ENVIRONMENTALLY COMPATIBLE PHYTO-CHEMICAL LARVICIDES FOR MOSQUITO CONTROL

Thesis submitted in partial fulfilment of the requirements for the Degree of

Doctor of Philosophy

Under
The Faculty of Environmental Studies

by

LATHA C.

SCHOOL OF ENVIRONMENTAL STUDIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI - 682 016

APRIL, 1998

Certificate

This is to certify that the results presented in this thesis entitled **Environmentally Compatible Phyto-Chemical Larvicides for Mosquito Control** are based on the original research carried out by Ms. Latha, C. under my guidance in the School of Environmental Studies, Cochin University of Science and Technology in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title or recognition.

Kochi - 682 016
April, 1998

Dr. Ammini Joseph,
Supervising Guide
Senior Lecturer
School of Environmental Studies
Cochin University of Science
& Technology, Kochi - 682 016

DECLARATION

I, Ms. Latha, C. hereby declare that the results presented in this Ph.D. thesis entitled Environmentally Compatible Phyto-Chemical Larvicides for Mosquito Control are based on original research work carried out by me under the guidance of Dr. Ammini Joseph, Senior Lecturer, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title or recognition.

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Kochi - 682 016

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INTRODUCTION

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- ★ Objective of the Present Investigation

CHAPTER 1

INTRODUCTION

The use of chemicals to control insect pests dates back to the time of ancient culture. Homer, the Greek epic poet, had mentioned the fumigant value of burning sulphur around 1000 B.C. The Romans treated themselves with hellebore to destroy infections of human lice. Dioscorides, the Greek physician, knew the toxic nature of arsenic. Before A.D. 900, the Chinese were applying arsenic to control garden pests.

A few truly effective pesticides known to the European agriculturists in the late 1700s were the botanicals and heavy metals. The botanicals were pyrethrum, derris, and tobacco leaf infusions. Arsenic and mercury were used for seed treatments. The unparalleled expansion of American agriculture during 1820-1860, and concomitant pest infestation of crops resulted in the introduction of Paris Green in the late 1860 to control colorado beetle of potatoes. This was followed by the production of a number of heavy metal-based insecticides to control a variety of insect pests.

The discovery of DDT, in 1939, revolutionized our concept of insecticides and insect control. It's low cost of production, high persistence and selective toxicity towards insects and mammals, and broad spectrum of contact insect activity made it the most widely manufactured insecticide ever produced. This was followed by the introduction of a number of synthetic organochlorine insecticides. During World War II the Germans opened the

field of organophosphorous insecticides which led to the development of parathion, coumaphos,dementon and many more. Over the years several analogs of organophosphorous reached the market. The carbamates were the third major group of synthetic insecticides to be discovered in the early 1950s (Hayes and Laws, 1991).

Pesticide use in developing countries is indispensable in the struggle against hunger and disease. Overload on the public services, poor sanitation facilities, pollution of air, drainage ditches, scarcity of housing, lack of water, and open dumps of garbage in the urban slums and slanty towns in developing countries, catalyse the proliferation of vectors and vector-borne diseases. The World Health Organization Report (WHO, 1997) states that despite the increase in life expectancy achieved in the 20th century, the health expectancy of the humanity, particularly in the developing world, is not impressive. Whatever be the mode of transmission, the infectious diseases continue to take heavy toll of life in the developing countries. Fleas and mosquitoes are the most important vectors in cities. Of all insects that transmit disease, mosquitoes represent the greatest nuisance.

Major Vector-Borne Diseases

The vector-borne diseases requiring greatest attention are malaria, dengue, dengue haemorrhagic fever, dengue shock syndrome, filariasis, Japanese encephalitis, yellow fever, schistosomiasis, onchocerciasis, trypanosomiasis, and plague. The data of prevalence of vector-borne disease is inadequate, but it is roughly estimated that more than five million children die of vector-borne diseases annually throughout the world (Das and Jumbulingam, 1997).

Malaria is essentially a disease of the poor countries, and included under 'Tropical Diseases' by WHO. Improper water storage methods are the main causes of the proliferation

of urban malaria vector *Anopheles stephensi*. The malaria situation in 1950s was very serious in India with over 75 million cases occurring, and resulting in the death of 0.8 million (Pattanayak *et al.*, 1994). In 1960s near elimination of malaria was possible in India through the extensive use of insecticides and anti-malarials. The enthusiasm was so great, and in 1963 research on malaria lost importance, and the publication of *Indian Journal of Malariology* was stopped. There were financial cuts and malaria was considered as a disease of no importance in the context of national priorities; but in a few years malaria struck back with vengeance. In 1976, National Malaria Eradication Programme (NMEP) reported 6.46 million parasite positive cases. Among various steps taken in 1977, research on malaria was reemphasized (Sharma, 1996). In the last three decades, there have been intensive control efforts against malaria. Still, it remains a major health problem in several countries. Malaria is endemic in 91 countries, with about 40% of the world's population at risk. Of the estimated 500 million infections, and two to seven million deaths from malaria every year, over 90% occur in the African sub-continent. In fact nine out of ten cases of malaria occur in sub-Saharan Africa, while two-thirds of the rest are concentrated in just six countries *i.e.*, India, Brazil, Sri Lanka, Vietnam, Combodia, and Solomon Islands (WHO, 1996).

Epidemic dengue haemorrhagic fever and dengue shock syndrome were first reported in Philippines in 1956. Since then, epidemics have occurred periodically in other South-East Asian countries (WHO, 1986a). Dengue fever and dengue haemorrhagic fever are closely related conditions caused by four distinct viruses transmitted by *Aedes aegypti*. Dengue is the world's most important mosquito-borne disease and the spreading of the disease is the result of improper water storage methods. A total of 2500 million people world wide are at the risk of infection. An estimated 20 million cases are added each year (WHO, 1996).

A number of species are involved in the transmission of yellow fever. Some of them are *Aedes aegypti, Ae.africans,* and *Ae.simpsoni*. The number of yellow fever cases reported to WHO from Africa increased in mid 1980s; but decreased in 1991. From America a total of 50-250 cases are reported annually (WHO, 1995a).

Japanese encephalitis is posing a threat to public health in recent years. From a sporadic form, the disease is showing trends of periodical outbreaks in the past few years in different parts of the world. About three quarters of the cases occur in the Western Pacific, China, and adjacent countries with the remainder occurring in South-East Asia, especially India. The transmission cycle of this mosquito-borne virus disease involves wild birds, and domestic pigs as amplifying hosts. The most common mosquito vectors are *Culex tritaeniorhynchus, Cx.vishunui* and *Cx.bitaeniorhynchus* (Reuban *et al.*, 1994). In Malaysia, *Culex sitiens* acts as the vector of Japanese encephalitis. An estimated 43,000 cases of Japanese encephalitis occur globally each year, with 11,000 deaths, and nearly 9,000 disabled (WHO, 1996).

Inadequate solid waste management and poor water management are often the major causes for the proliferation of the filariasis vector *Culex quinquefasciatus*. The spreading of the disease is mainly due to unplanned urbanisation and an all-round environmental degradation. About 120 million people are infected in tropical areas of Africa, India, South-East Asia, the Pacific islands, and South and Central America (WHO, 1996). Species of *Culex* transmit filariasis caused by *Wuchereria bancrofti*. *Culex sitiens* is also reported to be infected with *Wuchereria bancrofti* and *Brugia malayi* (Panicker *et al.*, 1981).

Schistosomiasis is transmitted by fresh water aquatic and amphibious snails that serve as intermediate hosts. Aquatic snails of the genus *Bulinus* transmit *Schistosoma haematobium* and the genus *Biomphalaria* transmit *S. mansonia*; amphibious snails of the genus *Oncomelania* transmit the *S. japonicum* group and the hybrid snails of genus *Neotricula* transmit *S.mekongi*. In most endemic areas, the highest prevalence and intensity of infection are found in children between five and fifteen years of age. The high prevalence of schistosomiasis in many parts of the world is directly related to human contact with natural water bodies (WHO, 1993).

Onchocerciasis is caused by *Onchocerca volvulus*. This nematode is transmitted by *Simulium* flies. The disease is a serious threat in many parts of tropical Africa, Latin America and Arabian peninsula. In Africa spread of onchocerciasis is a consequence of environmental changes such as deforestation, resulting in the conversion of forest habitats into savannah and the creation of artificial breeding sites (WHO, 1995b).

African trypanosomiasis or sleeping sickness is endemic in 36 countries of Sub-Saharan Africa. The World Health Organization Report estimates that some 50 million people are at the risk of acquiring the disease. Although 22 species of *Glossina* occur in the Sub-Saharan region of Africa only a few are known to be vectors of human trypanosomiasis. The parasites are *Trypanosoma brucei gambiense* and *T. b. rhodesiense* (WHO, 1986b).

Plague is regarded as a disease of medieval times. During 1995, 1,400 cases of human plague were reported from different parts of the world to WHO including at least 50 deaths. The disease occurs particularly in rodents. It spreads from rat to rat and from rat to humans mainly by rat fleas. Rapid rate of urbanization is the main cause of the high rate surveillance of the disease, despite the availability of effective drugs (WHO, 1996).

The two principal approaches to control vector-borne diseases are through pesticide application, either by larviciding or indoor spraying, and chemotherapy.

Among third world countries, India was one of the first countries to start large scale use of synthetic pesticides for control of insect pests of public health and agricultural importance. The modern era of vector control and plant protection in India started with the introduction of DDT in 1947 (Mehrotra,1985). This was followed by organophosphates in 1953 and later carbamates (Banerjee, 1979). It was widely believed that these powerful pesticides could be used to control malaria as well as other vector-borne diseases. Unfortunately, total reliance on these synthetic chemicals too often brought about unexpected side-effects. These include environmental contamination, toxicity to non-target organisms, evolution of resistance, and proliferation of both old and new pests. It is estimated that upto 90% of the pesticides being used never reach their intended targets. They reach several categories of non-target species, plant and animal hosts, and natural enemies living in or near the community. As a result many beneficial organisms are poisoned unintentionally (Hayes and Laws, 1991; Cunningham and Saigo, 1995).

Most organochlorines such as DDT, telodrin, aldrin, endrin, dieldrin, and hepatochlor are too toxic for use in environment. One of the best known characteristics of organochlorine pesticides is their persistence in the environment for decades, possibly centuries. Surveys conducted in different countries revealed contamination of organochlorine pesticides in milk and in fatty food. Contamination is quite high and widespread in developing countries, where these pesticides are still predominant (Singh and Dhaliwal, 1993). Experiments with birds proved that DDE, a persistent breakdown product of DDT, was responsible for the thinning of egg shells, and adverse effects on reproduction (Blus, 1995). Recent studies revealed that DDT and DDE can mimic the effect

of estrogen (Cunningham and Saigo, 1995). Organophosphorous and carbamate residues in seed grains, vegetation, and formulated pesticide granules have killed a large numbers of wildlife. Many of these are extremely toxic to birds and mammals (Hill, 1995).

Because of the immense insecticidal pressure on arthropods, a number of resistant varieties have evolved (Pedigo, 1989). Insects are all now resistant to commonly-used classes of compounds such as organochlorines, organophosphorous compounds, and carbamates. Eighty one arthropods were reported resistant to insecticides in 1962. By 1968 this number had increased to 102 (WHO, 1970). In 1987 the number was greater than 500 (Engel *et al.*, 1990). As WHO (1986c) reports, twenty species of *Culex* show resistance to one or the other kind of insecticides. Resistance to DDT and organophosphorous compounds is widespread in *Culex quinquefasciatus*. Specific malathion resistance has been reported in *Cx. annulirostris*. Nineteen species of *Aedes* are recorded to be resistant to these insecticides. *Aedes aegypti* has shown resistance to carbamates as well as DDT and organophosphorous compounds. At least fifty seven species of *Anopheles* have been reported to be resistant to one insecticide or the other. Although most of the reports are of resistance to organochlorines, there are instances of resistance to organophosphorous compounds and carbamates (WHO, 1986d). Apart from increasing insecticidal resistance by the mosquito vector, the malarial parasite has also demonstrated resistance to all the major anti-malarial drugs, including the newer drugs such as artemesinin and its derivatives (Kondrachine and Trigg, 1997).

The application of agricultural pesticides has led to the development of resistance in a number of anopheline vector species and field breeder *Cx.tritaeniorhynchus*, a vector of Japanese encephalitis (WHO, 1986c). The use of modern pesticides in agriculture has

eliminated many useful insects and hymenopteran parasites that helped in controlling vector population. For example, Coelomonyces, a fungal infection on mosquito, was quite common; but with the extensive use of insecticides the infection rate drastically reduced due to decline in cyclops and other ostracod population. It also induced resistance in the vector population (Das and Jambulingam, 1997).

The resurgence of many vectors and outbreaks of epidemics in 1970s, led to the simultaneous development of a new group of pesticides which are substantially safer to mammals and cause least environmental hazards. The organochlorine pesticides have fallen out of favour or banned for use in developed nations, and the organophosphorous and carbamate chemicals together with synthetic pyrethroids dominate the pesticide industry today (Hodgson and Kuhr, 1990). A gradual shift is also evident towards reshaping the policy of vector control, and agricultural pest control with greater emphazis on environmental management and integrated methods of control. It is essential to find suitable alternatives which are not only safe and effective but also available, renewable, and compatible with nature. In recent years there has been an increasing interest in the use of alternative methods of insect pests and vector control. Some of them are pheromones, repellents, antifedants, and biopesticides. The third generation pesticides such as, juvenile hormone analogues and ecdysone are also used against insect pests and vectors (Aidely, 1976). Biopesticides are considered to be an effective technique for controlling vectors. It is nothing but the control of harmful pests by using other insects, pests, plants or any such living body. Biopesticides have the potential to replace chemical pesticides and have a long history. Even in Neolithic times farmers used biopesticides prepared from the seeds of resistant plants (Chakraborty and Basu, 1997). Today, neem is regarded as nature's own answer to effective insect control.

Biological control is one of the most important techniques for controlling insect pests. De Bach (1974) stated that 120 different pest species are completely or partially controlled by natural enemies. Approximately 1,500 naturally-occurring microorganisms or microbial by-products have been identified as potentially useful insecticidal agents. The most widely-used insecticidal agents are the two subspecies of *Bacillus thuringiensis-israelensis (Bti)* and *kurstaki*. *Bti* has exhibited significant larvicidal activity against *Aedes aegypti, Cx. pipiens* and *An. stephensi* (Aizawa and Ohba, 1985). Most strains of *B. sphaericus* are potentially useful larvicides against populations of *Culex* and some species of *Anopheles*. The NPV and BV baculovirus sub-groups have received most attention as viral pesticides (Kawanishi and Held, 1990). Biological control agents suitable for controlling mosquito breeding are the larvivorous fishes, *Gambusia affinis* and *Poecilia reticulata*, the nematode *Romanomermis iyengiri*, the fungus *Lagenidium giganteum*, and *Toxorhynchites* (VCRC, 1989).

Biopesticides have been suggested as an effective technique in integrated vector control. The philosophy of integrated control envisioned by WHO (1983) is a combination of available methods to control vectors with minimum ecological damage. It involves the use of:

- pesticides and the simultaneous or sequential use of personal protection
- habitat management and source reduction
- biological control using fish
- public education
- prophylasis or treatment with drugs

The essential requirement for integrated control is the availability of more than one method; eg., a selective pesticide without detrimental effect on naturally occurring biological control agents, an adequate knowledge of biology, ecology, and behaviour of the associated non-target organisms and of human behaviour to ensure not only effectiveness in controlling the vector but also human safety and prevention of other unacceptable side-effects.

1.1. OBJECTIVE OF THE PRESENT INVESTIGATION

In the last decades considerable headway has been made in research and development of phyto-chemical pesticides. The most notable recent success is the commercial development of neem products for insect control. The present investigation on **Environmentally Compatible Phyto-Chemical Larvicides for Mosquito Control** was undertaken to identify plants of the locality with potential larvicidal activity on mosquito larvae. This has been achieved by screening 17 plant species against four mosquito species. The observation and data are compiled in the next six chapters to follow.

CHAPTER 2

COLONIZATION AND REARING OF THE TEST ORGANISMS - THE MOSQUITOES

- ★ Introduction
- ★ Life Cycle
- ★ Distribution of the Mosquito Species in Kochi City

- ★ Materials and Methods
- ★ Selection of the Test Organisms
- ★ Colonization and Rearing of Mosquito Species
- ★ Rearing Cages
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- ★ Results and Discussion

CHAPTER 2

COLONIZATION AND REARING OF THE TEST ORGANISMS - THE MOSQUITOES

2.1. INTRODUCTION

Mosquitoes have a world-wide distribution occurring through the tropical, temperate regions, and arctic regions. The only area where they are absent is Antarctica. They are found at elevations of 5500 m and in mines at depths of 1250 m below the sea level. There are about 3100 species of mosquitoes belonging to 34 genera (Knight and Stone, 1977). The most important man-biting mosquitoes belong to the genera *Anopheles, Culex, Aedes, Mansonia, Haemagogus, Sabethes,* and *Psorophora* (VCRC, 1989).

Among different mosquito species a few are vectors of the tropical diseases such as filariasis, yellow fever, Japanese encephalitis, dengue and dengue haemorrhagic fever, malaria etc. The medical importance of *Culex, Aedes, Anopheles* and *Mansonia* are given in Table 2.1.

Life Cycle

The life cycle of a mosquito has four stages, namely egg, larva, pupa, and adult. The eggs are usually laid in water or in places likely to be submerged later. The eggs may be laid singly as in *Anopheles*, or *Aedes*, or collectively in egg rafts as in *Culex*. When the eggs are laid on water they may hatch within a few days, but those laid out of water remain unhatched until submerged.

Table 2.1. Symptoms of mosquito-borne diseases

Mosquito species	Disease	Symptoms
Culex quinquefasciatus	St. Louis encephalitis	Fever and aches, headache
	Bancroftian filariasis	Fever, headache, rash, inflammation of lymph nodes and lymphatic vessels
Culex tritaeniorhynchus		
Culex vishunui	Japanese encephalitis	Severe headache, fever, vomitting, cerebral haemorrhage
Culex bitaeniorhynchus		
Aedes aegypti	Dengue	Fever, headache and severe pain in the limbs and joints
	Viral encephalitis	Headache, fever, nausea with subsequent convulsions
	Yellow fever	Headache,fever, congestion of conjunctiva, haemorrhage, black vomit, blood in stools
Anopheles stephensi	Malaria	Shivering, fever, headache and pain in the limbs
Mansonia annulifera	Brugian	Fever, headache, inflammation
Mansonia uniformis	filariasis	in hands, and genital organs
Mansonia indiana		

The larvae are always aquatic. They are active and voracious creatures feeding on bacteria, yeasts, algae, protozoa and organic matter. The larval period may be as short as 7 to 10 days,

but may extend to several months depending on the temperature and other environmental conditions. On the fourth moult the mosquito larvae transform to the pupae.

The pupae are unusual in that they remain mobile and responsive to external stimuli. The pupal period lasts for 1 to 5 days under favorable conditions. The pupal skin then splits along the back and adult mosquito extricates itself.

After the emergence of adults they rest on the water or the edges of the breeding habits during which their wings unfold and the body wall gets hardened. The life span of the adults varies with the species, but often extends from several weeks to months. The biting and feeding habits vary from species to species. Some species bite man (anthropophilic) and the females require blood meal for oviposition. Certain other species are animal biting (zoophilic). The male mosquitoes feed on nectar. Odour, temperature and carbon dioxide emanating from the body are the attraction of the females towards the host. After obtaining the blood meal the mosquitoes rest. By the time the blood is fully digested, the ovaries and the eggs are fully developed. The gravid mosquitoes choose appropriate habitat to lay the eggs. The eggs undergo further development in the aquatic habitat and thus the life cycle continues (VCRC, 1989).

Distribution of the Mosquito Species in Kochi City

The bionomics, ecology and species composition of mosquito population in Kochi have been extensively studied. On an average, single individual in Kochi is bitten by 20 to 2352 mosquitoes per night in different months of the year. It is estimated that if ten mosquitoes bite a man per night a human population of one million would be loosing approximately 50 liters of blood per night (Dhanda, 1996).

The important breeding places of mosquitoes in Kochi city are,

1. Drains/canals
2. Septic tanks
3. Cement tanks
4. Pools/ponds
5. Pit latrines
6. Marshy lands
7. Wells
8. Over-head tanks
9. Water meter chambers
10. Other miscellaneous household containers

The contribution of mosquitoes in terms of daily emergence from various habitats varies with season and locality. The larval population is minimum in drains and cannals during the rainy months due to flushing or flooding. Their contribution during the rest of the seasons is high, accounting for over 90% of the total mosquito production from all source. The next major breeding source is septic tanks. There are over 1.2 lakhs septic tanks in Kochi Corporation area and most of them are heavy breeding places of mosquitoes (Dhanda, 1996). The mangrove habitats found in different parts of Vypeen Island in the suburb of Kochi is infested with *Culex sitiens* (Mariappan *et al.*, 1996).

A total of 35 species of mosquitoes belonging to eight genera have been listed from Kochi city (Mariappan *et al.*, 1997). The mosquito menace in Kochi is mainly due to the

night biting mosquitoe species such as *Culex quinquefasciatus, Culex sitiens, Armigeres subalbatus, Mansonia* species and *Anopheles* species. The important day biting mosquitoes are *Aedes aegypti* and *Aedes albopictus*.

2.2. MATERIALS AND METHODS

Selection of the Test Organisms

Because of the importance in public health as vectors of different human diseases (Table 2.1), four important mosquito species which are widely distributed in and around Kochi city were selected as test organisms, namely *Culex quinquefasciatus, Culex sitiens, Aedes aegypti* and *Anopheles stephensi*. The breeding site of these species is given in Table 2.2.

Table 2.2. Breeding sites of test species

Test organism	Breeding site
Culex quinquefasciatus	Drains/Canals/ Cess pits
Culex sitiens	Mangrove pits/ Coastal areas/ Casuarina pits/Coir pits
Aedes aegypti	Domestic containers/ Cement tanks/ Coconut shells
Anopheles stephensi	Over-head water tanks

Colonization and Rearing of Mosquito Species

The larvae and pupae of the test species were collected from the densely populated breeding sites of Kochi city and Vypeen Island by means of standard dippers.

Cx.quinquefasciatus was located in the drains and canals of the city, whereas *Cx. sitiens* was collected from the mangrove pits of Vypeen Island. Water samples also were collected from the latter site to assess the salinity. Salinity was estimated using a salinity refracto-meter. The *Aedes* larvae and pupae were collected from various domestic containers and from discarded coconut shells. The Anopheline larvae and pupae were collected from over-head water tanks using standard nets. The larvae and pupae collected were transferred immediately to labelled containers.

The larvae and pupae were sorted in the laboratory. The late fourth instar larvae and the pupae of the mosquitoes collected were transferred separately to water containing test tubes and were plugged with cotton. These test tubes were carefully monitored, reared to adults, and properly identified according to Barraud (1934). After identification the adults of *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* were transferred into colony cages meant for them.

Rearing Cages

Culex and *Aedes* species were reared in colony cages of size 2x2x2 ft., and *An. stephensi* in cloth cage of size 1x1x1 ft. The rearing cages were provided with grapes, soaked blotting papers, and soaked cotton wool. The female mosquitoes were fed on 3 to 4 month old chicken every other day.

Care of Eggs

Water containing bowls were kept inside the cages of *Cx. quinquefasciatus* for laying eggs. Bowls containing saline water of salinity $15x10^{-3}$ were kept for egg laying by *Cx. sitiens* as the larvae were originally collected from saline habitat of Vypeen Island where the salinity of the water ranged from 12 to $20x10^{-3}$ In the case of *Ae. aegypti* soaked blotting

paper was introduced into the rearing cages for laying eggs. Blotting paper wrapped around water containing beakers were provided inside the cages of *An. stephensi*.

Egg rafts of *Cx. quinquefasciatus* and *Cx. sitiens* were transferred to water containing bowls kept outside the cage. The eggs of *Cx. sitiens* were allowed to hatch into the saline water of salinity $15x10^{-3}$. The single eggs of *Ae. aegypti* were removed into the water containing bowls kept outside the cage with the help of a brush. The anopheline eggs were allowed to hatch inside the beaker wrapped with blotting paper; as the eggs hatched, the first instar larvae were transferred to the water containing bowls.

Care of Larvae

Newly hatched larvae were fed on powdered dog biscuits and yeast powder in the ratio of 2:1. The larvae were observed to undergo four moults. In order to avoid any fungal infection the larvae were transferred to fresh tap water every alternate day.

Care of Pupae

The pupae as they formed were transferred into the colony cages meant for different species.

The females in the colony laid fertilized eggs which were transferred outside to hatch out into larvae. The loss of adults in the colony was made up by the emergence of pupae put into colony cage each day.

Experimental Conditions

Cx. quinquefasciatus, Cx. sitiens, and *Ae. aegypti*: Adults and immature stages were reared at 28 ± 2°C, over 60 -75% R.H.

An.stephensi: Adults were reared at 25 ± 1°C, over 80% R.H., and the immature stages were reared at 28 ± 2°C, over 60-75% R.H.

2.3. RESULTS AND DISCUSSION

After obtaining the blood meal, the gravid females laid eggs on the third day. The egg rafts of *Culex* species and the single eggs of *Aedes* and *Anopheles* hatched out on the second day. The first instar larvae underwent four moults and subsequently the fourth instar larvae pupated in six to seven days. The pupae emerged as adults within 24 to 48 hours. By successive replacement of pupae into the rearing cages a stable population of the four mosquito species could be maintained.

The laboratory colonization of the three species, *Cx. quinquefasciatus, Cx. sitiens,* and *Ae. aegypti* were achieved in large cages without any sophisticated equipments. These species prefer large cages for mating. When they were transferred to small cages they showed low rate of egg laying . The highest survival rate and egg laying of *An. stephensi* was achieved only in cloth cages. The relation between cage size and mating rate of mosquitoes and consequently the egg laying has been observed by the previous workers as well (Shono and Hirano, 1993).

Temperature is another crucial factor that influences the egg laying habit of these mosquito species. During the monsoon and post-monsoon seasons the rate of egg laying was high; as summer progressed there was drastic reduction in the number of eggs laid.

Culex sitiens is reported to breed in the brackish water (Horsfall, 1955). Panicker *et al.*(1981) studied the effect of salinity and oviposition, egg hatchability, larval duration and survival of *Cx. sitiens* by adding different quantities of sodium chloride to the larval rearing medium. It was observed that *Cx. sitiens* can readily adapt to tap water. In the present study the larvae of *Cx. sitiens* were reared in saline water to simulate the natural condition.

The mortality rate of larvae and pupae was very low in the laboratory. Since the mosquitoes produced enough eggs in ambient laboratory condition, the early fourth instar larvae for larvicidal screening was continuously obtained from the colony built in the laboratory.

CHAPTER 3

TOXICITY ASSAY OF PHYTO-CHEMICALS ON MOSQUITO LARVAE

- ★ Introduction
  - ★ Pyrethroids
  - ★ Nicotinoids
  - ★ Rotenoids
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- ★ Materials and Methods
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CHAPTER 3

TOXICITY ASSAY OF PHYTO-CHEMICALS ON MOSQUITO LARVAE

3.1. INTRODUCTION

Long before the advent of synthetic insecticides, naturalistic methods of pest control including the use of plants and their derivatives were used to kill pests of agriculture, veterinary, and public health importance. Plant natural products still have enormous potential to inspire and influence modern agrochemical research and integrated vector control programmes.

Plants are richest source of bioactive organic chemicals. These chemicals, often known as secondary metabolites are reported to have significant role in the defence mechanism of plants. Out of 2,75,000 plant species, only 2,400 have been tested for pesticidal activity (Singh, 1993). Historically, the commercial development of botanical insecticides is credited to a lady of Dalmatia, who noticed dead insects on a discarded bouquet of pyrethrin flowers. She began milling pyrethrum into powder and thus the pyrethrum industry was born (Sukumar *et al.*, 1991). Besides pyrethrin other commonly used commercial insecticides of plant origin are nicotine, rotenoids and neem.

Pyrethroids

The active principles, pyrethrins are present in the flowers of *Chrysanthemum* of the family Compositae. The most commonly exploited species is *Chrysanthemum cinerariaefolium*. The first detailed chemical study of the insecticidal principles of pyrethrum

flowers was reported by the Japanese chemist, Fjitani, in 1909. The insecticidal activity of pyrethrum is attributed to the action of six constituents—Pyrethrin-I, Pyrethrin-II, Cinerin-I, Cinerin-II, Jasmolin-I, and Jasmolin-II (Matsui and Yamamoto, 1971).

Pyrethrins cause a very rapid paralytic action in insects even when given in small dosages, but the effect is usually temporary. The dosage required to kill insects is much higher and needs longer time (Yamamoto, 1970). The attractive features as insecticide are the lack of persistence, rapid knock down activity on insects, and low mamalian toxicity. Prior to the discovery of DDT, natural pyrethrins were widely used for domestic and agricultural purpose. The domestic formulation contains about 0.5% active pyrethrum principles. The natural pyrethrins have been now replaced by more stable and cheaper synthetic pyrethrins (Ray, 1991).

Nicotinoids

The term nicotinoids includes nicotine, its structural analogues, and their optical antipodes. Nicotine has been isolated from at least eighteen species of *Nicotiana*, among which *N. tabacum*, and *N. rustica* are the most common (Quraishi, 1977).

Nicotine is highly toxic and in the presence of light decomposes easily. It is not volatile and is used as a contact insecticide against aphids and poultry mite, and as a fumigant in greenhouses. The formulations include sprays and dust (0.05-0.4%), and a concentrated solution of the sulfate (40% nicotine). One of the advantages of this insecticide is that it is completely devoid of toxic action against mammals (Ray, 1991).

Rotenoids

The rotenone and other related rotenoids are present in plants belonging to the family leguminosae and are widely used as a non-systematic pesticide against a wide variety of

insects, arachnids, molluscs and as fish poison. The commercial sources of rotenoids are *Derris, Lonchocarpus, Tephrosia,* and *Millettia*, of which *Derris* is the most exploited genus. The widely cultivated species are *D. elliptica* and *D. malaccensis* (Fukami and Nakajima, 1971).

Rotenone is considerably more toxic to fish, insects, and mammals. The slow action of rotenone as a contact insecticide and stomach poison is characteristic (Yamamoto, 1970). It's commercial use is limited by its rapid photodecomposition. Rotenone still finds use as domestic garden insecticide. Formulations include crystalline preparations, emulsified solutions and dusts of 0.75-5% concentrations (Ray, 1991).

Neem

The first report of pesticidal properties of neem in India dates back to 1927 when Mann and Burn observed that during the locust cycle adult locusts did not feed on neem leaves. Since then extensive research was conducted on the insecticidal property of this plant. Neem derivatives are known to exhibit insect control activity against more than 250 insect species. The insecticidal activity is reported from different plant parts such as seeds, roots, and leaves of the neem tree, *Azadiracta indica*. It shows relatively low mammalian toxicity, does not affect the non-target and beneficial organisms, is biodegradable, and is available in abundance.

The active principle to be isolated first from the plant was azadiractin. Following this a number of other compounds such as nimbandiol, salannin, gedunin, azadiradione and their derivatives have been isolated. Neem based formulations such as Margosan O, Azatin and Neem Azal are marketed widely. The National Chemical Laboratory (NCL), Pune, has isolated Neemrich-I and Neemrich-II from the seeds, and formulated dust and granules using permissible quantities of stabilizers (Nagasampagi, 1993).

The other insecticides of plant origin used traditionally are Quassia, Hellebore, Sabadilla and Ryania.

Current status of Phyto-Chemicals in Mosquito Control

Chemicals derived from plants have been projected as weapons in future mosquito control programmes as they are shown to function as general toxicants, growth and reproductive inhibitors, repellents and as ovipositional deterrants (Sukumar *et al.*, 1991). The results of laboratory tests and field trials of a series of plant extracts as well as purified phyto-chemicals conducted during the last decade are reviewed below.

The insect growth-regulating effect of neem seed kernel extracts, and crude and pure azadirachtin on mosquito larvae were investigated and compared with the effects of juvenile hormone analogue Altosid by Zebitz (1986). The results point out the strong interference of azadiractin with hormonal balance, most probably with ecdysteroid.

Hellpap and Zebitz (1986) observed that the combination of neem seed kernel extract and *B. thuringiensis* resulted in higher mortality compared to that exerted by each compound alone.

The effect of crude methanolic extracts of *Ajuga remota* and *A. reptans* was tested in the laboratory against the developing stages of *Aedes aegypti, Ae. togoi,* and *Culex quinquefasciatus* by Marcard *et al.* (1986). The extracts caused severe inhibition of development when young fourth instar larvae were exposed until adult emergence.

Larvicidal activity of oil from 10 plants against fourth instar larvae of *Anopheles stephensi* showed that maximum activity was in the case of *Cedrus deodara* which caused 50% mortality at the dose of 63.2 ppm (Anil kumar and Dutta, 1987).

Murty and Jamil (1987) studied the effect of South Indian Vetiveria oil (*Vetiveria zizinioides*) on the second, third, and fourth instar larvae of *Culex quinquefasciatus*. Malformations were found in all the concentrations. The 24 hour LC_{50} values for second, third, and fourth instar larvae were 0.053%, 0.062%, and 0.088%, respectively.

Crushed aqeous extracts of 24 plants showed that *Quassia amara* (leaves), *Thevetia nerifolia* (cotyledons), *Anacardium occidentale* (seed shells), *Carica papaya* (cell sap from fruits), *Hevea braziliansis* (cotyledons), and root of *Nerium indicum* were active against fourth instar larvae of *Culex quinquefasciatus*. Among these the activity of *Q. amara, A. occidentalis,* and *T.nerifolia* was found to be over 20 times higher than that of *Adathoda* (Evans and Kaleysa Raj, 1988).

Four new azadirachtin rich fractions-nemedin, vemidin, vepeidin and nemol- isolated from the fresh seeds of *Azadirachta indica* were tested for the larvicidal activity against early fourth instar larvae of *Anopheles culicifacies* and *Culex quinquefasciatus* by Rao *et al.* (1988).

Sujatha *et al.* (1988) studied the effect of the petroleum ether extracts of *Acorus clamus, Ageratum conyzoides, Annona squamosa, Bambusa arundanasia, Madhuca longifolia,* and *Citrus medica* on *Culex quinquefasciatus, Aedes aegypti,* and *Anopheles stephensi*.

Vasudevan *et al.* (1989) tested different concentrations of hot and cold water extracts of castor leaves against *An. stephensi, Cx. fatigans* and *Ae. aegypti*. The active component ricinin at 119 ppm in solution exhibited 100% ovicidal and larvicidal, but not adulticidal properties.

The extracts of two varieties of *Sorghum bicolor* seedling induced 90% mortality in second instar *Culex pipiens* larvae at 0.82 ppm and 90% mortality in third instar larvae at 1.12 ppm (Jackson *et al.*, 1990).

The synergistic action of synthetic pesticides in combination with various plant extracts have been illustrated by Thangam and Kathiresan (1990). The combination of synthetic pesticides with botanicals was suggested to reduce the environmental contamination.

The ethanolic extract of *Descurainia sophia* was tested for its activity against the mosquito *Culex quinquefasciatus*. The treatment of the late third instar larvae with 100-1500 ppm of the extract resulted in concentration dependent larval mortality and inhibition of emergence. At 1-100 ppm the extract induced ovicidal activity against zero hour old eggs and substantial mortality against newly hatched larvae (Mohsen *et al.*, 1990).

Zarroug *et al.* (1990) investigated the larvicidal activity of *Balanites aegyptiaca*. The larvae of *Anopheles arabiensis* were more susceptible than *Culex quinquefasciatus* and *Aedes aegypti* to its larvicidal effect. The saponin extract from fruit kernel was more active than water extract.

Evans and Kaleysa Raj (1991) has identified Quassin as the antilarval principal present in *Quassia amara*. This was effective against the mosquito larvae of *Cx. quinquefasciatus* at a concentration of 6 ppm.

The steam distilled oils of 3 species of marigold, *Tagetes patula, T. erecta* and *T. minuta* were tested for the larvicidal activity towards the third instar *Ae. aegypti*. The activity at 10 ppm was demonstrated only for *T. minuta* (Green *et al.*, 1991).

Plant samples of *Acanthus ilicifolius* (root), *Avicennia officinalis* (bark), *Excoecaria agallocha* (bark, fruit), *Lumnitzera racemosa* (bark), *Rhizophora apiculata* (stilt root), *R. lamarckii* (stilt root), *R. mucronata* (stilt root), *Salicornia brachiata* (shoot) and *Sonneratia apetala* (root) were extracted in acetone and were tested for mosquito larvicidal activity against *Aedes aegypti* (Thangam and Kathiresan, 1992).

Hebbalkar *et al.* (1992) fractionated the steam distillate of *Vitex negundo* by column chromatography. The mosquito repellent activity was evaluated against *Aedes aegypti*.

Saxena *et al.* (1992) observed the anti-juvenile activity of 15 plant extracts. Petroleum ether extract of *Eichhornia crassipes* and acetone extracts of *Ageratum conyzoides, Cleome icosandra, Tagetes erectes,* and *Tridax procumbens* showed growth inhibitory and juvenile hormone mimicing activity to the treated larvae of *Culex quinquefasciatus*. Biting behaviour was observed to be affected only in *Ageratum, Cleome* and *Tridax* extracts. Adults that emerged from larvae exposed to the plant extracts produced significantly shorter egg rafts.

Alkaloids isolated from *Annona squamosa* have shown larvicidal, growth-regulating and chemosterilant activities against *Anopheles stephensi* at concentrations of 50 to 200 ppm. Adults emerged from the larvae exposed to different treatments showed reduced fecundity and fertility in females (Saxena *et al.*, 1993).

The toxicological studies of the extract of *Indigofera tinctoria* against mosquito *Anopheles stephensi* larvae showed that rotenoids were very effective against mosquito larvae (Kamal and Mangla, 1993).

Laboratory studies were conducted with the crude ethanolic extract of *Cannabis sativa* an indigenous plant, to evaluate its insecticidal property against the larval stages of *Anopheles stephensi, Culex quinquefasciatus,* and *Aedes aegypti* mosquitoes by Jalees *et al.* (1993).

Insecticidal properties of essential oils and major constituents of aromatic plants, *Ocimun basilicum* and *O. sanctum* were evaluated against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* mosquito species. The bioassay tests revealed that the essential oil of *O. basilicum* and its major constituent, methyl chavicol are more effective as compared to *O. sanctum* (Bhatnagar *et al.*, 1993).

Petroleum ether extract of seaweeeds *Caulerpa peltata, C.racemosa, C. scalpelliformis, Chaetomorpha linum, Codium decorlicatum, Dictyota dichotoma, Enteromorpha clathrata, E. intestinalis, Halimeda opuntia, Hypnea valentiae, Sargassum tenerrimum, S. wightii, Turbinaria conoides, T. oranata* and *Ulva lactuca* were tested for the larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*. The extracts of *C. scalpelliformis, D. dichotoma, E. clathrata,* and *E. intestinalis* were found effective against larvae of *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} values below 100 mg/L (Thangam and Kathiresan, 1993).

Seeds of *Ocimum sanctum* were found effective in the control of the larvae of *Culex fatigans*. The seeds exude a mucilagenous substance to which larvae become attached firmly through the mouth parts, and die. One kilogram of Tulsi seeds has the capacity to bind 3-4 million fourth instar larvae. The method may be effective for control in small tanks, ponds and ditches (Hasan and Deo, 1994).

Cardenolides, secondary metabolites extracted from the leaves of *Calotropis gigantea*, have been tested for their toxicity on the larvae of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*. Cardenolides gave fifty per cent mortality on *An. stephensi* and *Ae. aegypi* at 2 mg/L , and on *Cx. quinquefasciatus* at 5 mg/L (Pugalenthi, 1995).

The activity of bioactive diterpene fraction of dichloromethane root extract of *Melantheria albinervia* was studied on *Aedes aegypti* (Slimestad, 1995).

In the present Chapter the larvicidal potential of a few locally available plants is investigated against the fourth instar larvae of *Culex quinquefasciatus, Culex sitiens, Aedes aegypti* and *Anopheles stephensi.*

3.2. MATERIALS AND METHODS

Selection of Plants

The plants for the extraction of phyto-chemicals were locally available. All of them were shrubs or herbs growing in the wild. Care was taken to select those plants having reported pharmacological properties or have been in use traditionally as fumigant or repellent against mosquitoes. Available literature was also made use of so that, as far as possible, they were unexploited species regarding their mosquito larvicidal property. Based on these criteria seventeen plant species were selected. They are listed in Table 3.1.

Preparation of Phyto-Chemical Extract

The plants collected were washed thoroughly in water and air dried at controlled temperature (40-60^{0}C). The aerial parts of the plant species were dried for 5 - 6 days, the finely chopped tubers were dried for 7 - 8 days and finely chopped roots were dried for 10 - 12 days. The materials were ground separately and stored in dry, labelled bottles at a temperature range of 15-20^{0}C.

Table 3.1. Selected plants with parts used

Selected plants	Family	Part used
Acalypha indica	Euphorbiaceae	Aerial part
Adenosma capitatum	Scrophulariaceae	Whole plant
Cassia alata	Papilionaceae	Aerial part
Cassia occidentalis	Papilionaceae	Aerial part
Cleome burmanni	Caparidaceae	Whole plant
Crotalaria straita	Papilionaceae	Aerial part
Curcuma raktakanda	Zingiberaceae	Leaves & Tuber
Eclipta alba	Compositae	Whole plant
Eupatorium odoratum	Compositae	Aerial & Root
Glycosmis pentaphylla	Rutaceae	Aerial & Root
Gliricidia maculata	Papilionaceae	Aerial part
Grewia microcos	Teliaceae	Aerial & Root
Heliotropium indicum	Boraginaceae	Whole plant
Hyptis suaveolens	Labiatae	Aerial & Root
Mikamia scandens	Compositae	Aerial part
Stachytarpheta indica	Verbinaceae	Aerial part
Tridax procumbens	Compositae	Whole plant

The powdered materials were continuously extracted using a soxhlet apparatus in petroleum ether, and in distilled alcohol at the boiling point of these solvents for 24 hours. These extracts were concentrated in a rotary vacuum evaporator. The residue was used to prepare stock solutions of 1 per cent (w\v)in acetone (for petroleum ether extraction) and in alcohol (for alcohol extraction).

Bioassay Procedure

Toxicity assays of all the extracts were conducted separately using the early fourth instar larvae of *Culex quinquefasciatus, Culex sitiens, Aedes aegypti,* and *Anopheles stephensi* at 28± 2^{0}C. The larvicidal activity of the extract was evaluated as per the method recommended by WHO (1981). The larvae for testing were released into a beaker containing tap water. The larvae showing a fuzzy appearance due to the presence of parasites on the body surface were discarded. The stock solutions of the plant extracts were volumetrically diluted to 250 mL with filtered tap water to obtain the test solutions of 10 mg/L, 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L. The test solutions for assaying the larvae of *Cx. sitiens* were prepared in saline water of salinity $15x10^{-3}$ Tween-80 was used as emulsifier at a concentration of 0.001 per cent in these test solutions. Two controls were maintained for each dilution, one consisted of the solvent used in the preparation of the stock solution, and the other consisted of tap water only. The test solutions were allowed to stand for 15-30 minutes. During this period 25 early fourth instar larvae were introduced to each of these test solutions and also to control sets.

For each dose four replica were run at a time. Dog biscuits and yeast powder (2:1) served as food throughout the larval period in the treated and in control sets. The test vessels were kept away from direct sunlight. The larval mortality was recorded after 24 hours. In recording the percentage mortality for each concentration, the moribund and dead larvae in four replicates were combined. The larvae were treated as dead if they could not be induced to move when probed with a needle in the siphon or cervical region. Moribund larvae were those incapable of rising to the surface within a reasonable period of time or of showing the characteristic diving reaction when the water was disturbed.

The larvae that pupated during the test were discarded. Tests with a control mortality of 20 per cent or more were considered unsatisfactory and discarded.

The bioassay was repeated three times with three different sets of stock solutions and with three different batches of mosquito larvae.

Data Analysis

The per cent mortality was calculated from the number of dead and moribund larvae. The difference in mortality between the applied doses was estimated by Analysis of Variance. The significance factor applied was:

SF = 0.0, No significance($p>0.05$)

SF = 0.5, significance($0.05>p>0.01$)

SF = 1.0, strong significance($p<0.01$)

The least significant difference (LSD) was computed to determine the overlap between dose pairs. The dose-response relationship was elucidated by Probit analysis and the results expressed as LC_{50} and LC_{90} (Finney, 1971).

3.3. RESULTS

Out of the seventeen plant species tested for mosquito larvicidal activity two species could induce 100% mortality in all the four mosquito species. The alcohol extract of these seventeen plant species was not lethal to any mosquito larvae. Larval mortality occurred only in petroleum ether extract of plants. There was no larval mortality either in water control or in solvent control. The result obtained for each plant species is detailed below.

Acalypha indica

Culex sitiens showed susceptibility to the petroleum ether extract of the aerial part of the plant. The other three mosquito species were not affected by the extract. The observations are given in Table 3.2. At 80 mg/L of the extract, *Cx. sitiens* larvae exhibited 100% mortality. Analysis of variance showed that the effects of various doses differed significantly. There was no overlap between the effects of any test doses as estimated by LSD (Table 3.2).

The LC_{50} value was 21.03 mg/L, and the LC_{90} value was 54.70 mg/L(Table 3.3).

Adenosma capitatum

The crude petroleum ether extract of the plant induced 100% mortality to *Cx. sitiens* at 80 mg/L. The other three species of mosquito larvae were not affected by this extract(Table 3.2). The analysis of variance and estimation of LSD (Table 3.2) showed that there was significant difference at 1% level in the effect of doses between 10 mg/L, 20 mg/L, 40 mg/ L, and 60 mg/L; but there was no significant difference in the effects of doses 60 mg/L and 80 mg/L.

The LC_{50} value of the extract was 10.79 mg/L, and the LC_{90} value was 44.57 mg/L (Table 3.3).

Cassia alata

The petroleum ether extract was lethal to the fourth instar larvae of *Cx. sitiens*. The extract failed to induce lethality on the other three species. The percentage mortality and analysis of variance against *Cx. sitiens* were given in Table 3.2. The larval mortality increased with the concentration of the extract. One hundred per cent mortality occurred at 80 mg/L of the

extract. The estimation of LSD showed statistically significant difference in the effect of doses 10 mg/L and 20 mg/L, 20 mg/L and 40 mg/L, and no significant difference in the effect of doses between 40 mg/L and 60 mg/L, and 60 mg/L and 80 mg/L. The calculated values of LC_{50} and LC_{90} were 14.79 mg/L, and 46.88 mg/L respectively(Table 3.3).

Table 3.2. Percentage mortality and *F* ratio between doses in *Culex sitiens* larvae after 24 hours exposure to the extract of *Acalypha indica* (aerial part), *Adenosma capitatum* (whole plant) and *Cassia alata* (aerial part)

Test dose (mg/L)	% mortality		
	A. indica	*A. capitatum*	*C. alata*
10	12	48	32
20	56	64	68
40	76	88	88
60	92	96	92
80	100	100	100
Control	nil	nil	nil
F ratio	3458.500	426.143	279.000
Probability	5.600E-13*	2.372E-09*	1.276E-08*
LSD	0.71	1.08	1.94

* Significant at 1% level

Table 3.3. Probit analysis of % mortality/test doses of the extract of *Acalypha indica*, *Adenosma capitatum* and *Cassia alata* against *Cx. sitiens* larvae following 24 hours exposure

Plant species	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
A. indica	Y=0.91+3.09x	21.03	17.06-25.00	54.70	2	1.28
A. capitatum	Y=2.85 +2.08x	10.79	9.23-12.35	44.57	2	1.09
C. alata	Y=2.09 +2.51x	14.79	12.93-16.65	46.88	2	0.47

Cassia occidentalis

The petroleum ether extract of this plant was not lethal to any of the test species of mosquito larvae irrespective of the test dose.

Cleome burmanni

The extract of the whole plant did not effect mortality at any test dose on any of the exposed mosquito larvae.

Crotalaria straita

The early fourth instar larvae of the tested mosquito species did not show any mortality in petroleum ether extract of the aerial part of the plant.

Curcuma raktakanda

The leaves and tuber extracted in petroleum ether exhibited 100% mortality to the early fourth instar larvae of all the test species of mosquito at 80 mg/L.

Leaves

The leaf extract of *C. raktakanda* was toxic to all the four mosquito species. One hundred per cent mortality was observed at 60 mg/L in the case of *Cx. sitiens*, whereas complete mortality was obtained at 80 mg/L, in the case of *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* (Table 3.4). The mortality of *Cx. sitiens* was 60% at the lowest test dose of 10 mg/L. Therefore an additional test dose of 5 mg/L was evaluated for larvicidal activity. It was observed that 40% of the larvae died following 24 hours exposure at this concentration of the extract.

Table 3.4. Percentage mortality and *F* ratio between doses in mosquito larvae after 24 hours exposure to the leaf extract of *Curcuma raktakanda*

Test dose	% mortality			
(mg/L)	*Cx. quinquefasciatus*	*Cx. sitiens*	*Ae. aegypti*	*An. stephensi*
5		40		
10	40	60	36	40
20	56	84	60	72
40	88	96	80	88
60	96	100	92	92
80	100	100	100	100
Control	nil	nil	nil	nil
F ratio	575.286	553.286	496.4	634.00
Probability	7.183E-10*	8.356E-10*	1.292E-09*	4.877E-10*
LSD	1.33	1.08	1.37	1.17

* Significant at 1% level

The *F* ratios were highly significant. According to the estimation of LSD, the lowest effective dose for the complete mortality of *Cx. quinquefasciatus*, *Ae. aegypti* was 60 mg/L, for *An. stephensi* it was 80 mg/L and for *Cx. sitiens* it was 40mg/L. The LC_{50} values for the four mosquito species ranged from 6.9 mg/L to 15.00 mg/L, and the LC_{90} values from 27.45 mg/L and 58.75 mg/L.

Table 3.5. Probit analysis of the % mortality/test doses of the leaf extract of *Curcuma raktakanda* against *Cx. quinquefasciatus*, *Cx. sitiens*, *Ae. aegypti* and *An. stephensi* larvae following 24 hours exposure

Test organism	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
Cx.quinquefasciatus	Y=2.10+2.51x	14.34	12.48-16.20	46.77	2	1.3
Cx.sitiens	Y=3.21+2.13x	6.90	5.92-7.88	27.45	2	1.1
Ae.aegypti	Y=2.46 +2.16x	15.00	12.86-17.14	58.75	2	0.12
An.stephensi	Y=2.41 +2.33x	12.91	11.18-14.64	45.81	2	0.57

Tuber

The tuber extract of *C. raktakanda* in petroleum ether was toxic to all exposed mosquito larvae. At 80 mg/L the extract effected complete mortality of *Cx. quinquefasciatus, Ae. aegypti*, and *An. stephensi*. Complete mortality of *Cx. sitiens* larvae occurred at 60 mg/L. At 10 mg/L the mortality of *Cx. sitiens* was 60%. Therefore, an additional test dose of 5 mg/L of the extract was evaluated for larvicidal activity wherein *Cx. sitiens* showed 36% mortality(Table 3.6). The *F* ratio indicated highly significant

difference between the effects of test doses. The effect of test concentrations 60 mg/L and 80 mg/L was found to overlap for *Cx. sitiens, Ae. aegypti* and *An. stephensi*, while the percentage mortality was significantly different for *Cx. quinquefasciatus* at 60 mg/L and 80 mg/L.

Table 3.6. Percentage mortality and *F* ratio between doses in mosquito larvae after 24 hours exposure to the tuber extract of *Curcuma raktakanda*

Test dose (mg/L)	% mortality			
	Cx. quinquefasciatus	*Cx. sitiens*	*Ae. aegypti*	*An. stephensi*
5		36		
10	40	60	32	40
20	60	80	60	72
40	88	96	84	92
60	96	100	96	96
80	100	100	100	100
Control	nil	nil	nil	nil
F ratio	437.125	1339.600	684.308	404.125
Probability	2.144E-09*	2.471E-11*	3.598E-10*	2.929E-10*
LSD	1.42	0.71	1.28	1.42

* Significant at 1% level

The LC_{50} values were estimated as 13.73 mg/L, 7.57 mg/L, 15.46 mg/L and 12.32 mg/L for *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* respectively, and the corresponding LC_{90} values were 44.88 mg/L, 29.11 mg/L, 48.08 mg/L and 37.49 mg/L (Table 3.7).

Table 3.7. Probit analysis of the % mortality/test doses of the tuber extract of *Curcuma raktakanda* against *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* following 24 hours exposure

Test organism	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
Cx.quinquefasciatus	Y=2.17+2.49x	13.73	11.93-15.53	44.88	2	0.61
Cx.sitiens	Y=3.08+2.19x	7.57	6.49-8.65	29.11	2	0.3
Ae.aegypti	Y=1.91+ 2.6x	15.46	13.56-17.36	48.08	2	0.03
An.stephensi	Y=2.11 +2.65x	12.32	11.18-14.64	37.49	2	0.03

Eclipta alba

The larvicidal activity of the extract was confined to *Cx. sitiens*. At 80 mg/L the extract exhibited 100% larval mortality (Table 3.8). The other three mosquito species did not show any susceptibility at any of the test concentrations. The variance was analyzed by two way ANOVA(Table 3.8). This showed strongly significant difference in the effects of doses while the replication did not differ. As revealed by the LSD values there was no significant difference in the effect of doses 60 mg/L and 80 mg/L. The values of LC_{50} and LC_{90} were 11.93 mg/L and 39.26 mg/L respectively(Table 3.9).

Table 3.8. Percentage mortality and *F* ratio between doses in *Cx. sitiens* larvae after 24 hours exposure to the extract of *Eclipta alba* (whole plant) *Eupatorium odoratum* (aerial part) and *Glycosmis pentaphylla* (aerial part)

Test dose (mg/L)	% mortality *E. alba*	*E. odoratum*	*G. pentaphylla*
10	40	48	44
20	76	76	72
40	88	88	88
60	96	96	96
80	100	100	100
Control	nil	nil	nil
F ratio	76.000	194.800	369.625
Probability	2.368E-10*	5.290E-08*	4.176E-09*
LSD	1.00	1.77	1.42

* Significant at 1% level

Table 3.9. Probit analysis of the % mortality/test doses of the extract of *Eclipta alba, Eupatorium odoratum* and *Glycosmis pentaphylla* against *Cx. sitiens* larvae following 24 hours exposure

Plant species	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
E.alba	Y=2.33+2.48x	11.93	9.48-14.04	39.26	2	0.49
E.odoratum	Y=2.79+2.18x	10.28	8.78-11.78	39.81	2	0.23
G.pentaphylla	Y=2.52+2.33x	11.56	9.95-13.17	41.02	2	0.10

Eupatorium odoratum

The petroleum ether extract of the aerial part was toxic to the larvae of *Cx.sitiens* (Table 3.8). The extract exhibited 100% mortality at 80 mg/L. Analysis of variance showed strongly significant difference in the effects of all the doses from 10 mg/L to 60 mg/L; but there was no statistically significant difference in the response of doses between 60 mg/L and 80 mg/L (Table3.8). The LC_{50} value of the extract was 10.28 mg/L, and the LC_{90} value was 39.81 mg/L (Table 3.9).

The root extract in petroleum ether was not lethal at any concentration to the exposed mosquito larvae.

Glycosmis pentaphylla

Aerial part

The petroleum ether extract exhibited hundred percent mortality to *Cx. sitiens* (Table 3.8); but the other three mosquito species were not affected by the extract. The results of analysis of variance are given in Table 3.8. As far as the mortality of the larvae was concerned there was significant difference in the effect of doses 10 mg/L and 20 mg/L, 20 mg/L and 40 mg/L, and 40 mg/L and 60 mg/L. There was no significant difference in the effect of doses between 60 mg/L and 80 mg/L (Table 3.8). The values of LC_{50} and LC_{90} were 11.56 mg/L, and 41.02 mg/L, respectively (Table 3.9).

Root

The petroleum ether extract induced 100% mortality at 80 mg/L to all tested mosquito species (Table 3.10). *Cx. sitiens* larvae were totally susceptible even at 60 mg/L of the extract. The extract produced 56% mortality to *Cx. sitiens* at the concentration of 10 mg/L. An additional

test dose of 5 mg/L was evaluated for larvicidal activity, and this effected 40% mortality. The analysis of variance by two way ANOVA and the estimation of the LSD is given in Table 3.10. There was significant difference between the effects of all test doses against *Cx. quinquefasciatus* and *Ae. aegypti*. The results at 60 mg/L and 80 mg/L were found to overlap in the case of *Cx. sitiens* and *An. stephensi*. The LC_{50} values were recorded as 15.64 mg/L, 7.71 mg/L, 17.38 mg/L and 15.27 mg/L against *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti,* and *An. stephensi* respectively. The corresponding LC_{90} values were 54.20 mg/L, 42.66 mg/L, 57.14 mg/L and 43.85 mg/L (Table 3.11).

Table 3.10. Percentage mortality and *F* ratio between doses in mosquito larvae after 24 hours exposure to the root extract of *Glycosmis pentaphylla*

Test dose (mg/L)	% mortality			
	Cx. quinquefasciatus	*Cx. sitiens*	*Ae. aegypti*	*An. stephensi*
5		40		
10	32	56	28	52
20	60	72	56	60
40	84	92	80	88
60	92	100	92	96
80	100	100	100	100
Control	nil	nil	nil	nil
F ratio	654.308	730.667	447.143	373.750
Probability	4.301E-10*	2.770E-10*	1.959E-09*	3.996E-09*
LSD	1.28	1.06	1.62	1.74

* Significant at 1% level

Table 3.11. Probit analysis of % mortality/test doses of the root extract of *Glycosmis pentaphylla* against *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* following 24 hours exposure

Test organism	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
Cx.quinquefasciatus	Y=2.17+2.37x	15.64	13.56-17.72	54.20	2	0.02
Cx.sitiens	Y=3.47+1.72x	7.71	6.41-9.01	42.66	2	0.53
Ae.aegypti	Y=1.93+2.48x	17.38	15.55-19.21	57.14	2	0.05
An.stephensi	Y=1.70+2.8x	15.27	11.18-14.64	43.85	2	0.15

Gliricidia maculata

This plant extract did not exhibit larvicidal activity against any of the exposed mosquito species.

Grewia microcos

The root and the aerial part of this plant were not toxic to the tested mosquito larvae.

Heliotropium indicum

The extract of this plant failed to induce any toxicity towards the exposed mosquito larvae.

Hyptis suaveolens

The early fourth instar larvae of *Cx. sitiens* was susceptibile to the extract of the aerial part of the plant. At 80 mg/L the extract induced 100% mortality (Table 3.12). The other three mosquito species were not affected by the extract.

Table 3.12. Percentage mortality and *F* ratio between doses in *Cx. sitiens* larvae after 24 hours exposure to the extract of the aerial part of *Hyptis suaveolens*

Test dose (mg/L)	% mortality
10	46
20	58
40	90
60	96
80	100
Control	nil
F ratio	490.462
Probability	1.356E-09*
LSD	1.20

* Significant at 1% level

The *F* ratio and probability values were calculated using two way ANOVA and the estimation of LSD showed statistically significant difference in the effects of the test concentrations between all doses except 60 mg/L and 80 mg/L (Table 3.12).

The LC_{50} and LC_{90} values were 13.59 mg/L, and 43.65 mg/L, respectively (Table 3.13).

Table 3.13. Probit analysis of % mortality/test doses of the extract of *Hyptis suaveolens* against *Cx. sitiens* following 24 hours exposure

Plant species	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
H.suaveolens	Y=2.37+2.39x	13.59	11.75-15.43	43.65	2	1.7

The root extract of this plant did not have larvicidal activity on the test organisms.

Mikamia scandens

This extract failed to induce any toxicity to the exposed mosquito species.

Stachytarpheta indica

The extract did not show any larvicidal activity against any of the exposed mosquito larvae.

Tridax procumbens

This extract did not exhibit any toxicity towards any of the test organisms at any of the tested concentrations.

3.4. DISCUSSION

Out of seventeen plants tested for mosquito larvicidal activity, the petroleum ether extracts of *Acalypha indica* (aerial part), *Adenosma capitatum* (whole plant), *Cassia alata* (aerial part), *Eclipta alba* (whole plant), *Eupatorium odoratum* (aerial part), *Glycosmis pentaphylla* (aerial part), and *Hyptis suaveolens* (aerial part) were effective against the larvae of *Culex sitiens*. These plants were not lethal to the other three mosquito species. Complete

mortality of *Cx. sitiens* larvae occurred at 80 mg/L of the crude extracts. The aerial part of *Glycosmis pentaphylla* was toxic to the larvae of *Cx. sitiens,* but not to the other three mosquito species. A comparison of the LC_{90} values (Fig.3.1) revealed that *E. alba, E. odoratum G. pentaphylla* (aerial part) and *H. suaveolens* are most toxic followed by *A. capitatum,* and *C. alata. A. indica* is the least toxic of the seven.

Curcuma raktakanda (tuber and leaves) and *Glycosmis pentaphylla* (root) are unique in that all the four mosquito species are susceptible to the petroleum ether extracts of these plants. The tuber extract of *Curcuma raktakanda* was relatively more toxic to the mosquito species than the leaf extract as indicated by the LC_{90} values ranging from 29.11 mg/L to 48.08 mg/L for tuber, and 27.45 mg/L to 58.75 mg/L for leaves (Fig.3.2). *Cx. sitiens* was the most sensitive species to the extract of *C. raktakanda* recording the minimum LC_{90} value of 27.45 mg/L. The least susceptible species among the four, was *Ae. aegypti*. The LC_{90} value obtained was 58.75 mg/L for the *C. raktakanda* leaf extract. The LC_{90} value for the extract of tuber was also the highest for *Ae. aegypti, i.e.,* 48.08 mg/L. The extract of *Glycosmis pentaphylla* was relatively less toxic than *C. raktakanda*, the minimum LC_{90} value recorded was 41.02 mg/L for *Cx. sitiens* against 27.45 mg/L of *C. raktakanda*. However, *Cx.sitiens* appears to be the most susceptible species to *G. pentaphylla*, and the most resistant species is *Ae. aegypti* with LC_{90} value of 57.14 mg/L. Considering the fact that eight plant species exhibited larvicidal effect on *Cx. sitiens* throughout the bioassays, it may be concluded that *Cx. sitiens* is the most susceptible species. At this point, the larval susceptibility status of *Cx. quinquefasciatus* and *Cx. sitiens* against fenthion is comparable. As reported by Dhanda, (1996), the LC_{50} values for *Cx. quinquefasciatus* and *Cx. sitiens*

Fig.3.1. Comparison of LC90 values

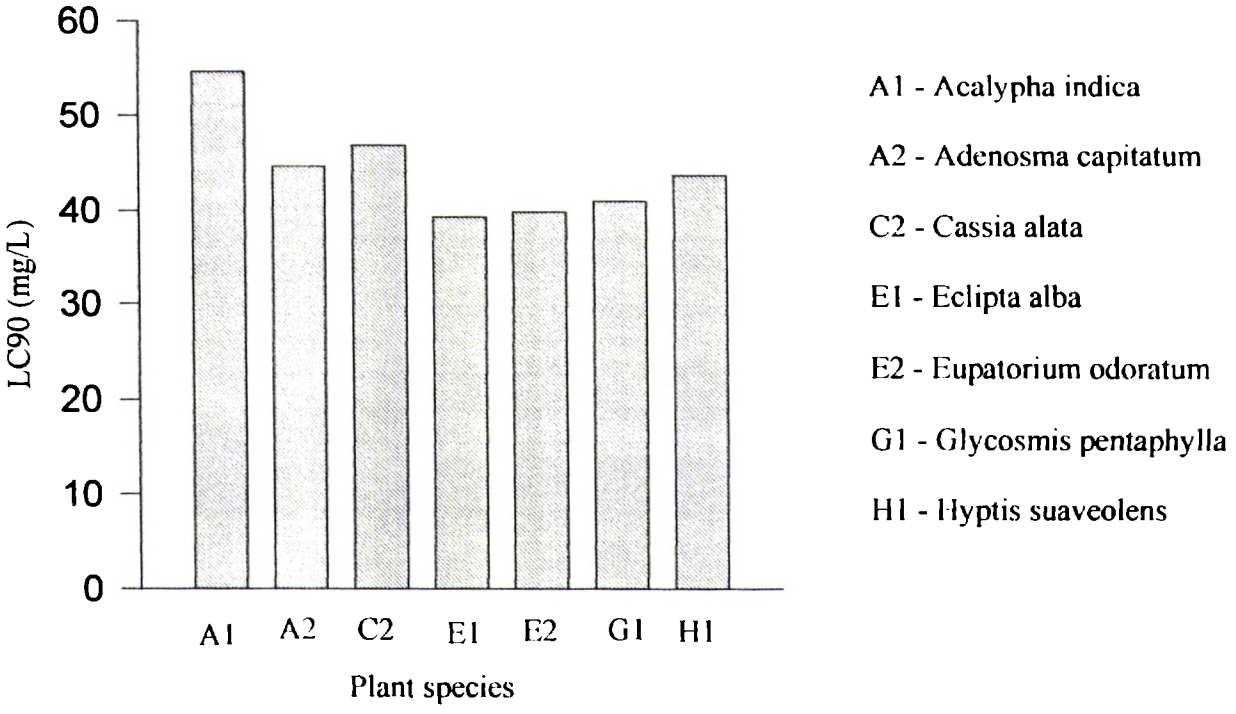
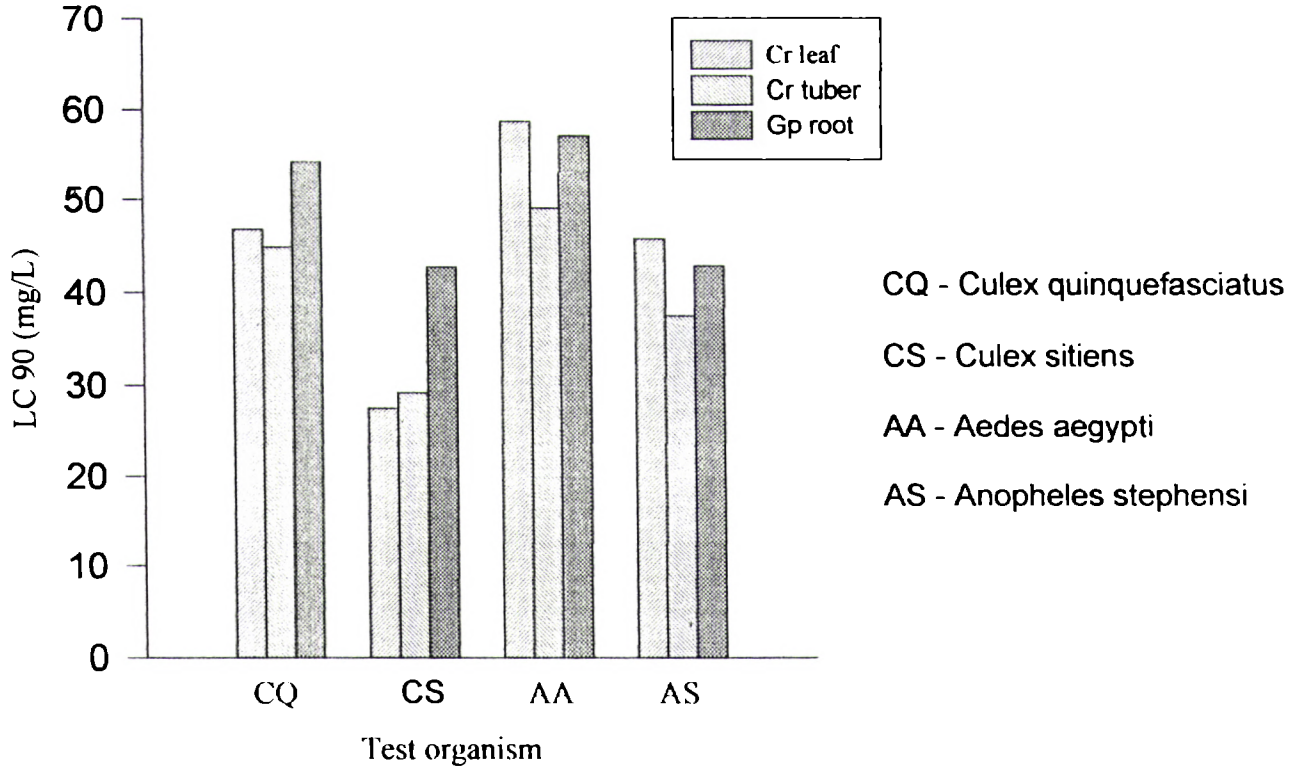


Fig.3.2. Comparison of LC90 values



against fenthion are 0.4132 mg/L and 7.0×10^{-6} mg/L respectively. The results obtained on the effect of *C. raktakanda* and *G. pentaphylla* on the four species reveal an order of susceptibility as *Cx. sitiens, An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*. Differential susceptibility of mosquito species to phyto-chemicals is well documented in the literature (Sukumar *et al.*, 1991). The phyto-chemicals usually act as general toxicants and the extent of toxicity is attributed to the intrinsic susceptibility of the species and its feeding behaviour. The feeding behaviour of the mosquito species tested is such that *Culex* feeds on the bottom, *Aedes* larvae feed indiscriminately, and *Anopheles* larvae feed on the surface (Zarroug *et al.*, 1990). As the extracts of *C. raktakanda* and *G. pentaphylla* are toxic to all the four species, it may be assumed that the phyto-chemical is well dispersed in the test medium to satisfy the feeding behaviour of all the four mosquito species and as such, it is the inherent susceptibility of the species that resulted in the differential toxicity.

Another distinct observation was that the alcoholic extract of the plant species did not affect the mosquito larvae. Larvicidal activity was exhibited only by petroleum ether extracts. This may be clearly attributed to the chemical nature of the compounds dissolved by these solvents. Alcohol is more polar solvent than petroleum ether. It may be assumed that a less polar compound may be the active larvicidal principle.

It was also observed that, the plant parts used to extract the phyto-chemical was decisive in the larvicidal action. The root of *E. odoratum* had no effect whereas the aerial part was toxic to *Cx. sitiens* larvae. Similarly, the aerial part of *G. pentaphylla* was toxic only to *Cx. sitiens* while the root induced mortality of all the mosquito species tested. The aerial part of *H. suaveolens* was toxic to *Cx. sitiens*. However, the root did not exhibit any

larvicidal property. The tuber and leaf extracts of *C. raktakanda* were toxic to the mosquito species; but the intensity of toxicity was slightly different. The toxicity of the plant species is related to specific phyto-chemicals produced by these plants. These compounds often designated as secondary metabolites are not required for normal growth and development of the plant; but has considerable role in the defence and environmental relationship. They are generally stored in specific tissues other than their sites of synthesis. For instance, alkaloids accumulate mainly in actively growing tissues, epidermal and hypodermal cells, vascular sheaths, and latex vessels (Goodwin and Mercer, 1983). This differential deposition in different tissues explains the variability of larvicidal action of the different plant parts.

Curcuma raktakanda is a species reported from the family Zingeberaceae. Many members of this family have earlier been found to have insecticidal activity (Pandji *et al.*, 1993). The insecticidal property of *Curcuma longa* has been recognized in many parts of the world especially in India and Pakistan. The rhizome of this plant is very effective as insect repellent particularly to *Tribolium castaneum* (Leal *et al.*, 1990). *Glycosmis pentaphylla* belongs to the family Rutaceae and has reported pharmacological properties (Kirtikar and Basu, 1933). In a previous study the benzene extract of the root bark of this plant was found to be toxic to the larvae of *Culex* (Chowdhury and Das, 1979). In another study Das *et al.* (1996) observed that the alkaloids, glycozoline and glycozolidine, obtained from *G. pentaphylla* were non-toxic to *Cx. quinquefasciatus* while the alkaloid glycosolone showed toxicity.

The findings of the present study reveal that *Curcuma raktakanda*, and the root of *Glycosmis pentaphylla* possess remarkable larvicidal activity against the four important mosquito species in Kochi. Hence, these plants are selected for further studies.

CHAPTER 4

EFFECT ON NON-TARGET ORGANISMS

4.1. INTRODUCTION

Most of the environmental concern about a mosquito larvicide is closely associated with aquatic ecosystems. Death of aquatic organisms and accumulation of pesticide residues in their tissues are the main hazards of pesticides in an aquatic environment. The use of DDT to curb the sudden outbreaks of vector - borne diseases should be regarded as a major environmental hazard on ecosystem function. The physicochemical properties of DDT and its metabolites enable these compounds to be taken up readily by organisms. High lipid solubility and low water solubility lead to the retention of DDT and its stable metabolites in fatty tissue. The retention of DDT metabolites means that toxic effects can occur in organisms remote in time and geographical area from the point of exposure. Accumulated DDT and its metabolites are transferred from aquatic organisms to consumers, including birds and mammals. Since fish is at the top of food chain, fish contaminated with pesticides might influence the pesticide levels in human body (WHO,1989).

New approaches of vector control from an excessive use of pesticides to an eco-friendly integrated vector management (IVM) programmes can cut down pesticide over use. At present IVM is the only ecologically based vector control strategy that uses various techniques applied at specific time. Biological control is an important tool in IVM to maintain an equilibrium between the host or prey, and parasite or predator.

Long before the discovery of mosquito transmission of malaria, fishes have been considered as a biological control agent of domestic pests and mosquitoes (Howard *et al.*, 1912). For many years several species of fishes have been used to clear tanks and ponds from mosquito larvae. The active interest began only from 1900, when studies were conducted to determine the utility of various species of fishes in mosquito control (Prasad and Hora, 1936). Although more than 40 different biocontrol agents for controlling mosquito larvae have been reported, only fishes have shown promising results (Wuneg *et al.*, 1987). Numerous literature have been documented concerning larvivorous fish, to obtain better progress in the utilization of fish alone or in combination of various methods in an integrated programme for the control of vector-borne diseases (Davey *et al.*, 1974). The WHO (1982) has recommended different varieties of fishes for large scale field trials in different countries. Some of them are *Gambusia punctata, Cubanichthys cubensis, Poecilia vitalata, Aplocheilus blochii, A. melastigma, Aphyocypris chinensis,* and *Oryzias latipes*. Sunder (1927) has reported the *Aplocheilus* species as being intensively used in malaria eradication programmes. Non-insecticidal integrated control methods in Bombay were described by Deobhanker (1986) and the use of *Poecilia reticulata* was highlighted. Najib *et al.*(1987) studied the efficacy of four fishes in controlling mosquito breeding. They were *Rasbora daniconius, Nuria danrica, Tilapia mossambica* and *Lepidocephalus thermates*.

Limited work has been conducted to assess the impact of natural products on aquatic system. The natural insecticide pyrethrins are lipophilic and long been reported to be toxic to fish (Elliott, 1989). Bhan (1989) suggested to the banning of synthetic pyrethroids like fenvalerate containing an α-cyano substitute in the benzyl alcohol moiety, in places where sensitive

fishes like rainbow trout are found. Rotenoids are reported to be used as a fish poison (Fukami and Nakajima, 1971). Evans and Kaleysa Raj (1991) observed that quassin was well tolerated by non-target organisms like *Aplocheilus blochii* and tadpoles of *Bufo melanosticus* at larvicidal concentration of 6 ppm.

The present investigation aims to define the toxicity of the crude extract of *Curcuma raktakanda* and *Glycosmis pentaphylla* to the three species of fishes namely *Aplocheilus lineatus, Poecilia reticulata* and *Oreochromis mossambicus*. The fish species used for the assay are larvivorous, and the relevance of the investigation is to demonstrate the compatability of the larvicide and the biological control when used simultaneously in an integrated mosquito control programme.

4.2. EXPERIMENTAL PROCEDURE

The toxicity of the tuber extract of *Curcuma raktakanda* and root extract of *Glycosmis pentaphylla* was tested against three species of fishes namely *Aplocheilus lineatus, Poecilia reticulata,* and *Oreochromis mossambicus.*

Acclimation

Aplocheilus lineatus and *O. mossambicus* were collected from ponds and paddy fields of Kochi. *P. reticulata* was collected from the drains and canals of the city.

In the laboratory, fishes were sorted to uniform length ranging between 3 ± 1 cm. These species were acclimated for one week in tap water. During this period mosquito larvae were provided as feed.

Experimental Design

One per cent stock solutions of the extracts were diluted up to 1000 mL with aerated tap water to obtain test doses of 40 mg/L, 60 mg/L, 80 mg/L, 100 mg/L and 120 mg/L. The test doses were allowed to stand for 20 to 30 minutes. During this period 10 fish were transferred to each test solution as well as to control sets. Two controls were maintained at a time, one consisted of aerated tap water and other consisted of the solvent acetone. The assays were conducted under ambient conditions: room temperature at 28±2^{0}C and pH between 6.5-7.

Data Analysis

The behaviour of the fish was observed for 24 hours following the application of plant extracts. Mortality was recorded after 24 hours of exposure. The LC_{50} and LC_{90} values were calculated by Probit analysis.

4.3. RESULTS

The three species of fishes upon exposure to the extracts of *C. raktakanda* and *G. pentaphylla* showed behavioural changes in the test doses above 80 mg/L such as excitation, hyperactivity, fast swimming, rigorous movements, inactive phase with occasional tremors and convulsions, and paralysis leading to death. There were no behavioural changes nor mortality in the control sets.

Toxicity to *Aplocheilus lineatus*

The extracts induced complete mortality at 120 mg/L. The LC_{50} values for *C. raktakanda* and *G. pentaphylla* were 59.42 mg/L and 52.60 mg/L, repectively. The corresponding LC_{90} values were 98.03 mg/L and 86.50 mg/L (Table 4.1).

Table 4.1. Toxicity of plant extracts to *Aplocheilus lineatus*

Extract	% mortality at (mg/L)					LC_{50} (mg/L)	LC_{90} (mg/L)
	40	60	80	100	120		
C. raktakanda	20	40	80	93	100	59.42	98.03
G. pentaphylla	30	47	93	96	100	52.60	86.50
Control	nil	nil	nil	nil	nil		

Toxicity to *Poecilia reticulata*

As given in Table 4.2, the *C. raktakanda* extract showed LC_{50} value of 62.81 mg/L and *G. pentaphylla* showed LC_{50} value of 55.59 mg/L. The LC_{90} values for the extract was 104.73 mg/L and 97.13 mg/L, respectively.

Table 4.2. Toxicity of the plant extracts to *Poecilia reticulata*

Extract	% mortality at (mg/L)					LC_{50} (mg/L)	LC_{90} (mg/L)
	40	60	80	100	120		
C. raktakanda	17	35	75	90	100	62.81	104.73
G. pentaphylla	26	45	80	93	100	55.59	97.13
Control	nil	nil	nil	nil	nil		

Toxicity to *Oreochromis mossambicus*

The *C. raktakanda* extract showed LC_{50} value of 68.91 mg/L and *G. pentaphylla* showed 65.76 mg/L. The LC_{90} values of these extracts were 105.19 mg/L and 100 mg/L, respectively (Table 4.3).

Table 4.3. Toxicity of extracts to *Oreochromis mossambicus*

Extract	% mortality at (mg/L)					LC_{50} (mg/L)	LC_{90} (mg/L)
	40	60	80	100	120		
C. raktakanda	8	25	65	90	100	68.91	105.19
G.pentaphylla	10	30	75	90	100	65.76	100.00
Control	nil	nil	nil	nil	nil		

4.4. DISCUSSION

The plant extracts evaluated for fish toxicity are lethal to the fishes at high doses. Comparison of the LC_{90} values as given in Fig.4.1 reveals that the range of toxicity of *Curcuma raktakanda* to the mosquito species is 6% to 10% whereas for the fish species it is 21% to 23%. Similarly, the LC_{90} values of mosquito species for *Glycosmis pentaphylla* is 9% to 12% and that for fish species is from 18% to 21% (Fig.4.2). This indicates that the plant extracts are twice more toxic to mosquito larvae than to fishes. Among the fish species, *Oreochromis mossambicus* shows higher survival rate than the other two species. Therefore, *Oreochromis mossambicus* is the least susceptible species.

Fig.4.1. Relative toxicity of C. raktakanda to fish and mosquito larvae

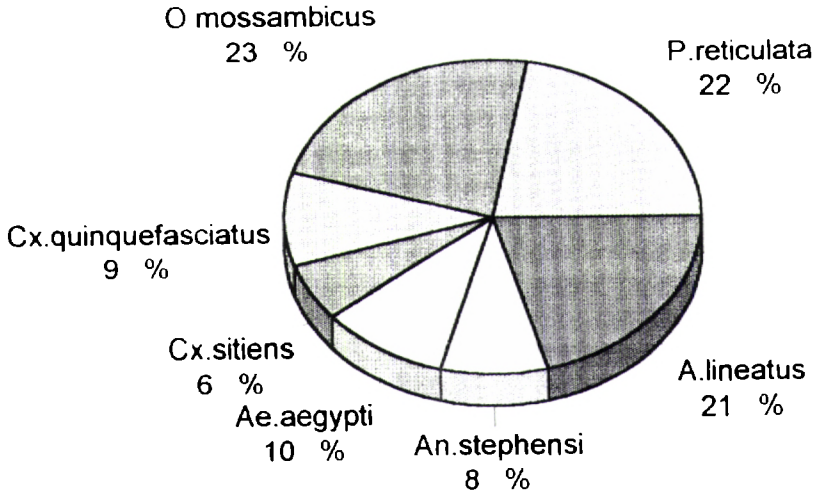
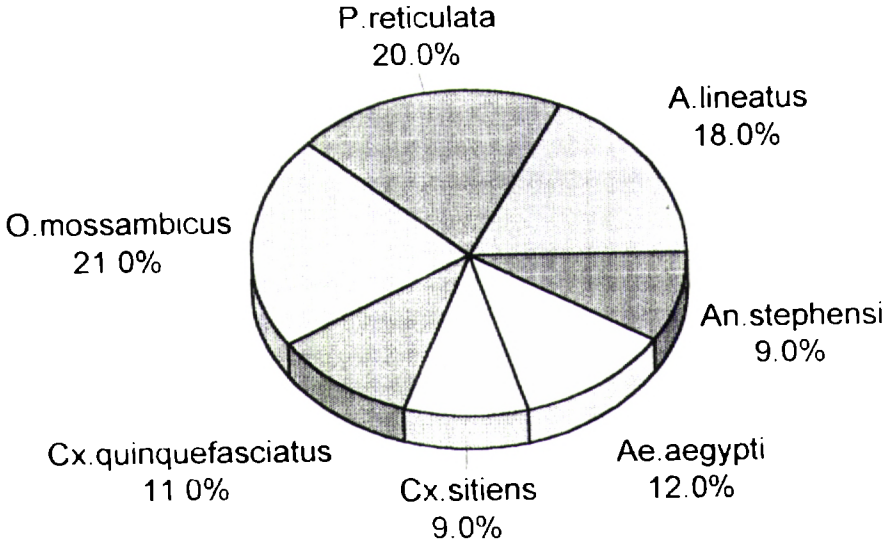


Fig.4.2. Relative toxicity of G. pentaphylla to fish and mosquito larvae



The impact of biocidal plant extracts on fishes and other non-target organisms has been studied earlier mainly with respect to neem products. Attri and Prasad (1980) observed that neem oil extractive is toxic to *Gambusia* and tadpoles at 0.04%, but at 0.005% it is safe to *Gambusia* species. This material was non-toxic to tadpoles at 0.01%. Bhuvaneshwari *et al.* (1994) assessed the toxicity of neem seed kernel (NSKE) extract and neem oil (NO) against different non-target organisms. Neem seed kernel extract showed complete mortality after 24 hours at 5%, 2.5%, 1.25%, 0.6% and 0.3% against two week old fingerlings of *Tilapia mossambica*. The authors concluded that neem products are relatively non-toxic to parasitoids and predators. However, the aquatic organisms were found to be affected by the oil content of neem products. Srinivasa Babu *et al.* (1993) have stated that botanical insecticides are relatively safer to aquatic organisms when compared to endosulfan and phoslone.

The low LC_{50} and LC_{90} values of extracts against mosquito larvae and comparatively high LC_{50} and LC_{90} values against fishes indicate that a larvicide based on *Curcuma ra ktakanda* and *Glycosmis pentaphylla* are less hazardous to the aquatic environment. The extracts of *C. raktakanda* and *G. pentaphylla* may be applied in the breeding sites of mosquitoes with minimum environmental disturbance. Therefore, a combination of biological control and biopesticide may help in the control of mosquito population.

CHAPTER 5

SHELF LIFE AND DEGRADATION STUDIES

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CHAPTER 5

SHELF LIFE AND DEGRADATION STUDIES

5.1. INTRODUCTION

Large amounts of pesticides reach soil by direct application or by aerial spraying or from plants or animal remains which become incorporated into the soil. The fate of pesticides after coming in contact with soil is governed by several factors such as its distribution in soil profile, its chemical structure and formulation, and to the physical, chemical and biological properties of the soil. Some pesticides may be lost from the soil environment by leaching, volatilization or chemical and biological transformations, whereas certain others remain in the environment. It is the extent of these transformations the majority of which are degradative, that determine the persistence of a pesticide in the soil environment. The pesticide fate and persistence in the water environment is governed by dilution, chemical and biological degradation, adsorption to living and non-living materials, absorption by living organisms with consequent accumulation and entry into food chain, sedimentation, volatalization and photodecomposition (Hill and Wright, 1978).

The organochlorine insecticides are persistent in the soil due to their low biodegradability. When DDT is applied to soil, 50% is lost within 16-20 days. The estimated time for the loss of 90% surface applied DDT is 1.5 to 2 years. When it is worked out in soil 50% loss occurs in 5 to 8 years, and 90% of the applied insecticide is lost in 25 to 40 years (WHO, 1989). DDT is stable to light in storage, and remains active even after prolonged exposure to light. Solid DDT

is scarcely affected by UV lamp, even after 35 hours of exposure. An alcoholic solution showed no change after exposure to sunlight for one year (West and Campbell, 1950). The half lives of organochlorine pesticides are in the order of DDT (3 to 10 years), dieldrin (1 to 7 years), aldrin (1 to 4 years), isodrin-endrin (4 to 8 years), and heptachlor (7 to 12 years) (Brooks, 1974).

The organophosphorous and carbamate insecticides are degraded in the soil faster than organochlorines. The principal route of decomposition of organophosphorous insecticides in the environment is the hydrolysis process by microorganisms (Matsumura, 1973). The vapour pressure of carbamate is low; they will evaporate or sublimate slowly at normal temperature, which may lead to volatilization of carbamates from water and soil. The light absorption characteristics of carbamates contribute to their rapid photodegradation or photodecomposition. In most soils the carbamates are readily degraded by soil microorganisms (WHO, 1986e).

Most of the degradation studies on natural products are limited to pyrethrum, nicotinoids, and rotenoids. Natural pyrethrins and earlier synthetic pyrethrins contain several light absorbing moieties. So, they are photochemically unstable and have poor storage life in formulation. Successive modifications of the basic structure of natural pyrethrin made it possible to obtain more photostable derivatives. In the field, the photostable pyrethroids persist on the crops for 7-30 days, while insect infestations are controlled. The residue then reaching the soil is metabolized by soil organisms and are further degraded. Therefore, residues do not accumulate to contaminate the environment. This behaviour contrasts with that of chlorinated hydrocarbons (DDT) whose decomposition products are non-polar and may persist for years (Elliot, 1989). The main route of degradation of pyrethroids is ester cleavage followed by the oxidation of the acid and alcoholic parts into carbon dioxide. Pyrethroids are powerful lipophilic insecticides readily degraded by

sunlight and microorganisms in the soil (Demoute, 1989). Nicotinoids and rotenoids are also photodecomposed by light (Quraishi, 1977).

It is very important to evaluate the stability, persistence and degradation ability of a larvicide in the natural condition. This chapter elaborates the methods adopted for the study of shelf life of the extracts of *Curcuma raktakanda* and *Glycosmis pentaphylla* at different storage temperature for a period up to one year, the residual effect and degradation of the extracts in the soil and water environment.

5.2. MATERIALS AND METHODS

Shelf Life

The shelf life of the extracts of *Curcuma raktakanda* and *Glycosmis pentaphylla* was studied with respect to their activity on the early fourth instar larvae of *Culex quinquefasciatus*.

Stock solutions of 1% were prepared from the residue obtained from the petroleum ether extraction. A 100 mLsample of the stock solution was transferred to sterilized, labelled bottles. Each stock solution was stored in replicate bottles. The samples were stored at temperatures of 0^0C, 4^0C, and room temperature (28 ± 2^0C). The samples were stored upto one year.

Larvicidal Activity

The larvicidal activity of the samples was evaluated at different time intervals of storage period. The test dilutions and the procedure of the assay were same as done before (Chapter 3).

Residual Effect

The residual effect of the extract were studied on the early fourth instar larvae of *Cx. quinquefasciatus* and *Cx. sitiens*. The test set up was similar to that followed earlier (Chapter 3).

The larvae were introduced into the test solutions and the mortality was recorded after 24 hours. The dead and surviving larvae were discarded from the test doses and from control sets. New batches of 25 larvae were introduced to the test vessels and control sets. Mortality of these newly introduced larvae was recorded after 24 hours. These larvae were then replaced by a fresh batch. The procedure was repeated until there was no mortality in any of the test concentrations.

Degradation in Water

The stock solution of the plant extract was diluted to 250 mL with tap water to obtain samples of 100 mg/L in the larval test vessels. These were kept undisturbed upto 12 weeks. Replicate samples of these test solutions were extracted weekly in chloroform using a separating funnel. The chloroform fraction was separated and concentrated. Thin layer chromatography was performed using this fraction to determine the degradation of the extract qualitatively. The chromatograms were compared with that of the fresh extract.

Degradation in Soil

Sandy loam surface soil was collected from different locations in the vicinity of the laboratory. The soil samples were combined, mixed thoroughly, sieved to remove larger particles, and allowed to dry at 28 ± 2°C. Soil portions of 70 gm were weighed out into individual 250 mL conical flasks. The plant extracts were made upto 100 mg/L and uniformly applied to the soil. The flasks were plugged with cotton and maintained at room temperature upto 12 weeks. The moisture content in the soil was maintained at 4 - 8% with tap water. The pH of the soil was 6.5.

Replicate soil samples were extracted weekly using soxhlet apparatus in chloroform for three hours. Qualitative estimation of the degradation of the extract was determined by thin layer chromatography and compared with that of the fresh extract.

Thin Layer Chromatography (TLC)

Thin layer chromatography was conducted in 10x20 cm glass plates, coated with silica gel of 0.5 mm thickness. The coated plates were activated at 100-105°C for half an hour. The fresh extracts of *C. raktakanda* and *G. pentaphylla* were concentrated and applied to the silica gel coated plates using capillary tubes. Several such TLC plates were prepared and introduced into saturated chambers containing different solvents.

The solvents used for developing silica gel plates were:

1. Petroleum ether:chloroform (3:1)
2. Petroleum ether:methylene dichloride (3:1)
3. Petroleum ether:diethyl ether (3:1)
4. Petroleum ether:ethyl acetate (3:1)
5. Petroleum ether:alcohol (4:1)
6. Petroleum ether:methanol (5:1)
7. Benzene: chloroform (3:1)
8. Benzene: methylene dichloride (3:1)
9. Benzene: diethyl ether (3:1)
10. Benzene: ethyl acetate (3:1)
11. Benzene: alcohol (4:1)
12. Benzene: methanol (5:1)

The chromatograms were visualized by spraying anisaldehyde-sulphuric acid reagent or by exposing the chromatograms to iodine vapours. The solvent system that exhibited the best resolution of the extracts was selected for the rest of the TLC analysis.

Data Analysis

The larvicidal activity of the stored extracts was evaluated in terms of the per cent mortality and LC_{50}. The residual effect was studied using per cent mortality at 24 hour intervals. The degradation of extract was qualitatively determined by counting the number of spots on the chromatogram and calculating the R*f* values.

5.3. RESULTS

Shelf Life

The LC_{50} value of the fresh extract of *C. raktakanda* against *Cx. quinquefasciatus* was 13.73 ± 1.8 mg/L and the LC_{50} value of *G. pentaphylla* was 15.64 ± 2.08 mg/L. Upon storage the LC_{50} values varied between 13.12 ± 1.72 mg/L and 14.04 ± 1.8 mg/L with respect to the extracts of *C. raktakanda* (Table 5.1) and from 14.15 ± 1.75 mg/L to 16.18 ± 1.97 mg/L with respect to the extracts of *G. pentaphylla* (Table 5.2).

Residual Effect

The extract of *C. raktakanda* showed a gradual decrease in the larvicidal activity after the second day. At 80 mg/L the extract exhibited 100% mortality up to 4th day against the larvae of *Cx. quinquefasciatus* (Table 5.3). However, at the lower test doses, mortality was reduced during this period. On the 5th day, there was only 40% mortality of the larvae at 80 mg/L of the extract, and on the 6th day there was no mortality in any of the test doses.

Total mortality of *Cx. sitiens* larvae occured upto seven days and complete survival of the species was observed on the 11th day of the application of the extract (Table 5.4).

Table 5.1. Shelf life studies of *Curcuma raktakanda* extract on *Cx. quinquefasciatus*

Period of Storage	Temperature °C	% mortality at (mg/L) 10	20	40	60	80	LC_{50} (mg/L)
0 day	28±2	40	60	88	96	100	13.73±1.8
7 days	0	42	60	88	96	100	13.37±1.89
	4	40	61	87	95	100	13.61±1.82
	28±2	41	60	87	98	100	13.61±1.63
15 days	0	41	62	87	96	100	13.34±1.71
	4	40	60	88	97	100	13.77±1.77
	28±2	40	61	86	96	100	13.761±1.8
30 days	0	39	60	86	98	100	14.04±1.8
	4	42	60	88	97	100	13.36±1.73
	28±2	40	59	89	97	100	13.8±1.78
45 days	0	40	62	87	98	100	13.65±1.76
	4	40	60	88	97	100	13.77±1.77
	28±2	41	61	89	97	100	13.39±1.75
60 days	0	40	61	88	97	100	13.55±1.76
	4	41	59	89	98	100	13.64±1.76
	28±2	39	64	86	97	100	13.54±1.56
100 days	0	41	62	89	97	100	13.27±1.72
	4	42	61	88	98	100	13.12±1.72
	28±2	40	60	88	96	100	13.82±1.8
120 days	0	40	61	87	95	100	13.61±1.82
	4	40	60	88	97	100	13.77±1.77
	28±2	39	64	86	97	100	13.54±1.56
Six months	0	40	60	88	96	100	13.82±1.8
	4	41	62	87	96	100	13.34±1.71
	28±2	39	64	86	97	100	13.54±1.56
1 year	0	41	59	88	96	100	13.71±1.82
	4	38	60	87	97	100	13.64±1.76
	28±2	42	59	87	96	100	13.52±1.8

Table 5.2. Shelf life studies of *Glycosmis pentaphylla* extract on *Cx.quinquefasciatus*

Period of Storage	Temperature °C	% mortality at 10 (mg/L)	20 (mg/L)	40 (mg/L)	60 (mg/L)	80 (mg/L)	LC_{50} (mg/L)
0 day	28±2	32	60	84	92	100	15.64±2.08
7 days	0	32	60	85	93	100	15.77±2
	4	30	58	85	93	100	15.84±1.96
	28±2	31	63	86	94	100	14.15±1.75
15 days	0	30	60	84	94	100	16.18±1.97
	4	33	61	85	92	100	15.84±1.82
	28±2	32	62	86	91	100	15.48±1.78
30 days	0	31	61	83	92	100	15.59±2
	4	32	62	84	92	100	15.24±1.75
	28±2	32	61	85	91	100	15.84±1.82
45 days	0	30	62	84	92	100	15.84±1.96
	4	31	61	84	91	100	15.52±1.99
	28±2	30	61	86	93	100	15.78±1.92
60 days	0	30	61	85	92	100	15.84±1.96
	4	33	62	84	92	100	15.24±1.75
	28±2	31	62	85	92	100	15.41±1.91
100 days	0	32	61	85	91	100	15.84±1.82
	4	31	61	86	92	100	15.84±2
	28±2	32	60	85	91	100	15.55±2.1
120 days	0	31	61	83	92	100	15.59±2
	4	30	62	84	92	100	15.84±1.96
	28±2	32	61	83	92	100	15.84±1.96
six months	0	31	62	85	93	100	15.70±1.84
	4	32	60	84	91	100	15.45±2
	28±2	30	61	84	93	100	15.55±2.1
1 year	0	30	61	84	93	100	15.55±2.1
	4	32	61	84	92	100	15.84±1.96
	28±2	32	62	83	92	100	15.24±1.75

Table 5.3. Residual effect of the extract of *Curcuma raktakanda* on *Culex quinquefasciatus*

Days	%mortality at (mg/L)				
	10	20	40	60	80
1	42	60	88	96	100
2	41	60	88	96	100
3	28	40	72	90	100
4	nil	nil	40	60	100
5	nil	nil	nil	20	40
6	nil	nil	nil	nil	nil
Control	nil	nil	nil	nil	nil

The extract of *G. pentaphylla* exhibited residual effect on *Cx. quinquefasciatus* upto eight days. One hundred per cent mortality was exhibited at 80 mg/L upto 4th day. Further, the per cent mortality decreased successively. On the 9th day there was no mortality in any of the test doses (Table 5.5). The larvicidal activity of the extract on *Cx. sitiens* was evident upto 15th day. The extract induced 100% mortality for 9 days when applied at a concentration of 80 mg/L. After 9 days the rate of mortality decreased gradually reaching 5% on the 15th day. On the 16th day of observation the larval mortality was nil (Table 5.6).

Table 5.4. Residual effect of the extract of *Curcuma raktakanda* on *Culex sitiens*

Days	%mortality at (mg/L)					
	5	10	20	40	60	80
1	41	62	84	96	100	100
2	40	62	82	95	100	100
3	39	62	85	97	100	100
4	42	60	84	96	100	100
5	36	50	75	90	100	100
6	20	30	50	75	100	100
7	10	15	28	55	100	100
8	4	8	10	35	56	95
9	nil	nil	nil	nil	25	95
10	nil	nil	nil	nil	nil	25
11	nil	nil	nil	nil	nil	nil
Control	nil	nil	nil	nil	nil	nil

Degradation Studies

The separation of the crude extracts of *C. raktakanda* and *G. pentaphylla* in different solvent systems is represented in Table 5.7. The chromatogram of the fresh extract (zero hour sample) showed 13 spots in petroleum ether:chloroform (3:1), visualized by spraying anisaldehyde-sulphuric acid reagent and that of *G. pentaphylla* resolved into 10 spots in benzene:chloroform (3:1), visualized in iodine chamber. The R*f* values of the compounds are given in Table 5.8.

Table 5.5. Residual effect of *Glycosmis pentaphylla* on *Culex quinquefasciatus*

Days	%mortality at 10 (mg/L)	20	40	60	80
1	32	60	84	93	100
2	32	60	82	90	100
3	20	52	76	80	100
4	12	40	52	76	100
5	nil	10	20	50	88
6	nil	nil	16	40	60
7	nil	nil	nil	10	20
8	nil	nil	nil	nil	8
9	nil	nil	nil	nil	nil
Control	nil	nil	nil	nil	nil

Table 5.6. Residual effect of the extract of *Glycosmis pentaphylla* on *Culex sitiens*

Days	%mortality at (mg/L)					
	5	10	20	40	60	80
1	36	60	80	96	100	100
2	38	60	81	96	100	100
3	36	60	81	94	100	100
4	35	61	82	96	100	100
5	36	59	81	95	100	100
6	36	62	80	95	100	100
7	35	60	81	96	100	100
8	28	52	78	85	96	100
9	15	40	65	75	85	100
10	5	30	50	65	75	90
11	nil	20	40	52	60	85
12	nil	8	20	38	50	75
13	nil	nil	nil	10	20	40
14	nil	nil	nil	nil	5	20
15	nil	nil	nil	nil	nil	5
16	nil	nil	nil	nil	nil	nil
Control	nil	nil	nil	nil	nil	nil

Table 5.7. Chromatograms of *Curcuma raktakanda* and *Glycosmis pentaphylla* in different solvent systems

Solvents	Number of Spots			
	C. raktakanda		*G. pentaphylla*	
	Anisaldehyde-Sulphuric acid	Iodine	Anisaldehyde-Sulphuric acid	Iodine
1. Petroleum ether: chloroform (3:1)	13	11	5	8
2. Petroleum ether: methylene dichloride (3:1)	8	6	5	6
3. Petroleum ether: diethyl ether (3:1)	8	6	5	6
4. Petroleum ether: ethyl acetate (3:1)	11	8	5	6
5. Petroleum ether: alcohol (4:1)	6	4	4	4
6. Petroleum ether: methanol(5:1)	6	4	4	5
7. Benzene: chloroform (3:1)	12	8	6	10
8. Benzene: methylene dichloride (3:1)	6	6	6	8
9. Benzene: diethyl ether (3:1)	6	5	6	8
10.Benzene: ethyl acetate (3:1)	10	5	6	8
11.Benzene:alcohol (5:1)	6	4	4	4
12.Benzene:methanol (5:1)	6	4	4	4

Table 5.8. Chromatograms of *Curcuma raktakanda* and *Glycosmis pentaphylla* showing R*f* values.

Spots	R*f* values of	
	C. raktakanda	*G. pentaphylla*
1	0.08	0.03
2	0.15	0.12
3	0.19	0.17
4	0.21	0.34
5	0.38	0.54
6	0.55	0.68
7	0.56	0.74
8	0.62	0.86
9	0.67	0.94
10	0.76	0.99
11	0.81	
12	0.92	
13	0.97	

When these extracts were exposed to soil and water environment, they were observed to degrade within a few weeks. After one week of exposure to water and soil media, the chromatogram developed from *C.raktakanda* exhibited only 12 spots indicating that one of the fractions had decomposed. The number of fractions that resolved on the chromatogram

decreased as the duration of exposure increased. The extract in water degraded completely in 7 weeks. In the soil media, even after 12 weeks, four fractions were remaining (Table 5.9).

Table 5.9. Qualitative estimation of the extracts of *Curcuma raktakanda* and *Glycosmis pentaphylla* during weekly intervals in water and soil

Period	Number of Spots in the Chromatogram			
	C. raktakanda		*G. pentaphylla*	
	Water	Soil	Water	Soil
0	13	13	10	10
1	12	12	9	10
2	10	12	8	9
3	6	10	5	8
4	4	10	3	6
5	2	9	1	6
6	1	9		5
7		8		4
8		6		3
9		6		1
10		6		
11		4		
12		4		

The chromatogram of *G. pentaphylla* developed after exposure to water and soil media indicated that there was a gradual disappearance of the constituents with exposure time. In water media, the extract degraded in six weeks and in soil environment within ten weeks.

5.4. DISCUSSION

The larvicidal activity of the extracts of *Curcuma raktakanda* and *Glycosmis pentaphylla* is not affected by temperature upto 28 ± 2^{0}C or over a period of storage, upto one year. The observation is in sharp contrast to that of natural pyrethrins which is usually stored in glasses wrapped in metal foil at a temperature of 25^0 C due to their instability (Ray, 1991). The natural pyrethrins are heat and light sensitive, and are easily oxidized with increasing concentration and purity.

It is observed from Fig.5.1 to Fig.5.4 that the residual activity of *C. raktakanda* lasts to a maximum of 10 days against *Cx. sitiens* and that of *G. pentaphylla* to a maximum of 15 days. In a similar study Chakravarty *et al.* (1969) observed that neem seed kernel extract (0.5-1.0%) sprayed on 35 day-old cucumber crop was active only for six days. Sunlight exposure of the plants treated with neem seed kernel extract showed activity against brown hopper only for eight days (Raguraman, 1987). The results obtained on the shelf life and residual activity of *C. raktakanda* and *G. pentaphylla* are comparable to those of neem seed kernel extract. However, there is a need to evaluate the residual effect of the extracts under different ecological conditions.

When coming to the natural degrading ability of these extracts in water and in soil, it was observed that the extracts degraded faster in water than in soil. In water the extract of *C. raktakanda* degraded completely within seven weeks, whereas in soil it showed longer duration. On the 12th week four compounds, compound 8 (Rf = 0.62), compound 10 (Rf = 0.76), compound 12 (Rf = 0.92) and compound 13 (Rf = 0.97), persist in soil. The extract of *G. pentaphylla* shows degradation in six weeks in water and ten weeks in soil. The

Fig.5.1. Residual effect of *C. raktakanda* on Cx. quinquefasciatus

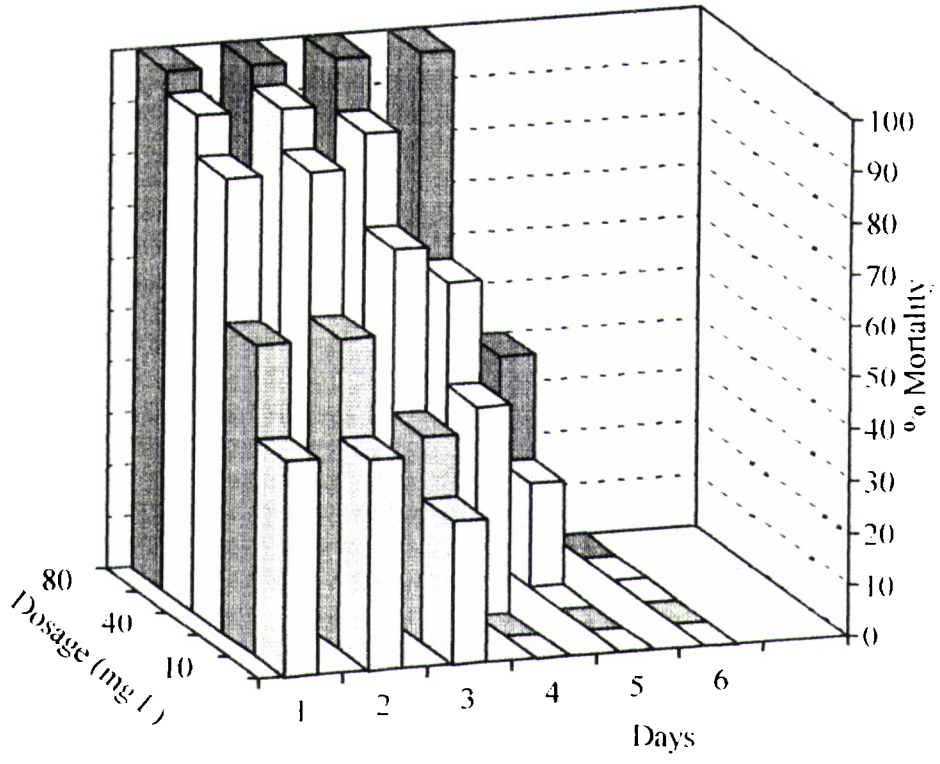


Fig.5.2. Residual effect of C. raktakanda on Cx. sitiens

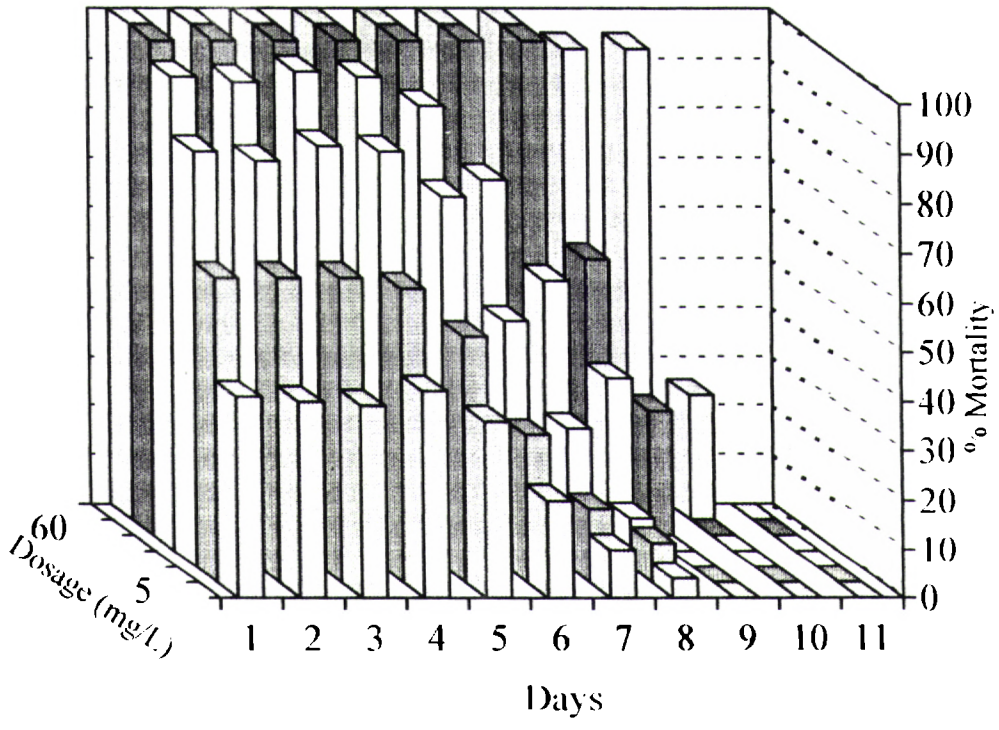


Fig.5.3. Residual effect of G. pentaphylla on Cx. quinquefasciatus

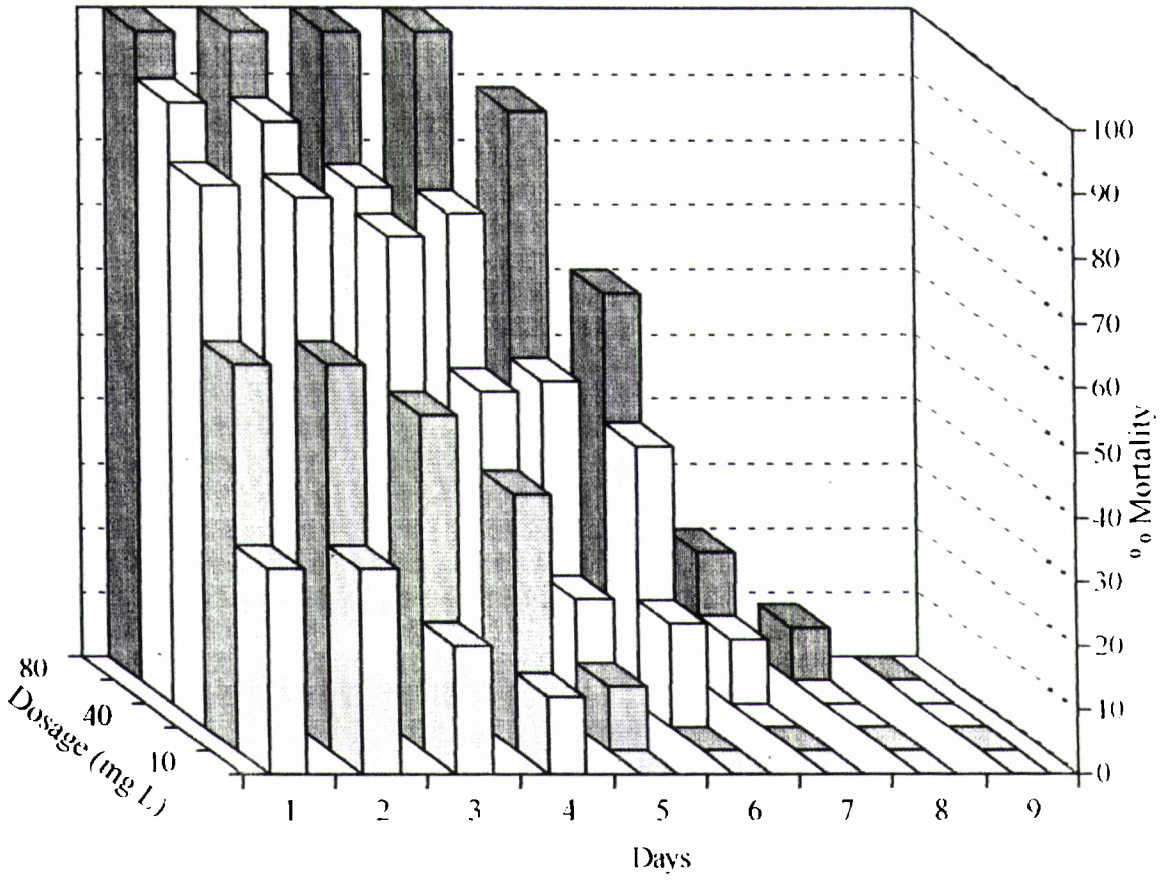
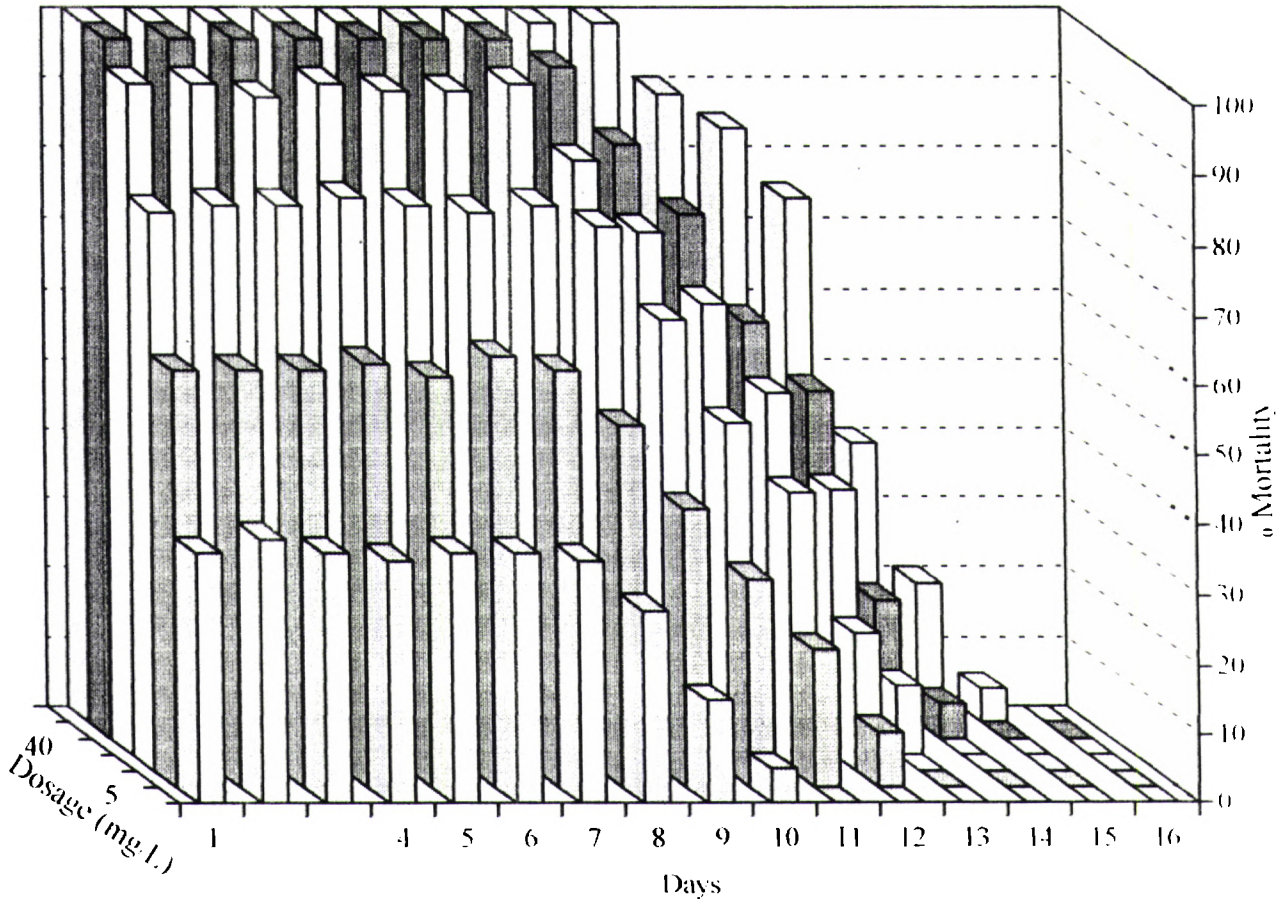


Fig.5.4. Residual effect of G. pentaphylla on Cx. sitiens



results clearly indicate that a formulation based on these plants will not be an environmental hazard as they are not persistent and hence has no possibility of accumulation. The entire mechanism of the degradation of these extracts is not studied, obviously because the nature of the compounds ~~are~~ is unknown.

World wide contamination and bioconcentration of DDT and its metabolites and the interaction of other synthetic insecticides to the ecosysten are well documented (Nimmo and Mc Ewen, 1994). Alternate methods of controlling agricultural and public health important pests are to be viewed in this context. In spite of the short life span of the extracts in water, the degrading ability in natural conditions, place *Curcuma raktakanda* and *Glycosmis pentaphylla* in a unique position in natural pesticide literature.

CHAPTER 6

ISOLATION OF ACTIVE FRACTION

- ★ Introduction
- ★ Materials and Methods
  - ★ Column Chromatography
  - ★ Collection of Elutants
  - ★ Larvicidal Screening
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  - ★ Toxicity of *Curcuma raktakanda* Fractions
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CHAPTER 6

ISOLATION OF ACTIVE FRACTION

6.1. INTRODUCTION

Plants contain a variety of bioactive compounds. Many of these compounds have been purified and characterized for use in pharmacology, as insect repellents, growth promoters and so on. Phyto-chemical analysis of the active components involves solvent extraction of the plant parts, purification and characterization of the compounds by Chromatographic and Spectroscopic methods (Ikan, 1969; Pavia *et al.*, 1976).

Phyto-chemical studies of *Curcuma longa* have been investigated from dates back, as the species is widely used for domestic purpose. In contrary, the phyto-chemical studies on *Curcuma raktakanda* is limited. However, insecticidal compounds including flavonoids and cinnamic acid derivatives have been isolated from other members of Zingiberaceae (Pandji *et al.*, 1993). *Curcuma longa* is widely used as insect repellent, and in food dye. The sesqueterpenes isolated from this plant was reported to be active against termites (Fernandez, 1990).

The phyto-chemical investigations on different parts of *Glycosmis pentaphylla* have been reported in literature. Chatterjee and Majumdar (1952) isolated two alkaloids, pentaphylline and glycosominin, from *Glycosmis pentaphylla,* and in 1953 a new alkaloid glycosin from this plant. Chakraborty *et al.* (1966) reported the structural studies on glycozolidne, the second carbazole derivative from *G. pentaphylla*. Desai *et al.* (1967) isolated arborinine, cycleanine,

isochondrodendrine and skimmianine from the leaves of *G. pentaphylla*. Govindachari *et al.* (1969) isolated arborinine from the stem. A new alkaloid glycophynine and an amide glycomide has been isolated from the flower heads (Sarkar and Chakraborty, 1977). Sarkar and Chakraborty (1979) reported a new quinazoline alkaloid from the flower heads. Das *et al.* (1982) isolated new 2-quinolone alkaloid, glycosolone from the root bark of the plant. Mukherjee *et al.* (1983) isolated a new carbazole, glycolinine from the seeds of *G. pentaphylla*. Glycozolinol, a new carbazole alkaloid has been isolated from the plant by Bhattacharyya *et al.* (1984). Bhattacharyya *et al.* (1985) reported another new carbazole alkaloid, glycozolidol from the roots of the plant. Bhattacharyya and Chowdhury (1985a) identified the structure of glycozolidal. Glycolone, a quinalone alkaloid, has been isolated from the leaves of *G. pentaphylla*. The structure of the compound has been established as 4, 8-dimethoxy-3-[3-methyl but-2-ethyl]-2-quinolone from physical and chemical evidence (Bhattacharyya and Chowdhury, 1985b). Chowdhury *et al.* (1987) isolated carbazole and 3 methyl carbazole from the root bark of *G. pentaphylla*. A carbazole alkaloid, mupamine has been isolated from the leaves of the plant (Kamaruzzman *et al.*, 1989). Jash *et al.* (1992) have isolated three carbazole alkaloids, glycozoline, 3-formyl carbazole and glycosinine from the roots of the plant. The structure of glycozoline and glycosinine has been established as 5-methoxy-3-methyl carbazole and 2-methoxy 3-formyl carbazole from the physical and chemical evidence. The alkaloids arborine, arborinine, skimmianine, glycorine, glycosmicine and an amide were isolated from the flower heads of *Glycosmis pentaphylla* (Sinhababu and Takur, 1995). The alkaloids, glycozoline and glycozolidine, isolated from *G. pentaphylla* were non-toxic to the larvae of *Culex quinquefasciatus*, whereas the alkaloid glycosolone isolated from this plant exhibited toxicity to *Cx. quinquefasciatus* (Das *et al.*, 1996).

The present investigation attempts to fractionate the crude extract of *Curcuma raktakanda* and *Glycosmis pentaphylla* by Chromatographic technique and locate the fraction inducing mortality on the mosquito larvae.

6.2. MATERIALS AND METHODS

The isolation of active fraction from the petroleum ether extract of the tuber of *C.raktakanda* and the root of *G. pentaphylla* was achieved by column chromatographic technique. The fractions obtained were subjected to larvicidal screening.

Column Chromatography

A glass column of 600x30 mm was packed with 150 gm of silica gel (60-120 mm) of column chromatographic grade in petroleum ether(60-80°C).

Preparation of Sample

A five gram residue of petroleum ether extract was dissolved in 10 mL chloroform, and approximately 12 gm silica gel was stirred with the extract until it was a free flowing powder. The sample was carefully introduced atop the silica gel packed column. The loaded column was kept undisturbed.

Collection of Elutants

In order to purify *C. raktakanda* sample, the loaded column was eluted initially with 100% petroleum ether (60-80°C). The polarity was gradually increased by 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85%, and 100% chloroform. Eleven fractions were collected in 500 mL volume.

To purify *G. pentaphylla* the loaded column was eluted initially with 100% petroleum ether (60-80°C), and then with petroleum ether:benzene (1:1). The polarity was gradually increased by 100% benzene, 25%, 50%, 75%, and 100% chloroform. Seven fractions of 500 mL were collected individually.

The solvent systems were selected based on the resolution obtained in the Thin Layer Chromatography (Chapter 5).

Larvicidal Screening

The fractions of 500 mL were evaporated separately in a rotary vacuum evaporator. The residue obtained was weighed and stock solutions of 1%(w/v) were prepared in acetone. For *C. raktakanda* eleven stock solutions, and for *G. pentaphylla* seven stock solutions were prepared in acetone.

The larvicidal activity of the fractions against early fourth instar larvae of *Culex quinquefasciatus, Culex sitiens, Aedes aegypti*, and *Anopheles stephensi* was determined by standard WHO methods, as described in Chapter 3. The activity of the fractions was computed in terms of LC_{50} and LC_{90}

Thin Layer Chromatography

The Column Chromatographic fractions that exhibited larvicidal activity were resolved on the Thin Layer Chromatogram. The procedure of TLC was similar to the one described earlier (Chapter 5). The solvent system used for *C. raktakanda* was petroleum ether:chloroform (3:1) and that for *G. pentaphylla*, was benzene:chloroform (3:1).

6.3. RESULTS

Toxicity of *Curcuma raktakanda* Fractions

Out of 11 column chromatographic fractions of *C. raktakanda*, the petroleum ether:chloroform (65:35) fraction exhibited larvicidal activity against all the test organisms. Hundred per cent mortality was obtained at 25 mg/L (Table 6.1). Since at 5 mg/L the fraction showed 56% mortality of *Cx. sitiens* larvae, an additional test dose of 2 mg/L was evaluated for larvicidal activity. The LC_{50} and LC_{90} values are given in Table 6.2.

Table 6.1. Percentage mortality of mosquito larvae after 24 hours exposure to petroleum ether:chloroform(65:35) fraction of *Curcuma raktakanda*

Test organism	% mortality at (mg/L)					
	2	5	10	15	20	25
Cx. quinquefasciatus		38	62	82	98	100
Cx. sitiens	35	56	81	96	100	100
Ae. aegypti		33	58	80	94	100
An. stephensi		40	65	84	95	100
Control	nil	nil	nil	nil	nil	nil

Table 6.2. Probit analysis of the % mortality/test doses of petroleum ether:chloroform(65:35) fraction of *Curcuma raktakanda* against *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* following 24 hours exposure

Test organism	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
Cx. quinquefasciatus	Y=2.61+2.9x	6.68	5.95-7.41	18.50	2	2.82
Cx. sitiens	Y=3.88+2.11x	3.38	2.93-3.83	12.82	2	1.54
Ae. aegypti	Y=2.42+2.96x	7.48	6.71-8.25	19.95	2	1.2
An. stephensi	Y=4.49+2.82x	6.49	5.77-7.21	18.19	2	0.91

The other fractions of *C. raktakanda* were not lethal to the mosquito species tested.

Toxicity of *Glycosmis pentaphylla* Fractions

Of the seven column chromatographic fractions collected from the residue of *G. pentaphylla*, benzene:chloroform (75:25)fraction showed larvicidal activity against all the mosquito species tested (Table 6.3). This fraction produced 100% mortality at 25 mg/L. The values of LC_{50} and LC_{90} are given in Table 6.4. At 5 mg/L the fraction showed 56% mortality against *Cx.sitiens* larvae. So, an additional test dose of 2 mg/L was evaluated for larvicidal activity and this induced 35% mortality.

The other fractions did not induce mortality to the exposed mosquito larvae.

Table 6.3. Percentage mortality of mosquito larvae after 24 hours exposure to benzene:chloroform(75:25) fraction of *Glycosmis pentaphylla*

Test organism	% mortality at (mg/L)					
	2	5	10	15	20	25
Cx. quinquefasciatus		30	58	78	94	100
Cx. sitiens	28	52	78	90	100	100
Ae. aegypti		28	55	78	90	100
An. stephensi		45	60	80	96	100
Control	nil	nil	nil	nil	nil	nil

Table 6.4. Probit analysis of % mortality/test doses of the benzene: chloroform (75:25) fraction of *Glycosmis pentaphylla* against *Cx. quinquefasciatus, Cx. sitiens, Ae. aegypti* and *An. stephensi* following 24 hours exposure

Test organism	Regression equation	LC_{50} (mg/L)	95% confidence limit	LC_{90} (mg/L)	DF	χ^2
Cx. quinquefasciatus	Y=2.27+3.06x	7.8	7.01-8.59	20.42	2	1.12
Cx. sitiens	Y=3.72+2.07x	4.16	2.26-6.06	16.98	2	0.38
Ae. aegypti	Y=2.29+2.96x	8.22	7.39-9.05	21.87	2	0.53
An. stephensi	Y=3.02+2.48x	6.71	6.00-7.42	19.95	2	2.9

Thin Layer Chromatography of Active Fractions

The chromatogram of *C. raktakanda* fraction in petroleum ether:chloroform (65:35) separated into five spots in TLC. These five spots were identified to the spots No. 4, 5, 6, 7 and 8 on the chromatogram of the crude extract. The chromatogram of the active fraction of

G. pentaphylla was resolved into four spots corresponding to the spots No. 3, 4, 5, and 6 of the chromatogram of the crude extract (Table 5.8).

6.4. DISCUSSION

The crude extract of *Curcuma raktakanda* could be separated into elevan fractions by column chromatography, out of which only one fraction exhibited larvicidal property. This larvicidal fraction resolved into five fractions on TLC. Similarly the crude extract of *Glycosmis pentaphylla* was fractionated into seven fractions by column chromatography. Only one fraction out of the seven had larvicidal property. This fraction was composed of four components as visualized in TLC. This clearly shows that the active fractions obtained from column chromatographic separation are mixtures of compounds. Absolute purity of the fractions was not obtained by column chromatography.

Eventhough there was lack of absolute purity of the active principle, the larvicidal dosage has been reduced. The LC_{90} values of the active fraction were in the range of 12.82 mg/L (against *Cx. sitiens*) to 19.95 mg/L (against *Ae. aegypti*), whereas the LC_{90} values of the crude extract were in the range of 29.11 mg/L (against *Cx. sitiens*) to 48.08 mg/L (against *Ae. aegypti*) for *C. raktakanda* (Table 3.7). Similarly, the LC_{90} values of the larvicidal fraction of *G. pentaphylla* were in the range of 16.98 mg/L (against *Cx. sitiens*) to 21.87 mg/L (against *Ae. aegypti*). The LC_{90} values of the crude extract were in the range 42.66 mg/L for *Cx. sitiens* and 57.14 mg/L for *Ae. aegypti* (Table3.11). The order of susceptibility of the mosquito species was identical in both the crude and fractionated sample. It is quite obvious that further purification of the fractions will reduce the larvicidal concentration substantially.

Although previous studies on *Curcuma raktakanda* are not available, a lot of work has been done on the other *Curcuma* species. The biological activity of *C. longa* is represented by sesquiterpenes (Fernandez, 1990). The insecticidal activities of *C. xanthorhiza* and *C. zedoaria* are due to flavonoids, sesquiterpenoids and cinnamic acid derivatives (Pandji *et al.*, 1993). On the contrary, different parts of *Glycosmis pentaphylla* have been thoroughly investigated by many. Das *et al.* (1996) investigated the toxicity of the alkaloids of *G. pentaphylla* against *Cx. quinquefasciatus* larvae. Though glycozoline and glycozolidine were non-toxic, glycosolone showed toxicity towards the larvae.

In the present study chemical nature of compounds in the active fraction can only be presumed on the basis of previous literature. It may be assumed that the activity of *Curcuma raktakanda* is due to terpenoids, and the activity of *Glycosmis pentaphylla* is due to its alkaloid content. Further investigations and standardization of the active principle of *C. raktakanda* and *G. pentaphylla* need to be done in future with HPLC and other sophisticated techniques. Even with the limited studies it is obvious that *Curcuma raktakanda* and *Glycosmis pentaphylla* will have a distinguished position in the biopesticide research.

CHAPTER 7

SUMMARY AND CONCLUSION

CHAPTER 7

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Until 1940, programmes to control vector-borne diseases were based on environmental management such as improving sanitation, and personal protection. With the advent of DDT all the measures based on sanitation were completely neglected. Though the history of chlorinated insecticides clearly points out their positive importance in public health improvement, the ecological backlashes of excessive spraying, in 1950s and 1960s have reemphasized the importance of control measures prior to DDT era, and the whole concept of Integrated Vector Management (IVM) was introduced. In fact, plant products were used as early as 17th century as contact insecticides. The success of neem and neem products based formulations has resulted in the introduction of biopesticides in integrated vector control programmes. Consequently, isolation of natural products from traditionally used medicinal plants or folklore medicines are reported from many parts of the world, especially from third world countries. The main advantage of botanicals are that they are as effective as synthetics, specific, compatible with several pesticides, have low toxicity towards mammals, birds and beneficial fauna, leave no residues in the environment, and are easily biodegradable.

Through the present investigation the larvicidal activity of 17 locally available plant species against four important mosquito species of Kochi, namely *Culex quinquefasciatus*, *Culex sitiens*, *Aedes aegypti* and *Anopheles stephensi* was tested. The mosquitoes were reared in the laboratory and larvicidal screening were conducted on the fourth instar larvae. Out of 17 plants screened for

mosquito larvicidal property six plants showed larvicidal activity against *Culex sitiens*, with no effect on the other three species. They were *Acalypha indica* (aerial part), *Adenosma capitatum* (whole plant), *Cassia alata* (aerial part), *Eclipta alba* (whole plant), *Eupatorium odoratum* (aerial part), and *Hyptis suaveolens* (aerial part). The aerial part of *Glycosmis pentaphylla* was toxic to *Culex sitiens*, but not to the other three species. However, the root of *G. pentaphylla* was lethal to all the species of mosquito larvae tested. The tuber and leaves of *Curcuma raktakanda* were effective against all the four mosquito species. The larvicidal activity was quantified as LC_{50} and LC_{90} values based on 24 hour mortality data. The LC_{50} values for *Curcuma raktakanda* (tuber) and *Glycosmis pentaphylla* (root) were in the range of 7.57 ± 1.08 mg/L to 15.46 ± 1.9 mg/L, and 7.71± 1.3 mg/L to 17.38 ± 1.83 mg/L respectively. The corresponding LC_{90} values ranged from 29.11 mg/L to 48.08 mg/L, and 42.66 mg/L to 57.14 mg/L.

The essential feature of an eco-friendly pesticide is that it should not affect non-target organisms and should not leave any environmental residues. The effects on non-target organisms were studied with respect to larvivorous fishes *Aplocheilus lineatus, Poecilia reticulata,* and *Oreochromis mossambicus*. The LC_{90} values obtained for fishes were twice that of the LC_{90} values obtained for mosquitoes. The persistence and degradability of the extracts of *Curcuma raktakanda* and *Glycosmis pentaphylla* in water and soil media were investigated. In water the extract of *Curcuma raktakanda* degraded completely within seven weeks, whereas in soil four fractions of the extract persisted after 12 weeks. The extract of *Glycosmis pentaphylla* degraded in six weeks in water and within ten weeks in soil. For a comparison it is well to remember that the persistence of synthetic pesticides, particularly chlorinated hydrocarbons in

the ecosystem is in the order of years and therefore has resulted in environmental accumulation and related issues.

A long shelf life is one of the criteria that decides the marketability of a pesticide. The biopesticides investigated remained active for one year. As the observation could not be continued for a longer period, it is assumed that the shelf life could be more than one year.

Another important finding of present investigation was the isolation of active fraction from the extracts. Within the limitation of the experimental frame work, the compound isolated showed remarkable larvicidal activity against four mosquito species. The LC_{90} values of the active fraction were in the range of 12.82 mg/L to 19.95 mg/L whereas the LC_{90} values of crude extract were in the range of 29.11 mg/L to 48.08 mg/L for *Curcuma raktakanda*. The LC_{90} values of the crude extract of *Glycosmis pentaphylla* and the active fraction were in the range of 42.66 mg/L to 57.14 mg/L and 16.98 mg/L to 21.87 mg/L respectively. These active fractions are not absolutely pure as resolved on thin layer chromatographic plates. Still, the larvicidal dosage had been considerably reduced. Further purification of these fractions need to be done in future with HPLC and other sophisticated techniques . The chemical nature of the bioactive compound can be presumed by comparing previous literature of *Curcuma* species and *Glycosmis pentaphylla*. Extensive research has to be undertaken for the characterization, and structure elucidation of the bioactive compounds in these plants. Though this is the main limitation of the present investigation, the identification of potential larvicidal plants from the local flora will definitely open the field for further research on these biopesticides.

In the light of present investigation it is borne out that *Curcuma raktakanda* and *Glycosmis pentaphylla* can be used as a major environmentally viable integrated control technique against

mosquitoes. The success of the programme would depend on devising a method to commercially produce the plant extract and make it more user-friendly.

At the fag end of the 20th century it is inevitable to introduce environmentally sound control agents in integrated vector control programmes. Considering the vastness of flora and the massive efforts that are now underway to catalogue all the flora at Panchayat level along with the traditional wisdom of the farmers and ayurvedics, it could be possible to bring forth more meaningful and comprehensive Intellectual Property Rights (IPR) laws for a sustainable and judicious use of precious natural resources of the third world countries.

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