in such areas to reduce soil erosion and at the same time provide timber which are resistant to insect attack for the building industry and firewood. in addition to the extraction of the active compounds which are distributed in all parts of the plant (Mwangi and Kabaru, 1991).

4.2 CONCLUSIONS

1. It is apparent that there are two groups of the active principals in *M. volkensii* extracts:
   (a) One group has acute toxicity at higher concentrations within 48 hours on the second instar larvae of *Ae. aegypti*.
   (b) The other group has growth inhibiting effects at lower concentrations.

2. Apart from good growth inhibition effect observed in the test solutions of fraction A, generally it had very little effects on all the other aspects which were investigated.

3. Azadirachtin A does not exhibit any acute toxicity at higher concentrations within 48 hours on the second instar larvae of *Ae. aegypti*, but it has good growth inhibiting effects at lower concentrations just like *M. volkensii* extracts.

4. The overall physiological effect of azadirachtin A at lower concentrations on growth and development against various larval stages of *Ae. aegypti* is better than that of *M. volkensii* test substances.

5. *M. volkensii* test substances and azadirachtin A have stronger larvicidal and slight pupicidal effects on various developmental stages of *Ae. aegypti*.

6. The newly formulated pellets from the acetone extract fraction of *M. volkensii* can be the most effective strategy for the control of various larval stages of *Ae. aegypti*.

7. *M. volkensii* extracts can be used as a source of potent mosquito growth inhibitor, larvicide, pupicide, ovicide and antiovipositant of plant origin.

8. Based on the results of this investigation, *M. volkensii* extracts seem to have greater advantages over azadirachtin A in the control of *Ae. aegypti*.

9. The use of extracts from *M. volkensii* and azadirachtin A for the control of various developmental stages of *Ae. aegypti* (mosquitoes) should not be considered on short term effects, e. g. mortality after 48 hours, but should be based on the post-treatment effects after a period of time.

10. Since *M. volkensii* extracts can be effectively used in the control of *Ae. aegypti*, further research should be carried out to find the possibilities of using these extracts for the control of some insecticide-resistant populations of mosquitoes and other disease vectors.

11. Naturally derived insecticide products from *M. volkensii* offer promising possibilities for the third world countries, where the tree is indigenous, as an alternative source to the inorganic and synthetic pesticides which are associated with multiple ecological, health and social problems arising as a result of their improper application in the control of insect disease vectors and crop pests.

APPENDIX I

Chi-square (X^2) values testing for homogeneity of the data (n = 20).

Test substance	X^2 value	Degrees of freedom (d.f.)	Probability (p)
Fraction A	0.085	6	p > 0.99
Fraction B	0.418	6	p > 0.99
Fractions (A+B)	0.070	6	p > 0.99
Fraction (4:6&1:1)	0.084	6	p > 0.99
Fractions (4:6&1:1+B)	0.211	6	p > 0.99
Compound D_2	0.067	6	p > 0.99
Olive oil	0.050	5	p > 0.99
Fraction B +Olive oil	0.061	5	p > 0.99
Azadirachtin A	0.032	6	p > 0.99

Tabulated X^2 values (Fisher and Yates, 1963):

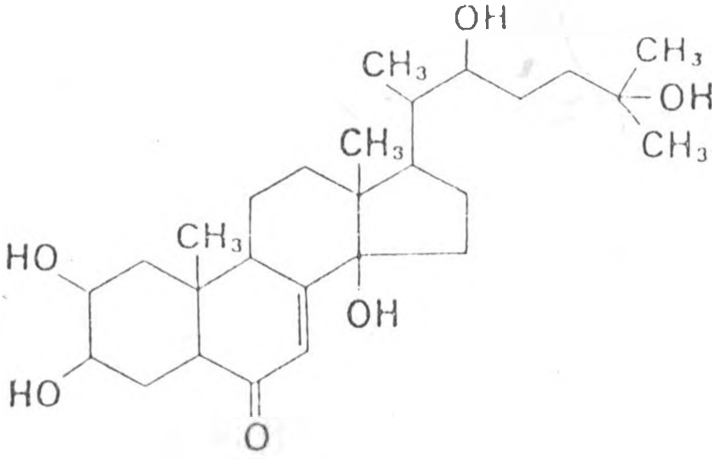
$X^2_{0.05,\ 5} = 11.070$ $\quad$ $X^2_{0.99,\ 5} = 0.554$

$X^2_{0.05,6} = 12.592$ $\quad$ $X^2_{0.99,\ 6} = 0.872$

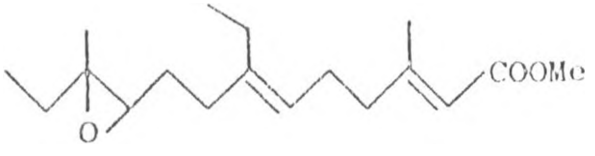
From the appendix 1 above, there is no significant difference in homogeneity of the data in all cases statistically, p > 0.99, d.f. = 6 and/ or 5. This therefore shows that the data points are linear and hence fit probit analysis.

APPENDIX II

(a): Structural formulae for α- ecdysone and juvenile hormone I.



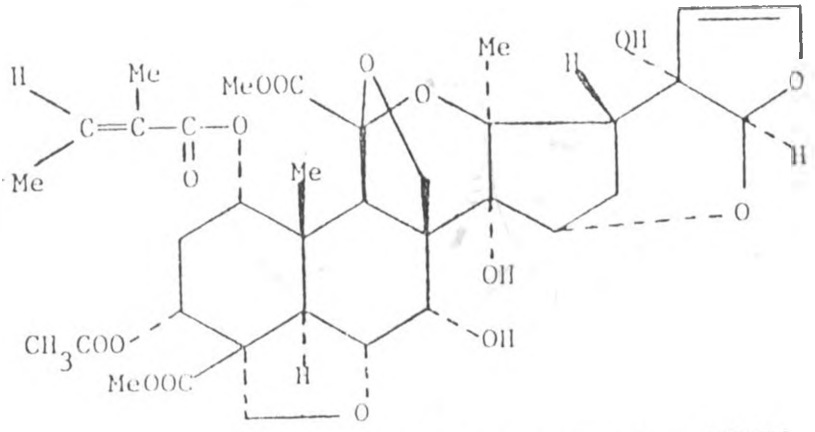
α-ecdysone (Schneiderman, 1971)



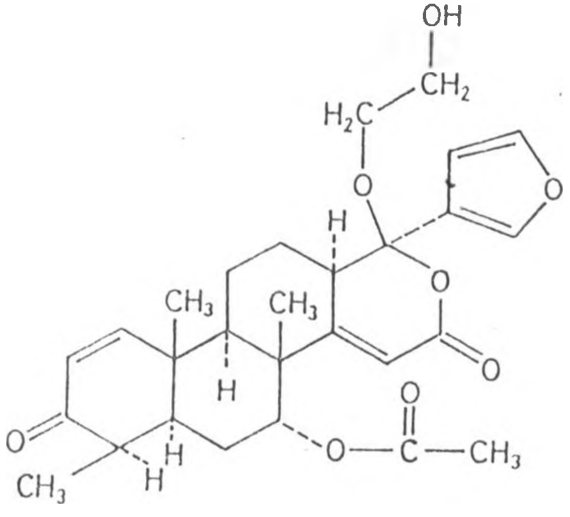
juvenile hormone I (Schneiderman, 1971)

(Appendix II cont.).

(b): Structural formulae for some compounds obtained from the the Meliaceae family (*A. indica*, *M. azedarach*, *M. toosendan* and *M. volkensii*).

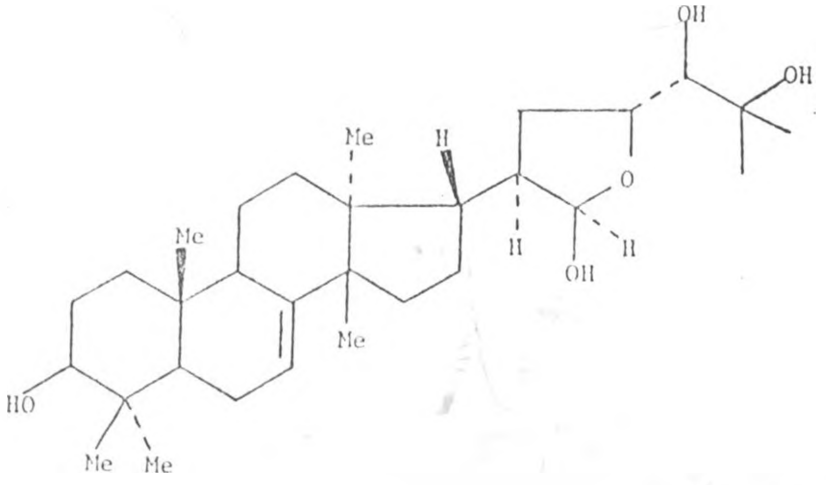


azadirachtin (Zanno *et al.*, 1975)

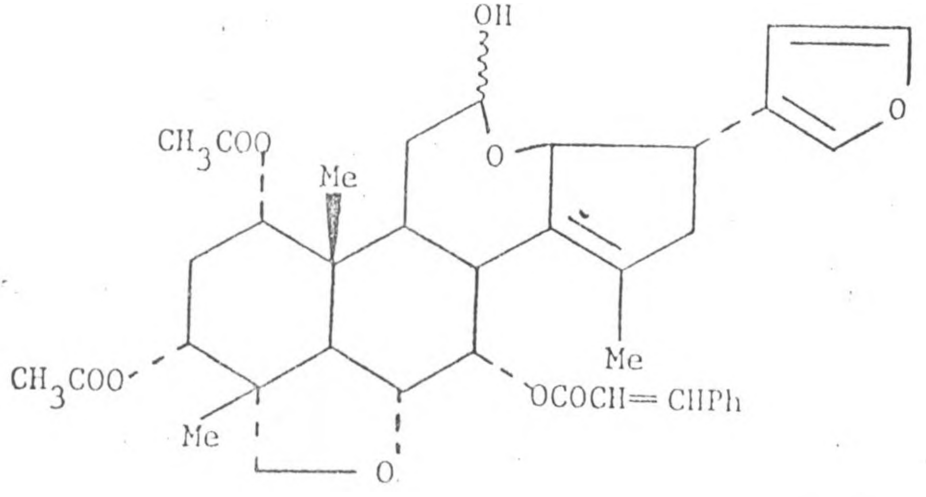


Mahmoodin (Siddiqui *et al.*, 1992)

(Appendix II (b) cont.).

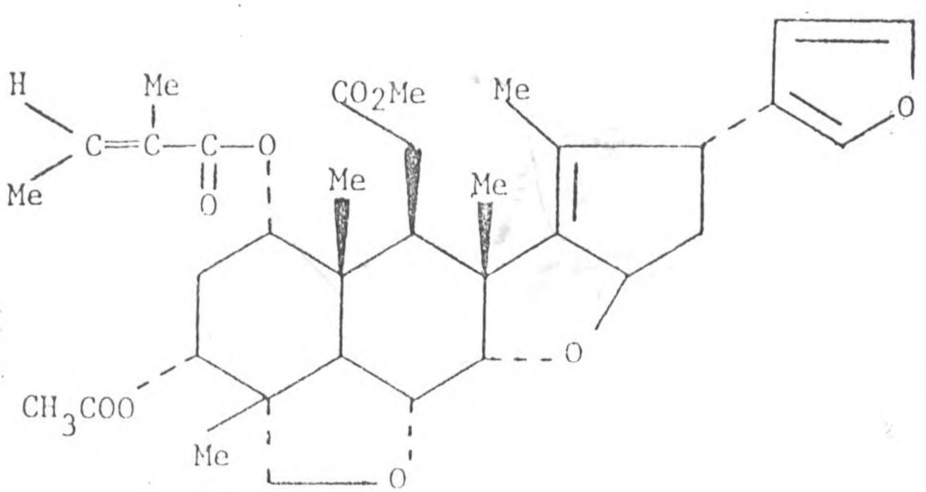


Meliantriol (Levie *et al.*, 1967)

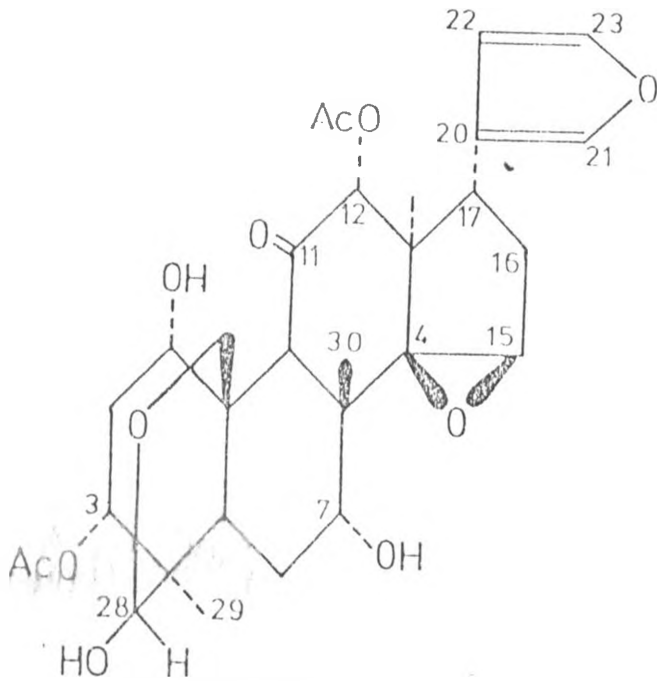


nimbolin A (Siddiqui et al., 1988)

(Appendix II (b) cont.).

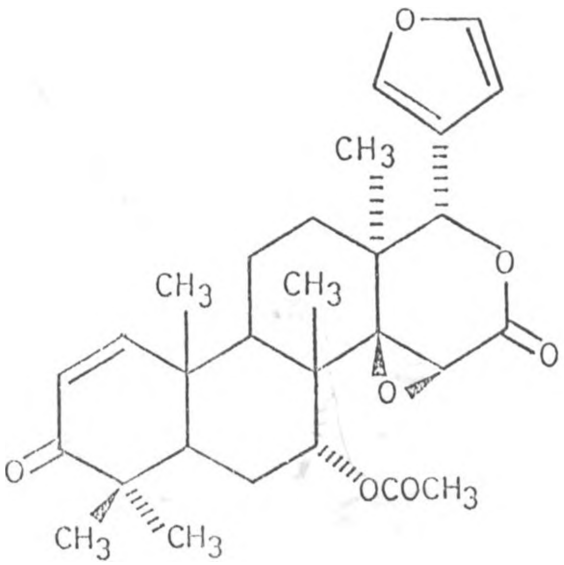


Salannin (Henderson *et al.*, 1964; Rajab *et al.*, 1988)

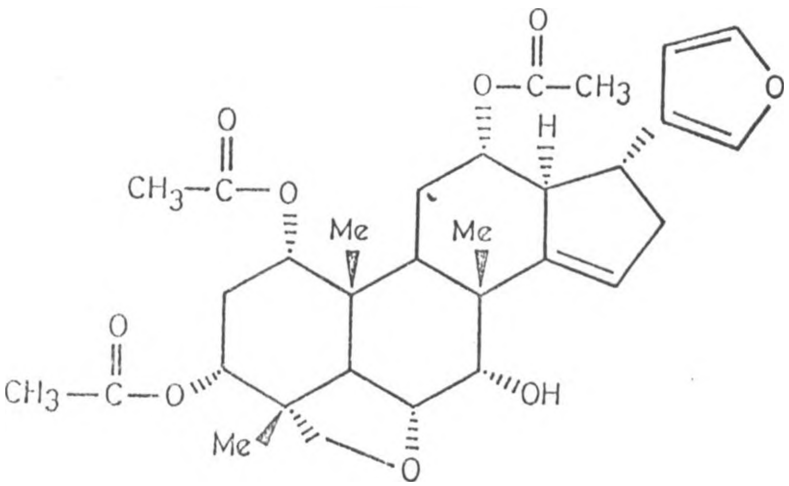


Toosendanin (Shu and Liang, 1980)

(Appendix II (b) cont.).

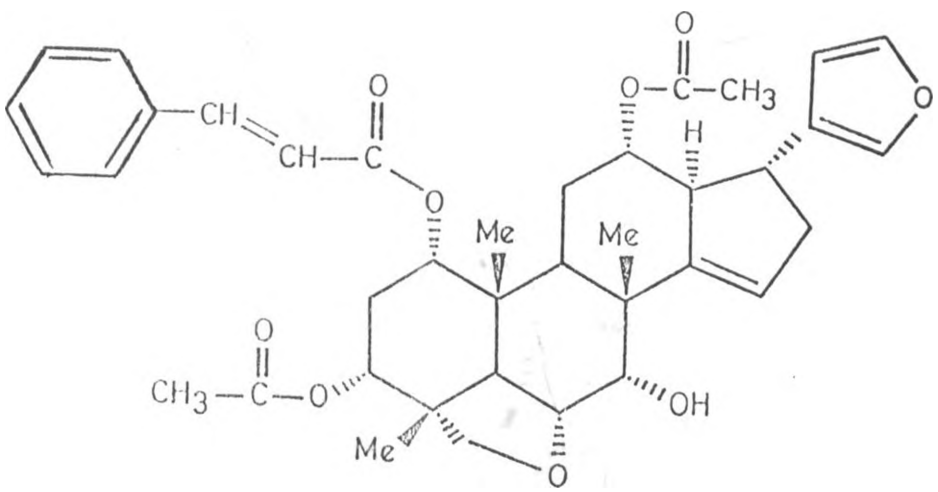


gedunin (Khalid et al., 1989)

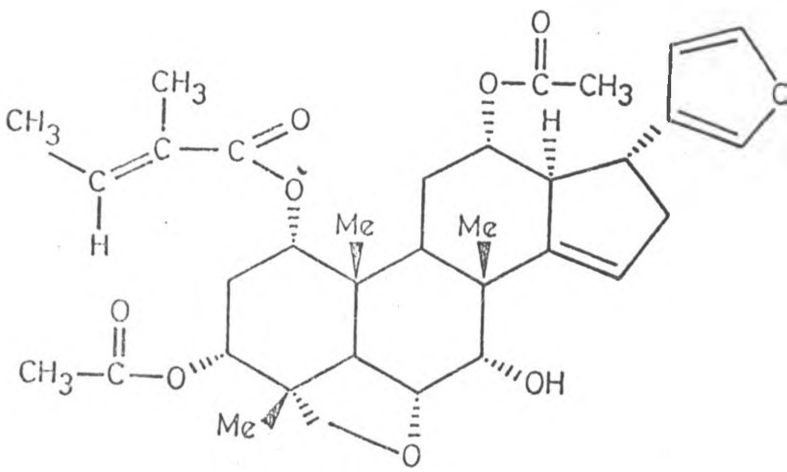


1-acetyltrichilinin (Rajab and Bentley, 1988)

(Appendix II (b) cont.).

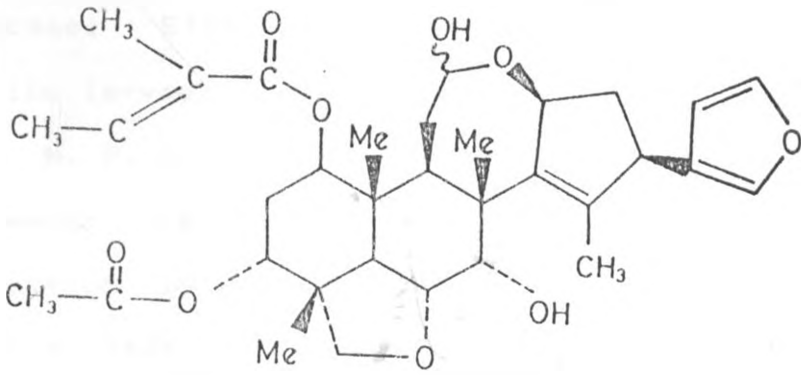


1-cinnamoyltrichilinin (Rajab and Bentley, 1988)



1-tigloyltrichilinin (Rajab and Bentley, 1988)

(Appendix II (b) cont.).



Volkensin (Rajab *et al.*, 1988)

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