1. INTRODUCTION

Mosquitoes are common flying insects in the family Culicidae that are found around the world. There are about 3,500 species of mosquitoes that have been around for more than 30 million years. And it seems that, during those millions of years, mosquitoes have been honing their skills so that they are now experts at finding people to bite. They have medical importance as vectors for the transmission of serious diseases that cause morbidity, mortality, economical loss and social disruption. Disease transmitted by mosquitoes include, Rift Valley fever, Elephantiasis, Japanese Encephalitis, Dengue fever, Yellow fever, Chikungunya and Malaria in African and Asian countries. There are three main mosquito vectors namely, Anopheles, Culex and Aedes which are responsible for millions of death worldwide.

Rapid increase in population, limited funds, and know-how together with environmental change and an increase in the resistance of vectors and pathogens to insecticides and drugs and a shift in vector-control operations from long-term preventive measures to on-the-spot responses have led to an increase in vector-transmitted diseases (Gubler, 1997). Vector control is an important strategy in controlling and preventing vector-borne diseases such as dengue.

Vector control is of serious concern in developing countries like India due to lack of general awareness, development of resistance and socio-economic reasons. Every year a large part of the population is affected by one or more vector-borne diseases. Mosquito control, in view of their medical importance, assumes global importance. In the context of ever increasing trend to use more powerful synthetic insecticides to achieve immediate results in the control of mosquitoes, and alarming increase of physiological resistance in the vectors, its increased toxicity to non-target organism and high costs are noteworthy (WHO, 1975).

1.1. Mosquito Borne disease-Dengue

Dengue is a mosquito-borne infection that causes a severe flu-like illness – dengue fever (DF). Symptoms of infection usually begin 4 - 7 days after the bite of an
infective mosquito and typically last for 3 - 10 days. Clinical features of DF vary according to the age of the patient with infants and young children having a fever with rash. Older children and adults may have a mild fever or a high fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. Sometimes the disease manifests as a potentially lethal complication – dengue hemorrhagic fever (DHF) – which is characterized by high fever that continues for 2-7 days and can be as high as 41° C. The fever is accompanied by other complications such as convulsions, enlargement of the liver, and in severe cases circulatory failure. DHF often begins with a sudden rise in temperature accompanied by facial flush and other flu-like symptoms. DHF is a leading cause of serious illness and death among children in some of the affected countries, especially in Asia.

1.1.1. Epidemiology

The causative organism of DF is any of four distinct but closely related viruses, or serotypes: dengue 1-4. Recovery from infection by one provides lifelong immunity against that virus but confers only partial and transient protection against subsequent infection by the other three viruses. Sequential infection by the different viruses puts people at greater risk for DHF. Dengue is endemic in at least 100 countries in Asia, the Pacific region, the Americas, Africa, and the Caribbean. The World Health Organization estimates that 50 to 100 million infections occur yearly, including 500,000 DHF cases and 22,000 deaths, mostly among children. The global incidence of dengue has grown dramatically in recent decades due to a number of factors including: continued unplanned urbanization leading to inadequate delivery of municipal services such as those related to water and sanitation, and solid waste disposal; expanding geographic distribution of the four dengue viruses and their mosquito vectors - the most important of which is the predominantly urban species Aedes aegypti; increase in production and use of non-biodegradable food, water and other product containers which when discarded provide ideal larval habitats; the import of used tyres by countries at risk for dengue; lack of national programmes for dengue prevention.

Recently there have been concerns about increasing dengue epidemics. An outbreak in Venezuela in 2007 is reported to have led to over 80,000 cases, including
more than 6,000 cases of DHF. Infection rates among those who have not been previously exposed to the virus are often 40 to 50 during epidemics but can reach 80 to 90. It is estimated that 500,000 people with DHF require hospitalization each year, the majority of who are children. About 2.5% of those affected die, but better access to medical care can reduce death rates to below 1%. However, without proper case management, DHF fatality rates can exceed 20%.

1.1.2. Dengue-Transmission

Dengue viruses are transmitted to humans through the bites of infective female Aedes mosquitoes. Aedes aegypti is the major vector but Ae.albopictus is also becoming increasingly important due to expansion in its geographic distribution. Mosquitoes generally acquire the viruses while feeding on the blood of an infected person. In order for transmission to occur the mosquito must feed on a person during a 5-day period when large amounts of virus are in the blood. This period usually begins a little before the person becomes symptomatic. Some people never have significant symptoms but can still have enough viruses to infect mosquitoes. Inside the mosquito the virus undergoes an incubation period of 8-12 days before it gets transmitted to another person. The mosquito remains infected for the remainder of its life, which might be days or a few weeks. Infected female mosquitoes sometimes pass the virus to their offspring via their eggs in what is known as transovarial transmission. Infected humans are the main carriers and multipliers of the virus, serving as a source of the virus for uninfected mosquitoes. In rare cases dengue can be transmitted in organ transplants or blood transfusion from infected donors.

1.1.2. Biology of vector, Aedes aegypti

Aedes aegypti, the major vector of dengue viruses is closely associated with people and their dwellings. In Asia and the Americas, the mosquito readily breed in man-made containers such as the concrete cisterns used for domestic water storage, metal drums, flower vases and planter dishes, discarded plastic food containers, used automobile tyres and other items that collect rainwater. In Africa the mosquito also breeds extensively in natural habitats such as tree holes, leave axils and other microhabitats within groves of leafy vegetation and banana plants. The mosquito lays
her eggs on the sides of the various water receptacles. Eggs hatch into larvae after a rain or flooding. A larva changes into a pupa in about a week, which then becomes an adult mosquito after two days. The eggs of *Ae. aegypti* are adapted to withstand desiccation and to survive without water for several months on the inner walls of containers. This makes it very difficult to control the mosquito since its populations quickly recover as a result of eggs that remain dormant for a long time before hatching until the onset of rainfall or the addition of water to containers.

Mosquito eggs hatch into larvae or "wrigglers," which live at the surface of the water and breathe through an air tube or siphon. The larvae filter organic material through their mouth parts and grow to about 0.5 to 0.75 inches (1 to 2 cm) long; as they grow, they shed their skin (moult) several times. After the fourth moult, mosquito larvae change into pupae, or "tumblers," which live in the water anywhere from one to four days depending on the water temperature and species. The pupae float at the surface and breathe through two small tubes (trumpets). Although they do not eat, pupae are quite active. At the end of the pupal stage, the pupae encase themselves and transform into adult mosquitoes. Inside the pupal case, the pupa transforms into an adult. The adult uses air pressure to break the pupal case open, crawls to a protected area and rests while its external skeleton hardens, spreading its wings out to dry. Once this is complete, it can fly away and live on the land.

One of the first things that adult mosquitoes do is seek a male, mate and then feed. Male mosquitoes have short mouth parts and feed on plant nectar. In contrast, female mosquitoes have a long proboscis that they use to bite animals and humans and feed on their blood (the blood provides proteins that the females need to lay eggs). After they feed, females lay their eggs (many need a blood meal each time they lay eggs). Adult *Ae. aegypti* mosquitoes bite people in and around houses during the day or night. There are usually distinct peaks of biting within a 24-hour period but these may vary according to weather conditions and geographic location. Females continue this cycle and live anywhere from many days to weeks. Adult mosquitoes preferentially rest in dark cool areas indoors and outdoors.

### 1.1.4 Global strategy for prevention and control
There is no vaccine or specific treatment for dengue, but appropriate clinical management frequently saves the lives of patients with the more serious DHF. Preventing mosquito bites, either through the use of self-protection measures or vector control at community level is currently the only practical way to stop dengue virus transmission in an endemic area. At the national level, many countries especially in the WHO- South East Asia and Western Pacific regions have action plans based on the Global Strategy for prevention and Control of Dengue Fever and Dengue Hemorrhagic Fever. The Global Strategy emphasizes the following five key elements depending on a country’s available infrastructure and resources:

- Selective, integrated mosquito control with community and intersectional participation.
- Active disease surveillance based on strong health information systems:
- Emergency preparedness:
- Capacity-building and training:
- Vector control research.

A number of larval and adult mosquito control methods are available which can effectively be used to control Aedes aegypti mosquitoes using an integrated vector management (IVM) approach. Hence review of naturalistic methods of control of mosquito larvae is gaining importance.

1.2. **Advantages of bio pesticides**

The chemical measures in public health programs were initially decreased the mosquito population, but these have failed because, the constant use of chemical insecticides has often led to disrupt natural biological control system and outbreaks of insect species. The conventional insecticides are based on a single active ingredient, but
plant-derived insecticides comprise an array of chemical compounds which act concertedly on both behavioural and physiological processes (Saxena, 1988). The botanical insecticides are generally insect-specific and are relatively harmless to non-target organisms including humans. They are also biodegradable and harmless to the environment (Jacobson, 1971). The failure of chemical insecticides to control the insect and growing public concern for safe food and a healthy environment have catalyzed the search for more environmentally benign control methods for the management of the vectors. To overcome these problems, it is necessary to search for alternative methods of vector control. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans, and other advantages (Liu et al., 2000).

A large number of plant extracts have been reported to have mosquitocidal or repellent activity against mosquito, but few plants have shown practical utility for mosquito control. Plant products, can be obtained either from the whole plant or from a specific part by extraction with different type of solvents. Some phytochemicals act as general toxicant both against adult as well as larval stages of mosquitoes, while others interfere with growth and development (growth inhibitors) or with reproduction (chemosterilant) or produce olfactory stimuli thus act as repellent or attractant.

### 1.2.1. *Strychnos nux-vomica* L (Etti tree)

The Strychnine tree (*Strychnos nux-vomica* L.) also known as Nux vomica, Poison Nut, Semen strychnos and Quaker Buttons, is a deciduous tree native to India, southeast Asia, a member of family Loganiaceae. It is a medium-size tree growing in open habitats. Its leaves are ovate and 2–3.5 inches (5.1–8.9 cm) in size. The fruit are about the size of a large apple with a smooth and hard shell which when ripened has a lovely orange color. The meat of the fruit is soft and white with a jelly-like pulp containing five seeds covered with a soft woolly substance. The seeds are removed from the fruit when ripe. The seeds have the shape of a flattened disk completely covered with hairs radiating from the center of the sides. This gives the seeds a very characteristic sheen. The seeds are very hard, with a dark gray horny endosperm where the small embryo is housed that give off no odour but possess a very bitter taste. The seeds contain alkaloid strychnine and brucine, traces of
strychnine, glucoside loganine 3% fatty matter, caffeotannic acid and a traces of copper. However, the tree’s bark also contains brucine and other poisonous compounds.

In herbal medicine, *Strychnos nux-vomica* is recommended for liver cancer, upset stomach, vomiting, abdominal pain, constipation, intestinal irritation, hangovers, heartburn, insomnia, certain heart diseases, circulatory problems, eye diseases, depression, migraine headaches, nervous conditions, problems related to menopause, and respiratory diseases in the elderly. In folk medicine, it is used as a healing tonic and appetite stimulant. *Strychnos nux-vomica* is used in Chinese herbal medicine to unblock channels and obstructions, reduce swelling, alleviate pain, and to treat abscesses and yin-type ulcers. In traditional Chinese treatment of cancer, it can be used in combination with other herbs. Strychnine is sometimes used as a rodenticide to control rats, mice and rodent pest of agriculture.

1.2.2. *Azadirachta indica* (Neem)

*Azadirachta indica* (Neem) is a tree in the mahogany family Meliaceae. It is one of two species in the genus *Azadiracta*, and is native to India, growing in tropical and semi-tropical regions. Indian scientists were the first to bring the plant to the attention of phyto pharmacologist in 1942 while working at the Scientific and Industrial Research laboratory at Delhi University. Three bitter compounds were extracted from neem oil, which were named *nimbin*, *nimbinin*, and *nimbidin* respectively and the seeds contain a complex secondary metabolite azaddiractin. Products made from neem tree have been used in India for over two millennia for their medicinal properties. Neem products have antihelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative. Neem products are also used in selectively controlling pests in plants. It is considered a major component in Ayurvedic and Unani medicine and is particularly prescribed for skin disease. All parts of the tree are said to have medicinal properties (seeds, leaves, flowers and bark) and are used for preparing many different medical preparations. Part of the Neem tree can be used as a spermicide.
Neem-based bio pesticides and neem extracts have a wide range of effects against insect pests including repellence, feeding, toxicity, sterility and growth regulator activity and are relatively safe towards non-target biota with only minimal risk of direct adverse effects on aquatic biota from contamination of water bodies. Allelochemicals such as azadirachtin, nimbin, nombidin, nimbolides, nimbolic acid, salannin, meliantritol, azadirachtol present in neem affect the biochemical and physiological processes of insect system and nullify the insect detoxification mechanism thereby not allowing the pest to develop resistance. As an emulsifiable concentrate, the neem oil formulation had greatly reduced sized particles and evenly mixed within the water column with a few suspended particles on the water surface. The spread of these fine particles probably increased the efficacy of formulation. Furthermore, this could potentially be a sustainable component of Integrated Vector Management programmes.

1.2.3. *Bacillus thuringiensis* var. *israelensis*

Microbial larvicides are formulated to deliver a natural toxin to the intended target organisms. Bacteria are single-celled parasitic or saprophytic microorganisms that exhibit both plant and animal properties and range from harmless and beneficial to intensely virulent and lethal. *Bacillus thuringiensis* (*Bt*), is the most widely used agricultural microbial pesticide in the world. *Bt* products have been available since the 1950s. Mosquito control agents based on *Bt* are the second most widely registered group of microbial pesticides. *Bacillus thuringiensis* is a bacterium which occurs naturally in soils and aquatic environments globally. In 1976, Goldberg and Margalit isolated *Bti* from *Culex pipiens* collected in an Israeli river bed. In 1977, de Barjac designated this *Bt* strain as H14, noting that it is toxic to mosquito and black fly larvae. Over the last three decades, a number of other strains have been investigated, some with desired larvicidal effects. Two strains, SA3A and FM65-52, are currently utilized for commercial products.

The active ingredients in *Bti* formulations are delta-endotoxin (d-endotoxin) crystals separated from bacteria near the end of manufacturing processes. These toxic crystals are incorporated into various products which allow their release into water so that they may be ingested by mosquito larvae. The d-endotoxin crystals are activated
by the alkaline environment and enzymes of the mosquito midgut. The alkaline gut environment allows hydrolysis of the crystal’s protein coating and the release of pro-toxins. Gut enzymes then activate the pro-toxins and facilitate their binding to the gut epithelium of the mosquito larva. Cells rupture and are destroyed at the binding sites, leading to a loss of body fluids which results in death. This rapid action typically controls larvae in 4-24 hours.

*Bti* products are safe for non-target organisms in the environment. The crystalline d-endotoxins are not activated in the acidic guts of humans or other animals or in the alkaline guts of animals which do not contain the enzymes necessary for activation and binding of released pro-toxins. This specificity accounts for the highly selective nature of *Bti* larvicides which is limited to Dipterans, notably mosquitoes, black flies, and some midges. When insects ingest toxin crystals, the alkaline pH of their digestive tract activates the toxin. *Bacillus thuringiensis* produces many parasporal crystal toxins during sporulation that on proteolysis bind with the specialized mid gut receptors, thereby causing disruption of gut epithelium, gut paralysis, toxemia and eventual death of the host insect. It is effective on most mosquito species in a very wide variety of habitats; *Bti* formulations are thus ideally suited for IPM. They are now used as specific insecticides under trade names such as Dipel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on human, pollinators and other animals. *B. thuringiensis*-based insecticides are often applied as liquid sprays on crop plants or in the mosquito breeding sites, where the insecticide must be ingested to be effective.

1.3. Natural Bio control agents

To protect the safety of the environment, the use of biological agents in mosquito control operation to control vector borne diseases has been emphasized during the last two decades. Biological control, using the natural enemies of *Aedes* appears to be an alternative approach to the systematic failure of use of insecticides (Lardeux *et al.*,1992). There are several types of biological control including the direct introduction of parasites, pathogens and predators to target mosquitoes. Predatory copepods are one of the natural enemies that feed on mosquito larvae.
These agents are microcrustacea, present in fresh water worldwide. *Mesocyclops* is one of the genus of copepods that has most been studied as an antagonist of mosquito larvae and whose effectiveness has been demonstrated in different countries, including the United States (Marten, 1990), Honduras (Marten *et al.*, 1994), Vietnam (Nam *et al.*, 2000), and the French Polynesia (Lardeux *et al.*, 1992). Cyclopoids are more effective for *Aedes* control than other aquatic invertebrates that prey on mosquito larvae because their high reproductive capacity and broad diet including phytoplankton, protozoa, and small animals enable them to maintain abundant populations independent of mosquito larval abundance. Their small size and high reproductive capacity also make cyclopoids inexpensive and convenient for large-scale production and distribution (Riviere *et al.*, 1987; Marten 1990c; Suarez *et al.*, 1992). A high reproductive capacity and a generation time of 2 to 4 weeks (depending upon temperature), plus the fact that a female is inseminated for life, ensure that a large population will be established within a few weeks after introducing only a few adult female cyclops population can persist for long periods in a container, sometimes years. Many species can survive when a container dries up, as long as some soil or detritus remains. The eggs, which are carried in sacs on each side of a female’s body until they hatch, are not resistant to desiccation. Cyclops can survive in dryness as adults or copepodids, which are active within hours after there is water in the container again.

### 1.3.1. Biology of Copepods

The body is tear drop shaped with large antennae. Although like other crustaceans they have an armored exoskeleton, they are so small that in most species this thin armor and the entire body is almost transparent. Copepods do not have eyes; the eyespot in the middle of the forehead detects light but does not form an image. Copepods move by means of rapid oar like movements with the help of their large antennules (the long structures extending to each side of the body from the front). The antennules contain mechanical sensory organs that detect vibrations in the water so that copepods know when small animals such as mosquito larvae are close enough to be captured as food. The male produces an adhesive package of sperm and transfers it to the female’s genital opening with its thoracic limbs. Female copepods carry egg
sacs on both sides of their body for about three days until young copepods emerge from the eggs. The eggs hatch into nauplius larvae, which consist of a head with a small tail, but no thorax or no true abdomen. The nauplius moults five or six times, before emerging as a “copepodid larva”. This stage resembles the adult, but has a simple, unsegmented abdomen and only three pairs of thoracic limbs. After five moults, the copepod finally takes on the adult form.

Copepods eat small animals up to twice their own size. The total capacity of a copepod population to kill mosquito larvae is enormous. About 10 percent of areas with water where mosquitoes might breed have natural populations of *Mesocyclops* or other large copepods, which drastically reduce the survival of mosquito larvae. The development of copepods for dengue control has been much more successful because copepods are effective and easy to use in the simple container habitats where *Aedes aegypti* breeds. It is unusual for copepods to get into man-made containers; but they thrive in many kinds of containers when introduced, and they do so independently of the supply of mosquito larvae. Copepod populations range from hundreds in a rainwater-filled tyre to thousands in a water storage tank. The largest species usually kill more than 99 percent of the *Aedes aegypti* larvae, and they usually stay in a container for as long as there is water. The simple life cycle of copepods and their ability to thrive on a diet of protozoa make mass production easy and inexpensive. The system is simple, inexpensive and highly resilient, functioning in open containers of any size or shape. One hundred adult female mesocyclops produce about 25,000 new adult females within a month. Females are inseminated during adolescence and require no further contact with males to produce 50 to 100 eggs weekly during their several-month life span.

1.3.2. Copepods - *Mesocyclops thermocyclopoides*

*Mesocyclops thermocyclopoides* Harada is a common, and sometimes dominant, cyclopoid copepod found in the shallow ponds and lakes of India. This copepod feeds on the 1st and 2nd instars of the mosquito larvae, fatally wounding about seven individuals per day (Hernández and Schaper, 1999). It is one of the prevalent species in India and it has been used as a means of biological control in other countries also. If a copepod and a larvicide are applied together, the larvicide
can produce an immediate kill of all larval stages, and the cyclops can take over as the larvicide diminishes in effectiveness.

Of several approaches the use of plant extracts, microbial pesticides and predatory organisms are safer in use, non toxic to non-target organism and non-residual nature. Larviciding is a general term for killing immature mosquitoes by applying agents, collectively called larvicides, to control mosquito larvae and/or pupae. Larval Source Management (LSM) involves both the modification of water habitats, often referred to as source reduction and the direct application of larvicides to control mosquito production. Most mosquito species spend much of their life cycle in the larval stage and they are highly susceptible to both predation and control efforts. So, targeting mosquito at larval stage with natural agents would be more effective than any other strategy. The efficacy of the bio pesticides increases if they are used in combinations of two or more.

In this context, an attempt has been made to evaluate the combined effect of *Strychnos nux-vomica, Azadirachta indica, Bacillus thuringiensis* and copepod, *Mesocyclops thermocyclopoides* in controlling dengue vector, *Aedes aegypti*. 