CONTROL OF MOSQUITOES WITH BILE AND HERBAL EXTRACTS AND ASSESSMENT OF RESISTANCE OF POPULATION OF MOSQUITOES IN PUDUCHERRY

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INDEX

Introduction

Mosquitoes (means "little fly" in Spanish), the dipteran or two winged blood sucking ectoparasite that found in all over the world except Antarctica, are serious health hazardous biting insect of both men and animals. Besides nuisance biters, the different species of genera *Anopheles, Aedes* and *Culex* are also the obligate vectors of many pathogens of men and animals like malaria, dengue fever, arboviral encephalitis, chikungunya, West Nile virus, yellow fever, filariosis etc., which are not only the emerging health diseases in India including Puducherry but also in many other countries in the world, that leads to high morbidity and mortality in human and livestock, leading to major economic and health problems (WHO 2010, Bureau of Epidemiology 2011). Besides blood sucking activity and annoyance, the diseases spread by the mosquitoes lead to death of million of people and animals through out the world. Even today, mosquitoes transmitting malaria kill 2 million to 3 million people and infect another 200 million or more every year. Presently mosquitoes transmitted malaria is endemic in 104 countries and about 219 million cases of malaria (ranging154 – 289 million) and 660000 death (range 610000 – 971000) are reported in 2010 (WHO 2012). Mosquitoes follow the holometabolus life cycle and comprises of eggs, larvae, pupa and adults. There are about 490 species of *Anopheles* mosquitoes including sibling species are existing worldwide. Approximately 60–70 species can transmit malaria worldwide and of these, about 30 are vectors of major importance (WHO 2013). More than ten millions people are usually killed by other mosquito-borne diseases, like filariosis, yellow fever, dengue, encephalitis etc. The threat of encephalitis from mosquitoes is far alarming than the threat of malaria. Encephalitis, meningitis and other diseases can develop from the bites of mosquitoes infected with certain viruses like viruses of West Nile, St. Louis encephalitis, La Crosse (California) encephalitis, and Eastern equine and Western equine encephalitis etc. Therefore, mosquito control is an important component of the WHO Global Strategy for 'Malaria and Mosquito Borne Diseases' – whose objective is to break the transmission of mosquito born diseases.

The conventional strategies for controlling the mosquitoes menace includes using of long lasting insecticidal nets (LLINs) (Lengeler, 2004), indoor residual spraying (IRS) (Tarser *et.al*., 2007) or both. LLINs and IRS are most effective for late night anthrophilic

(feeds on human), endophagic (feed indoors) and endophilic (prefer to rest indoor) mosquitoes (Lengeler 2004, Tarser *et.al.,* 2007). But many mosquitoes are zoophilic in nature (feed on animal) or feed early in the evening outside the animal sheds or human houses – where the LLINs and IRS are least effective. As biological tools, *Gambusia* fishes (*G. affinis, G. holbrooki*) are widely used through out the world as larviphagous predators but these fishes may adversely affect the native fishes and other organisms. At present about 315 larviphagous fishes belonging to 32 genera under 7 families are used for mosquitoes control, but family *Cyprinodontidae* contributes the highest number of genera (15) and species (300) (Goutam *et.al*., 2013). Other promising larviphagous fishes are *Aphanius, Valencia, Aplocheilus, Oryzias, Epiplatys, Aphyosemion, Ropoffia, Nothobranchius, Pachypanohax, Rivukus, Fundulus, Cynolebias* etc. (Walton, 2007). However larviphagous fishes may not be randomly available to all the water resources preferred by mosquitoes for egg laying activities like fresh or stagnant water marshes, rice fields, grassy ditches, small temporary rain pools, foot prints, hoof prints, tree holes etc. More over *Aedes* spp. prefers to lay eggs on moist surface that suppose to be submerged with water in future.

During 'Global Malaria Eradiation Programme' in 1950s and 1960s, dieldrin resistance involving a specific resistance mechanism (gamma-aminobutyric acid (GABA) receptor) was recorded in *Anopheles gambiae* followed by DDT resistance in 1967, that develops either due to specific detoxification mechanisms (glutathione-s-transferase) or to nerve insensitivity resulting from a modification of a target site (sodium channel). From 1970, pyrethroids have been extremely in use throughout the world for mosquito control because they are safe, cheap, effective and long lasting. Alternatives such as organophosphates and cabamates are available for indoor spraying but they are more costly and less effective. Pyrethroids are the only insecticide approved by WHO for use in bed nets, curtains etc. because of their low mammalian toxicity and rapid degradation in the environment. Over the past decades, billions of dollars have been spent on distributing long lasting pyrethroid treated bet nets and indoor spray but its extreme use leads to development of resistant strain (Elissa *et. al*., 1993), thus key weapons to fight against mosquitoes, pyrethroid insecticides are loosing their edge. Regarding pyrethroid, Jo Lines, Head of Vector Control at Global Malaria Programme of WHO, Jeneva,

Switzerland, says "data are coming in thick and fast, indicating increase levels of resistance and also of resistance in new places". It is because of gene mutation that helps the mosquito's nerve cells to withstand pyrethroid attack and also due to increase level of enzymes that destroy the pyrethroids before they reach to their target (Ranson, *et. al.*, 2000). Thus, control of mosquitoes by use of chemical insecticides are becoming less effective not only due to the development of insecticides resistance but also due to biological magnification of toxic substances through the food chain, and adverse effects on environmental quality and non target organisms including human health and is alarming for alternative control strategies. However, in a developing country like India, the strategic control of mosquitoes cannot be imagined without the use of chemical based acaricides because of economic status of rural people and farmers, despite the probability of increasing trend of emergence of resistant mosquito population.

One of the earliest synthetic chemical, used as insecticide was lindane, but because of resistance problem and residual effects, its use along with other organo chlorines were curtailed. Malathion was first used in 1960s against insects and was used extensively for long time that lead to development of resistance in most of the countries, It has been observed that resistance to chemical acaricides can be developed in as few as 10 generations when the circumstances are very conducive (Hill, 1990) like use of sub lethal doses, improper adaptation of methodology or formulations or improper application of acaricides in improper time that lead to rapid evaporation by sunlight and leaves the traces of insecticides in the environment.

Insect growth regulators (IGRs) and microbial insecticides are intrinsically non-toxic, biologically specific, and environmentally safe compared to conventional chemical larvicides. However, only a limited number of IGRs such as juvenoid methoprene, chitin synthesis inhibitors such as diflubenzuron and triflumuron have been approved for use in mosquito control by WHO Pesticide Evaluation Scheme (WHOPES) (WHO 1997). Among these, resistance to methoprene has already been reported in some vector mosquito species (Amin and White, 1984, Dame *et. al.*, 1998, Cornel *et al.*, 2000). Therefore, there is a need to identify and evaluate newer IGR compounds or formulations to be used in vector control programmes (Sadanandane *et.al.*, 2012).

Because of resistant problem, environmental pollution and rising price of new synthetic insecticides, the search for new eco-friendly vector control tools has stimulated the use of phytochemicals in recent years, due to its target specificity, eco-friendly, readily biodegradable, cost-effectiveness and non toxicity to non target agents and they induce multiple effects against mosquitoes like growth regulations, fecundity, suppression, mal-sterility, larvicidal, ovicidal and deterrence of oviposition activity etc. (Su and Mulla, 1999).

From the onset of human civilization it was known that certain plants posses some insecticidal properties. About 2000 species of plants produces certain chemicals (trepans, tannins, certain alkaloids etc.) that have either repellent or controlling properties or some appears to be either juvenile hormone analogues or mimics (Hill 1990). For thousands of years mankind is using plant source to alleviate or cure illnesses or insect infestations. Application of active toxic agents from plant extracts as an alternative mosquito control strategy was available from ancient times. Owing to the fact that the application of synthetic larvicide has envenomed the surroundings as well as non-target organisms, natural products of plant origin with insecticidal properties have been tried as an indigenous method for the control of a variety of insect pests and vectors in the recent past. Insecticides of plant origin have been extensively used on agricultural pests and, to a very limited extent, against insect vectors of public health importance. The use of plant extracts for insect control has several appealing features as these are generally more biodegradable, less hazardous and a rich storehouse of chemicals of diverse biological activities. Plants constitute a source of novel chemical compounds which are of potential use in medicine and other applications. Plants contain many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which are deposited in their specific parts such as leaves, flowers, bark, seeds, fruits, root, etc. WHO has estimated that about 80 % of the more than 4000 million inhabitants of the world rely mainly on the traditional medicines (plant extracts or their active principles) for their primary health care needs. In 1985 Farnsworth *et al.* identified 119 secondary plant metabolites which were used as drugs. Out of 255 drugs which are considered as basic and essential by the World Health Organization (WHO), 11% are

obtained from plants and a number of synthetic drugs are also obtained from natural precursors.

Photochemical derived from plant origin have been used rationally either to repel or kill mosquitoes or their developmental stages since antiquity in India and many parts of the world. The synthetic chemicals and insecticides used for control of various insects are causing irreversible damage to the eco system since many of these are non degradable in nature. Various solvents are in use for extraction of various chemicals like chloroform (terpenoids, flavonoids, alkaloids, aglycones), exane (fat, waxes), dichloromethane (terpenoids, alkaloids, aglycones), dietylether (aglycones, alkaloids), ethyl acetate (alkaloids, aglycones,glycicides), acetone (alkaloids, aglycones, flavonols), ehanol (tannins, polyphenol, flavonol, terpenoids, sterols, alkaloids, propolis, polyacetylene), methanol (saponins, tannin, sugar, flavonols, phenones, aminoacids, anthocynins, terpenoids, xanthoxyllines, totarol, lactones, polyphenols) and water (suger, aminoacids, tannins, lactins, terponoids, anthocynins, starches, polypeptides)

Bile from bears and domestic animals like cattle and pig has long been used as traditional home remedies in many countries. In Japan, bile is used as component of digestive medicine and in China, bear bile is used as traditional medicine for about 1300 years (Shiro and Koichi, 2012). Animal bile is mainly composed of bile acids with highly hydrophobicity such as glycine and taurine conjugates of cholic acids and deoxycholic acids (Watanebe, *et al*., 2009), which are highly hydrophibic and cytotoxic in nature (Rode, *et al*., 1998 and Iwaki, *et al*., 2007). Though it has medicinal property but no work has been conducted on arthropods. Preliminary laboratory trials of bile of fish and ruminants are found promising candidate against developmental stages of housefly and mosquitoes and probably bile would be the promising alternative candidate for control of mosquitoes along with the conventional herbs.

The knowledge of resistance status of mosquitoes in Puducherry may provide an effective means for the development of alternative integrated vector control strategies. Therefore, it is the early dawn to launch extensive research to explore the eco friendly, health friendly biological products to overcome the mosquitoes menace and also to monitor the degree of resistance that might be developed in mosquitoes due to

prolonged use of chemical acaricides. With this back ground the present study was conducted with the following objectives:

1. **Monitoring of insecticide resistance of mosquitoes in Puducherry by CDC Bottle test and WHO insecticide resistance monitoring kits against larvae and adults.**

2. **Search for other effective means for control of mosquitoes by use of few unconventional herbs and few unusual chemical acaricides, not attempted till now against mosquitoes or its developmental stages**

Common mosquitoes from Puducherry

a) *Anopheles* spp. :

The females are only the blood suckers and prefer to suck blood from different mammals, including humans. At rest adult keeps head, thorax and abdomen upwards in diagonal position at a discrete angle to the surface. Species in the genus *Anopheles* have long palps equal in length to the proboscis. It is dark brown to black in colour, covered with dark brown to black hairs. *Anopheles quadrimaculatus* bears dark scales on the wings with patches of scales forming four darker spots on the wing The female mosquito mates several times in her short lifespan (few weeks) and after each meal produces large number of eggs preferably on surface of stagnant water resources or some times fresh water streams, ponds, and lakes with aquatic vegetation. The eggs are laid singly and bears float on either side. It cannot survive drying/desiccation and hatch within two days to two weeks after oviposition. The species of *Anopheles* mosquito is universally known as the Malaria Mosquito since it is a prime vector for human malaria. It also transmits heartworm in dogs. They were collected monthly from April 2013 to March 2014 using a mouth aspirator from human dwellings and cattle sheds,the artificially dug pit shelters and pot. In different pockets of Puducherry, *Anopheles* (*An. stephensi* ?) were found very common.

b. *Culex* spp:

Culex are medium-sized brown coloured mosquitoes having whitish markings on the abdomen and at rest keep the thorax and abdomen parallel to ground. They prefer to bite at dusk and after dark . *Culex pipiens and C. quinquefasciatus* commonly found in houses are more common in urban areas but *C. tarsalis* (western encephalitis mosquito) are more common in rural areas. *C. quinquefasciatus* was commomly available in different parts of Puducherry. They lay eggs in rafts in water filled containers, tree holes, ditches, sewage and septic system water, catch basins (storm drains), non-chlorinated swimming and wading pools, decorative ponds, bird baths, flower pots, buckets, clogged gutters, abandoned tires, and water-retaining junk and debris of all sorts. They cannot develop in running water and water that is present less than a week.

Adult *Culex* mosquitoes do not fly far from where they develop as larvae and die with onset of winter but those staying in house can "over-winter" in protected places like sewers, crawlspaces and basements. It transmits avian malaria, lymphatic filariosis caused by *Wuchereria bancrofti* and West Nile virus world wide.

c). *Aedes* spp.

Aedes aegypti is responsible to transmit dengue fever, chikungunya, bird malaria, yellow fever etc. and is very common in India. It is also called as tiger mosquito, because of presence of white bands on legs and other body parts. The mosquito probably originated in Africa (Mousson , 2005) but is now found in both tropical and subtropical regions throughout the world. Although *Aedes aegypti* mosquitoes most commonly bite at dusk and dawn, indoors, in shady areas, or when the weather is cloudy, but can bite throughout the year and at any time of day. It prefers to breed areas of stagnant water such as flower vases, uncovered barrels, buckets, discarded tires, toilet tanks or even in moist soil and may go for hibernation for 3 years. Research has shown that certain chemicals emanating from bacteria in water containers stimulate the female mosquitoes to lay their eggs. They are particularly motivated to lay eggs in water containers that have the correct amounts of specific fatty acids associated with bacteria involved in the degradation of leaves and other organic matter in water. The chemicals associated with the microbial stew are far more stimulating to discerning female mosquitoes than plain or filtered water in which the bacteria once lived.

CHARACTERISTIC FEATURES OF MOSQUITOES

ADULT STAGES:

Aedes spp

1. Wing with thin scale
2. Tip of abdomen is pointed
3. In abdomen, white bands are present laterally and basally (medially)
4. Prescapular bristles or setae are absent
5. Antennae are about same size as the proboscis
6. Sits on surface almost in parallel manner
7. Palps are very short than proboscis in female but in male palps are longer than proboscis with tapered tips

Anatomical structures	*A. aegypti*	*A. albopictus*	*A. vexans*
Wings	dark	dark	dark
Proboscis	dark in colour	dark in colour	dark in colour
Palps	dark with white tip; clypeus white	dark with white tip; clypeus black	dark with a few white scales on tip
Scutum	dark with white lyre shaped pattern stripe	dark with white median stripe	brown with golden brown scales
Thorax	dark with patches of white scales	dark with patches of white scales	brown with a few patches of whitish scales
Abdomen	dark with narrow white basal bands	dark with narrow white basal bands	dark with white basal bands shaped like an inverted 'V'
Hind legs	dark with white basal bands	dark with white basal bands	dark with narrow white basal bands

***Anopheles* spp**

1. Palps are as long as proboscis in both sexes but in male palps are club shaped at tips
2. Sits on surface as diagonal manner
3. No white bands on body surface
4. Wing vein no 2,4,6 are forked, covered with brown/black/white//cream coloured scale
5. Posterior border of wings have fine scale in fringed manner.
6. Wings may bears dark and pale spots

Anatomical structures	*A. crucians*	*A.quadrimaculatus*	*A. walkeri*
Wings	light & dark scales; costa dark, wing tip white; 3 dark spots on 6th vein	light & dark scales; 4 distinct darker spots on 6th vein	dark scales, often with 4 more or less distinct dark on 6th vein
Palps	dark with white rings	dark	dark with narrow white rings
Occiput	frontal tuft pale	frontal tuft pale	frontal tuft dark
Halter	knob dark scaled	knob dark scaled	knob dark scaled
Hind legs	dark with pale 'knee' spots	dark with pale 'knee' spots	dark with pale 'knee' spots

***Culex* spp**

1.No prespiacular or post spiracular setae present

2. Antennae are about same size as the proboscis

3.Legs are dark but no bands

4. Sits on surface almost in parallel manner

5. Palps are very short than proboscis in female but in male palps are longer than proboscis with tapered tips

Anatomical structures	***C.biscaynensis***	***C. declarator***	***C. pilosus***	***C.quinquefasciatus***
Wings	dark	dark	dark	dark
Thorax	pale, no patches of scales	dark brown spots create striped pattern; patches of white scales	dark with few patches of white scales	small patches of white scales
Scutum	brown	brown	dark.	straw
Palps	long & dark	long & dark	long & dark	long & dark
Oocciput				dark with a narrow line of flat, ovate scales bordering eye
Hind legs	dark	dark	dark	dark
Abdomen	dark with lateral white patches	dark with lateral white patches	dark, with bronze or blue-green reflection	dark with white basal bands shaped like an 'M'
Hind legs	dark	dark	dark	dark

LARVAL STAGES

Aedes spp.

1.Siphon present with pectin
2. Single hair tuft beyond pectin
3. Anal segment is completely ringed by seddle or if ringed not pierced by ventral brush
4. Swims in faster speed
5. Palps are very short than proboscis

Anopheles spp

1. Abdomen has palmate hairs
2. Siphon tube absent
3. Remains parallel to water surface in resting phase and resembling a twig
4. Swims in a stiff manner

Culex spp

1.Comb scales are present in last segment of abdomen and is more than one raw
2.Siphon present with pectin
3. Two or more hair tufts beyond pectin
4. Swims very fast

Review of literature

Work done in India on phytochemicals against mosquitoes:

To overcome problem of resistance and environmental safety, two formulations of Insect growth regulators (IGRs), viz. diflubenzuron, 2 per cent granular (GR) and 2 per cent tablet (DT) were tested in Puducherry for its efficacy against *Culex quinquefasciatus*, in comparison to its 25 per cent wettable powder (WP) formulation. WP, GR and DT formulations were tested in cesspits, street drains and abandoned wells each at four dosages, 25, 50, 75 and 100 g ai/ha. Additionally, the DT formulation was tested at a higher dosage of 1 tablet/m2 (equal to 400 g ai/ha). The WP and GR formulations yielded >80 per cent inhibition of adult emergence (IE) for 7-10 days in cesspits, 4-7 days in street drains and 7-21 days in abandoned wells at all dosages tested. The DT formulation was effective only at higher dosage 100 g ai/ha and or 1 tablet/m2 for 7-15 days at all habitats (Sadanandane *et.al*., 2012).

The ovicidal, larvicidal and adulticidal activities of crude hexane, ethyl acetate, benzene, chloroform and methanol extracts of root of *Asparagus racemosus* were assayed for their toxicity against three different mosquitoes species in Tamil nadu, India, viz., *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* The mean percent hatchability of the eggs was observed after 48 h post-treatment. The percent hatchability was inversely proportional to the concentration of extract and directly proportional to the eggs. All the five solvent extracts showed moderate ovicidal activity; however, the methanol extract showed the highest ovicidal activity. The methanol extract of *Asparagus racemosus* against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* exerted 100 % mortality (zero hatchability) at 375, 300 and 225 ppm, respectively. Control eggs showed 99-100 % hatchability. The larval mortality was observed after 24 h of exposure. All extracts showed moderate larvicidal effects; however, the highest larval mortality was found in methanol extract of root of *Asparagus racemosus* against the larvae of *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* with the LC50 and LC90 values were 115.13, 97.71 and 90.97 ppm and 210.96, 179.92, and 168.82 ppm, respectively. The adult mortality was observed after 24 h recovery period. The plant crude extracts showed dose-dependent mortality. At higher concentrations, the adult showed restless movement for some times with abnormal

wagging and then died. Among the extracts tested, the highest adulticidal activity was observed in methanol extract against *Anopheles stephensi* followed by *Aedes aegypti* and *Culex quinquefasciatus* with the LD50 and LD90 values were 120.44, 135.60, and 157.71 ppm and 214.65, 248.35, and 290.95 ppm, respectively. No mortality was recorded in the control (Govindarajan and Sivakumar, 2014).

Culex quinquefasciatus, the vector for filariasis in India, was attempted to eradicate using nanoemulsion of eucalyptus oil. The nanoemulsion was formulated in various ratios comprising of eucalyptus oil, tween 80 and water by ultrasonication. The stability of nanoemulsion was observed over a period of time and 1:2 ratios of eucalyptus oil (6%) and surfactant (12%) was found to be stable. The formulated eucalyptus oil nanoemulsion was characterized by transmission electron microscopy and dynamic light scattering. The nanoemulsion droplets were found to have a Z-average diameter of 9.4 nm and were spherical in shape. The larvicidal activity of eucalyptus oil nanoemulsion and bulk emulsion was tested and compared. Nanoemulsion showed higher activity when compared to bulk emulsion. The histopathology of larvae-treated and untreated nanoemulsion was analyzed. Furthermore, biochemical assays were carried out to examine the effect of nanoemulsion on biochemical characteristics of larvae. The treated larval homogenate showed decrease in total protein content and a significant reduction in the levels of acetylcholinesterase. The levels of acid and alkaline phosphatase also showed reduction as compared to control larval homogenate (Sugumar *et.al.*, 2014).

Larvicidal efficacy of an emulsified concentrate of neem oil formulation (neem oil with polyoxyethylene ether, sorbitan dioleate and epichlorohydrin), was evaluated against late 3rd and early 4th instar larvae of different genera of mosquitoes. The larvae were exposed to different concentrations (0.5–5.0 ppm) of the formulation along with untreated control. Larvicidal activity of the formulation was also evaluated in field against *Anopheles*, *Culex*, and *Aedes* mosquitoes. The formulation was diluted with equal volumes of water and applied @ 140 mg *a.i.*/m^2 to different mosquito breeding sites with the help of pre calibrated knapsack sprayer. Larval density was determined at pre and post application of the formulation using a standard dipper. Median lethal concentration (LC_{50}) of the formulation against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* was found to be 1.6,

1.8 and 1.7 ppm, respectively. LC_{50} values of the formulation stored at 26°C, 40°C and 45°C for 48 hours against *A. aegypti* were 1.7, 1.7, 1.8 ppm while LC_{90} values were 3.7, 3.7 and 3.8 ppm, respectively. Further no significant difference in LC_{50} and LC_{90} values of the formulation was observed against . *A. aegypti* during 18 months storage period at room temperature. An application of the formulation at the rate of 140 mg *a.i.*/m^2 in different breeding sites under natural field conditions provided 98.1% reduction of *Anopheles* larvae on day 1; thereafter 100% reduction was recorded up to week 1 and more than 80% reduction up to week 3, while percent reduction against *Culex* larvae was 95.5% on day 1, and thereafter 80% reduction was achieved up to week 3. The formulation also showed 95.1% and, 99.7% reduction of *Aedes* larvae on day 1 and day 2 , respectively; thereafter 100% larval control was observed up to day 7. It was concluded that neem oil formulation would be effective in controlling mosquito larvae in different breeding sites under natural field conditions (Dua *et.al.*, 2009).

The larvicidal potential of solvent leaf extracts of *Leucas aspera* and synthesized silver nanoparticles using aqueous leaf extract was tested against fourth instar larvae of *A. aegypti*. Larvae were exposed to varying concentrations of plant extracts and synthesized AgNPs for 24 h. The results were recorded from UV-Vis spectra, X-ray diffraction (XRD), Fourier transform infrared (FTIR), and scanning electron microscopy (SEM), and were used to characterize and support the biosynthesis of silver nanoparticles. The results suggested that the synthesized AgNP from leaf extracts had a higher larvicidal potential as compared to crude solvent extracts thus making them an effective combination for controlling *A. aegypti* (Suganya *et. al.*, 2013).

The crude leaf extracts of *Gymnema sylvestre* (Retz) Schult (Asclepiadaceae) and purified gymnemagenol compound were studied against the early fourth-instar larvae of *Anopheles subpictus Grassi* and *Culex quinquefasciatus*. Bioassay-guided fractionation of petroleum ether leaf extract of *G. sylvestre* led to the separation and identification of gymnemagenol as a potential new antiparasitic compound. The results suggested that the larval mortality effects of the compound were 28%, 69%, 100% and 31%, 63%, 100% at 6, 12 and 24 h against *A. subpictus* and *C. quinquefasciatus*, respectively and the per cent mortality were 100, 86, 67, 36, 21 and 100, 78, 59, 38 and 19 observed in the concentrations of 1,000, 500, 250, 125 and 62.75 ppm against the fourth-instar larvae of

A. subpitcus and *C. quinquefasciatus*, respectively. The purified compound gymnemagenol was tested in concentrations of 80, 40, 20, 10 and 5 ppm, and the per cent mortality were 100, 72, 53, 30 and 15 against *A. subpitcus* and 100, 89, 61, 42 and 30 against *C. quinquefasciatus*, respectively. The larvicidal crude leaf extract of *G. sylvestre* showed the highest mortality in the concentration of 1,000 ppm against the larvae of *A. subpictus* (LC(50) = 166.28 ppm, r (2) = 0.807) and against the larvae of C. quinquefasciatus (LC(50) = 186.55 ppm, r (2) = 0.884), respectively. The maximum efficacy was observed in gymnemagenol compound with LC(50) and r (2) values against the larvae of *A. subpictus* (22.99 ppm, 0.922) and against *C. quinquefasciatus* (15.92 ppm, 0.854), respectively. The control (distilled water) showed nil mortality in the concurrent assay (Khanna *et. al.*, 2011).

Repellent properties of three plant extracts--essential oil (steam distillate) of *Zanthoxylum limonella* (fruits), *Citrus aurantifolia* (leaf) and petroleum ether extract of Z. limonella (fruits) were evaluated as repellent against *Aedes albopictus* mosquitoes in mustard (Dhara) and coconut (Parachute) oil base under laboratory conditions. Three concentrations--10, 20 and 30% of the repellents were evaluated. Repellents in mustard oil afforded longer protection time against the bites of *Aedes albopictus* mosquitoes than those in coconut oil. At 30% concentration, 296-304 min protection time was achieved by the test repellents in mustard oil base while repellents in coconut oil exhibited 223.5-245 min protection time at the same concentration. Oil of *Z. limonella* gave the highest protection time against the bites of *Aedes (S.) albopictus* mosquitoes at all the concentrations than other herbal repellents tested both in mustard and coconut oil (Das *et.al.*, 2003).

Resistance test in India by WHO KIT

A study on susceptibility status of aquatic and adult stages of *Aedes aegypti* and *A. albopictus* was undertaken at International Airports of Thiruvananthapuram and Cochin located in southern India. The standard test kits of WHO were used to conduct insecticide susceptibility tests against various organophosphates, organochlorines, carbamates and synthetic pyrethroids. The results indicate that adult *Aedes aegypti* and *A. albopictus* were resistant to DDT and dieldrin, but susceptible to propoxur, fenitrothion, malathion, deltamethrin, permethrin and lambdacyhalothrin. The

susceptibility test conducted on immature stages of *A. aegypti* and *A. albopictus* revealed that they are susceptible to the larvicides commonly used under the National Vector Borne Diseases Control Programme viz. Temephos (0.02 ppm), Fenthion (0.05 ppm) Malathion (1.0 ppm) and Fenitrothion (0.06 ppm) (Sharma *et. al.*, 2004).

Susceptibility tests were carried out with insecticides like organochlorine organophosphorus and synthetic pyrethroids using the WHO test kits against *Anopheles stephensi* larvae and adults, collected from malaria endemic wards of Calcutta in December, 1995 and January, 1996 *Anopheles stephensi* adults were found resistant to DDT, propoxure, malathion but susceptible to fenthion and deltamethrin (Mukhopadhyay *et. al.*, 1996).

To obtain the base line data, a study conducted in district Patna, Bihar to note the susceptibility status of adult *Culex quinquefasciatus* against organochlorine, organophosphorous and synthetic pyrethroids. It was found that adult of *C. quinquefasciatus* developed double resistance to DDT and dieldrin but susceptible to O.P. compounds and pyrethroids. Cx.quinquefasciatus larvae are also found susceptible to O.P compounds like malathion, temophos, fenthion and fenitrothion (Mukhopadhyay *et. al.*, 1993).

Susceptibility studies of malaria vectors *Anopheles stephensi* and *A. subpictus* collected during 2004–2007 from various locations of Arid and Semi-Arid Zone of India (Rajasthan, Gujarat, Punjab) were conducted by adulticide bioassay of DDT, malathion, deltamethrin and larvicide bioassay of fenthion, temephos, chlorpyriphos and malathion using diagnostic doses. Both species from all locations exhibited variable resistance to DDT and malathion from majority of location. Adults of both the species were susceptible to deltamethrin. Larvae of both the Anopheline species showed some evidence of resistance to chlorpyriphos followed by fenthion whereas susceptible to temephos and malathion.

Monitoring the bioassay following protocol of WHO test kit, the DDT and deltamerhrin susceptibility was tested in Sibagar district in Assam. A total of 7655 mosquitoes were sampled under 3 genera viz. *Anopheles*, *Culex* and *Mansonia.* All the species were suspected to DDT but sensitive to deltamethrin except *M. indiana* which was suspected to be deltamethrin resistance. KDT 50 and KDT 90 values were significantly higher than deltamethrin (Dhiman *et. al.* 2013).

Materials and methods

A field trial was conducted in replicated moods to study the larvicidal, pupicidal and adulticidal efficacy of some herbal extracts / oils and new chemical acaricides that mainly used for agricultural pests, against the mosquitoes in and around Puducherry city, during one year duration from April 2013 to March 2014. To determine the resistance status of mosquitoes, WHO acaricides resistance monitoring kits and CDC Bottle test were performed as per WHO guide lines, CDC protocol, respectively.

Study area

Geographical location

Puducherry, a Union Territory, has a total area of 429 sq km covering Puducherry, Mahe, Yanam and Karikal. Puducherry and Karikal are in the pocket of Tamil Nadu, Yanam in Andhra Pradesh and Mahe in Kerala. Puducherry having quaternary cainozoic geology, tropical dry and wet climate, is situated between 11° N latitudes and 13° S longitudes, enclave in South Arcot district of Tamil nadu, at above average elevation of 15 mt (0-30) above the sea level. It is interacted by deltic channel of river Gingee and Pennaiyer and other streams forming the two main drainage basins and further porosed with lagoons, lakes and tanks. River Gingee crosses the region diagonally from north to south east.

Rain fall

Puducherry mainly gets rain from the north east monsoon during October to December. The normal mean rainfall is 1250mm. The mean rainfall distribution for last few decades indicated that from January to May less than 100 mm rainfall was received. From May to August, distribution of rainfall was 50- 150 mm per month and in September to December 200-300mm with usually maximum in October. The minimum number of rainy days during March and April on an average was 1-2 days and there was a decline trend of rainfall days in month preceding October.

Temperature

From end of February to mid June an increasing trend of temperature is noticed with mean daily temperature of 25 - 32 °C. In month of May and early June, constant hottest phase is seen with maximum 37 °C and mean daily minimum temperature of

27 ºC, The mean maximum temperature during the South Wet Monsoon is 35 ºC and mean minimum is 25 ºC. In December and January the temperature decreases to 21 ºC.

Humidity

Since Puducherry region is enclaved on western side by Bay of Bengal, the relative humidity is high during July – August (82%) and low in February – March (68%).

Soil

Mainly four types of soils in coastal alluvial plains zones are noticed viz. alluvial soil, coastal sandy soil, red ferruginous and calcareous soils on lime clay stones. About 50-60% area in Puducherry is covered with alluvial soils. It occurs in entire zone except the coastal belt where a longitudinal strip of coastal sandy soil is seen. Red ferruginous soil is seen in Mannadipet Communne.

1. Collection of mosquitoes:

The samples of immature mosquito populations (larvae and pupae), commonly encountered a mixed population of *Anopheles, Culex* and *Aedes* (composed of approximately 10 : 30 : 60 ratio, respectively) were collected from different resources like drainage, marshes, cesspits, cesspools, water filled tyres and discarded baskets, house constructing sites within Puducherry city limit (Fig.1&2). In different places in Puducherry city had both open and closed drainage system which were blocked at some places and in some places water got stagnated since the drain had no proper gradient and both the situations were found suitable for mosquito breeding spots (Fig.3&4). Some localities had also submerged shallow water plane. These were found potential source of developmental stages (larvae, pupa) of mosquitoes. The collected larvae were identified, cleaned with water and separated in the laboratory (Fig. 5, 6 &7).

Adults were mainly collected from human dwellings, domestic animal habitations and cattle shed during day time or after the dawn. Collection was made as per method described by Kaliwal *et. al.* (2010), using aspirator tube fortnightly from different places like shoe racks, walls of rooms, bathrooms, hanging objects (cloths, bags), objects on floor like furniture (cots, shelves, wooden planks) etc. The mosquitoes were brought alive in the laboratory in cages made up of mosquito net ((20x 20 x20 cm). They were

transferred into glass tubes and were anaesthetized with either ether or chloroform for making permanent mounting or for instant identification with out mounting, using the standard descriptive keys (Christopheres, 1933; Barraud 1934, Snell, 2005, Cutwa and O'Meara2008). Using the hand lens, the females were grouped as unfed, partially engorged, gravid, or semi gravid (WHO, 1975) to determine the gonotropic relationships of females (co relationship of frequency of feeding with the digestion of blood, maturation of eggs and ovipositon (Rao, 1981).

The unfed mosquitoes collected from human dwellings and animal sheds and larvae from breeding sites were subjected to insecticide sensitivity bioassay, which was performed according to WHO protocol, using Insecticide Resistance Monitoring Bioassay Kits, CDC Bottle bioassay test and larval Resistance Monitoring Bioassay Kit for adult and larvae, respectively. Knock down time (KDT) was monitored at 10 min interval and mortalities were recorded after 24 hr of exposure to different concentration of both chemical insecticides and herbal oils or extracts.

2. Killing and preservation of larvae:

The larvae of mosquitoes of different genera killed and preserved as per WHO technique (WHO, 2013). The larvae were killed by holding the vial containing the larvae over a flame of a burning cotton wool soaked with alcohol over a rock, for about 30–60 seconds or by transferring the larvae into hot water (50°C–70°C) with a pipette. Water was removed and was replaced with 70% alcohol to the vial and sealed properly after labeling.

3. Oviposition:

The induced oviposition was done according to the technique described by Thomas and James (1997). Blood engorged female mosquitoes were kept in a test tube having moist cotton at the bottom for oviposition. The female *Culex* and *Anopheles* were stressed to induce oviosition by cutting the wings at the base under dissecting microscope. The females were initially made motionless in a glass tube using ethyl acetate or chloroform socked cotton plug for a very short duration before removing the wings. The females were than placed in small uncovered plastic cups (200 ml capacity) filled with

dechlorinated water or rain water. The attention was paid to place the mosquitoes on the water surface by its legs but not on its back. Water level was maintained one inch below the brim and few old dry leaves of plants added to facilitate the quick oviposition and kept in dark places away from fans or winds since little movement. The ova were collected (Fig. 8) and identified (Fig.15&16). The ova and larvae were reared in insectory using sucrose as food (Fig. 9 & 10) and subsequent developmental stages were collected (fig. 11,12,13 &14) for further process. The field collected and laboratory reared larvae, pupae and adults were identified both in DPX/Canada balsam mounted (Fig.17,18,19,20,21,22,23,&24) as well as in unmounted natural states (Fig. 25,26,27& 28).

4. Collection of biles of ruminants fishes

Bile from sheep, goats and cattle were collected from Government slaughter house, Puducherry, immediately after slaughtering the animals and were preserved in refrigerator at -4° C without adding any preservatives. Bile from sweet water fishes were collected from the fresh fish at the time of evisceration and stored in refrigerator (Fig.31).

5. Herbal seeds, leaves, petals and oils

The aquatic extracts obtained after boiling of different herbs (viz. fruits, flowers and seeds of neem (*Azadirachta indica*), seed of Abrus precatorius, (Fig.36,37 & 38), leaves of *Nicotina tabacum* (Fig.39), datura seeds (Datura inoxia), seeds of ripen papaya (*Carica papaya*) and patels of Merry gold (*Calendula officinalis*) were tried against developmental stages of mosquitoes. Similarly effects of different oils of herbs, purchased from the local market and Ooty (Tamil Nadu) (Fig.29) viz castor oil (*Ricinus communis*), eucalyptus oil (*Eucalyptus globus*) , neem oil (*Azadirachta indica*), Mahua oil (*Madhuca indica*), black sed oil (*Nigella sativa),* mastered oil (*Sinapis alba / Brassica nigra*), pongam oil (*Millettia pinnata*) were also tried on larvae and pupa of mosquitoes of genera *Anopheles, Aedes* and *Culex* .

6. Soxlet's extraction:

Soxlet's extraction of herbs was done according to the procedure described by Shabbudin *et. al.*(2012) with some modification. Fresh leaves and seeds of neem (*Azadirachta indica*) fruits of *Abrus precatorius*, collected from different sites, were washed thoroughly to remove the dirt and soil and dried in shade for 1-2 weeks or in hot air oven at 60ºC. The flower of Marry gold and neem were also dried in shade without washing. The dried samples were subsequently ground into fine particles, sieved through tae strainer and stored in containers, labeled for future analysis. The ground samples were weighed and about 100gm were transferred into a thimble and were extracted with different organic solvents like n-hexane, ethyl acetone, methanol etc. in ratio of 1: 4(w/v) of dried sample (w) and solvent (ml), in sequence of increasing polarity at room temperature. The extracts were concentrated by evaporation by further processing at high temperature. The percolate was filtered, if needed, through Whatman filter paper (no 1), until became clear. A concentrated sample of 100 mg from each extract was dissolved in 1 ml of dimethyl sulphoxide. The extract was finally stored as aliquots until further use as insecticides (Fig.40).

7. Chemical compounds

Along with the insecticide resistance monitoring WHO kits for adults and larvae. dieldrin 4%, malathion 5%, propoxur 0.1% and cyfluthrin 0.15% were supplied by WHO. Three more unconventional chemical compounds, viz. Butox (deltamethrin, MSD Animal Health), Pyricon (chlorpyriphos 20%, Jayakrishna Pesticides (P) Ltd.) and Target (monocrotophos 36 %, Jayakrishna Pesticides (P) Ltd) were purchased from local market. For larval susceptibility test, different concentration of WHO recommended compound malathion 781.25 mg/l, 156.25 mg/l,31.25 mg/l and 6.25 mg/l were also were supplied by WHO (Fig.29 &30).

8. Effect of bile on larvae and pupae:

Late 3rd instar or 4^{th} instar larvae of mosquitoes were exposed to seven different concentrations of bile of ruminants (cattle, sheep, goats) (Fig.34 & 35) and fishes (*Labeo rohita* and *Catla catla*) as larvicide and pupicide (Fig. 32 & 33). At each concentration, a

total of 100 larvae and pupae in four replicates of 25 larvae each, were tested. A batch of 25 larvae was used as control for each test and mortality counts were made 24 h after exposure. Moribund larvae (showing tremors, rigidity or immobility or incapability to reach water surface on repeated stimuli) were considered as dead. Abbott's formula was used to correct the observed mortality of larvae, if the control mortality was ranging between 5 and 20% (Abbott, 1965). Control mortality was corrected using Abbotts' formula. A statistical analysis of LD50 and LD90 was based on overlap of 95% confidence intervals.

9. Bottle Bio Assay:

The bottle bioassay (BBA) was used as an indicator of insect response to a particular dose of insecticide to determining the specific time of exposure to kill the significant number of mosquitoes by that specific dose. The 250 ml capacity of Wheaton bottle (Borocil) were pre heated at 200°F in BOD for 15 min. to dry completely and then allowed to return to room temperature. The stock solution of active ingredient was prepared in light proof amber coloured bottle and kept in refrigerator until used. Before test to be performed, stock solution was brought to room temperature (fig.41).

a. Bottle preparation:

Bottles were prepared the day before used for testing. About 1 ml of acetone and active ingredient (to be tested) was pipetted into the test bottles and mouth was secured by screw cap so that no acetone was evaporated. [For deltamethrin (12.5 mg/ml), 0.1ml (1.25 mg) was added to 1 ml of acetone, for chlorpyriphos 20%, 0.1 ml (0.02 parts) was added to 1 ml of acetone and for monocrotophos 36%, 0.1 ml (0.036 parts) was added to 1 ml of acetone]. The bottle was swirled top to bottom as well as side wise so that all the inner surfaces of the bottle and inner wall of the cap were coated with the active ingredient. Now the bottle was gently vented by unscrewing the cap and the bottle was rolled to evaporate the acetone. The acetone free bottle, after secured with cap, was allowed to dry for at least 2 hours in a cool dry place, before test procedure to be performed. After the determination of diagnostic dose, the experiment was performed in the same manner with wild collected mosquitoes.

b. Control bottle:

The control bottle was prepared in similar fashion using only acetone.

c. Bioassay:

A diagnostic dose is an amount of insecticide required to kill 100% susceptible population of insects within a specific time (30 min-1 hr) which is called as diagnostic time. A group of twenty-five non-blood fed adult mosquitoes was aspirated in to the bottle by aspirator in test bottles (replicates of four) and control bottle. If any mosquito died during transfer into the bottle, the numbers were recorded and were subtracted from the test result number (those actually died by exposure to test acaricides). The number of mosquitoes that died at certain interval was recorded and continues until all mosquitoes died and percentage of mortality at each time interval was recorded.

d. Cleaning of bottle:

After the test, the bottles were cleaned by ringing with acetone followed by ringing in detergent for 24 hr. before final washing with water for 4-5 times. To ascertain weather the bottle was cleaned or not, few mosquitoes were released into the dry bottle and their mortality was observed up to 3 hr, if so the bottles were cleaned again.

10. Susceptibility tests by WHO kits:

The purpose of the susceptibility test was to detect the resistance population of mosquitoes, if any, to adopt alternate control strategy. All the susceptibility assays were carried out according to the method of WHO using the insecticide resistance monitoring WHO kits for adults and larvae. The toxicity of dieldrin 4%, malathion 5%, propoxur 0.1% and cyfluthrin 0.15% (supplied by WHO : ref no: WHO/VBC/81.806) were tested against from field-collected population of adult mosquitoes of genera *Aedes, Culex* and *Anopheles* , frequently encountered in Puducherry city limit. In the laboratory, three more unconventional chemical compounds, purchased from local market, viz. Butox (deltamethrin, MSD Animal Health), Pyricon (chlorpyriphos 20%, Jayakrishna Pesticides (P) Ltd.) and Target (monocrotophos 36 %, Jayakrishna Pesticides (P) Ltd) were also tried for both adults and larvae. For larval susceptibility test, different concentration of WHO recommended compound (ref no: WHO/VBC/81.806) malathion 781.25 mg/l, 156.25 mg/l,31.25 mg/l and 6.25 mg/l were used. In the laboratory,

different herbal oils like Eucalyptus oil, Neem oil, Mahuwa oil, Punga oil, Black seed oil, Bhringa oil and Castor oil, purchased from local market, were tried against larvae and pupae besides the water or chloroform/ ether extracts of leaves of neem (*Azadirachta indica*) fruits of *Abrus precatorius*, flowers of merry gold and neem, stem extract of merry gold etc.

A. Susceptibility tests for adult mosquitoes by WHO kit:

The tests were carried out on 2-3 days old sugar fed adults collected from fields as well as on 2 – 3 days old unfed adults those emerged from field collected larvae and pupae, maintained in water inside insectaries in the laboratory. The insecticide impregnated paper sheet (supplied with kit) was rolled into the 'exposure cylinders' and fastened with spring wire clip. Twenty five female mosquitoes were gently transferred in to the 'holding cylinder' with an aspirator. Death of mosquitoes that happened during the handling or transferring were counted and were subtracted from the total dead numbers died due to exposure to impregnated insecticides. The holding tube was attached with the exposure tube through the sliding plate. From the holding cylinder, all the mosquitoes were transferred to exposure cylinder through the holes of the sliding plate. After proper sealing the opening, the holding cylinder was detached from the groove and was kept upright position with screen end top under diffuse illumination and adequate humidity. After the required exposure period, the mosquitoes were transferred to holding tube and were detached from sliding plate groove (Fig.41). The tube was allowed to stand in cool environment (not exceeding 30° C) on the sliding plate or inside a cup or beaker and a pad of moist cotton wool was placed on the top of screen- closed opening end for 24 hr. The affected mosquitoes that were unable to walk or move were considered as dead. To count the live specimens, they were either stunned by a sharp jerking of the tube or were stupefied by chloroform or ether. A total of four replicated tests were performed using 4 controls using coconut oil based papers. All the impregnated paper could be used upto 20 times with in 3 weeks time. Tests with control mortality in excess of 20% were only taken into account and if control mortality was between 5-20 %, the percentage of mortality was corrected by Abbott's formula (1965):

$$\text{Abbott's formula} = \frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

The dosage - mortality regression line from observed data was drawn on logarithmic-probability paper and lethal time (LD 50, LD 90) etc. was determined accordingly.

Calculation of knock down rates:

The assessment of knockdown (KD) effect was done within 60 min. post exposure. A mosquito was considered knock downed, if it was unable to stand or fly in a coordinated way. Initial KD was detected after 10 min followed by 15, 20,30,40,50 and 60 min. and after 60 min. If KD rate was less than 80%, then another count was made for up to 80 min. If no resistance was recorded, than for confirmation of susceptibility, KD was recorded every 3 min. The knock down rates according to time exposure was adjusted to a log time- probit model to obtain the KD time for 50 % mosquitoes.

B. Susceptibility tests for larvae by WHO kit:

Sufficient number of late 3^{rd} or early 4^{th} stage larvae of mosquitoes were maintained in same water collected from the breeding sites and were maintained in darker place in the laboratory at room temperature (heavy metal in distilled water led heavy mortalities of larvae. Hard or chlorinated water were not recommended by WHO for organophosphorous compound). Any abnormal larvae (fuzzy appearance (fluffy or hairy appearance due to presence of any parasites), sluggish activity etc) were discarded from the lots. Initially the larvae were lightly ringed in normal water with a hair brush. Batches of 20 larvae were then distributed in each of 6 smell beakers by a dropper, each containing 30 ml of insecticide treated water (pipetting 1 ml of insecticide solution to 249 ml water and than stirred vigorously with a glass rod to achieve following concentration of malathion. The tests were done in cool place at about 25° C (not below 20 °C or above 30°C). The control groups were treated with alcohol only.

Malathion

Sl. no	Stock conc (mg/l)	stock quantity (ml)	water quantity (ml)	Achieved conc (mg/l)
1.	781.25	1.0	249.0	3.125
2.	656.25	1.0	249.0	0.625
3.	31.25	1.0	249.0	0.125
4.	6.25	1.0	249.0	0.025

(stock solution was supplied along with WHO kit)

All the serial concentrations were made in duplicate along with 2 sets of control (adding only alcohol in water) (Fig. 43). After 6 hrs of exposure if any pupa developed, was separated from the test beaker(s). After 24 hours of exposure, the percentage of mortality was counted for each concentration after taking into account the moribund (incapable to climb upto surface within a reasonable time or showing characteristic diving attitudes (tremors, rigour, incoordination etc.) and dead larvae (did not show symptoms of movements even after proving with a needle in siphon or cervical region under dissection microscope) in samples of both the replicates. If more than 10% larvae of control group pupate or if mortality in control group was 20% or more in 24 hours, the test was repeated. When the control morality in control group was between 5-20 %, the percent mortality was corrected by Abbott's formula:

$$\text{Abbott's formula} = \frac{\text{\% test mortality - \% control mortality}}{100 - \text{\% control mortality}} \times 100$$

Dosage- mortality regression line from observed data was drawn on logarithmic-probability paper and LD 50, LD 90 etc were determined.

C. Susceptibility tests for adult and larval stages of mosquitoes by other unconventional chemicals:

Fresh Whatman filter papers (no 1) were impregnated with Butox (deltamethrin - 0.0125 mg/ml), Pyricon (chlorpyriphos 0.2%) and Target (monocrotophos 0.036%) using acetone as media and dried in BOD incubator at 30 C. for 4-5 hours. The dried papers were packed into the WHO kit tubes and same protocol was followed as above

and mortality of adult mosquitoes were recorded maximum up to 24 hrs as per WHO recommendation. Similarly same techniques were followed for larvae too.

11. **Establishment of base line susceptibility for adults:**

To establish the base line susceptibility for mosquitoes for insecticides, batches of mosquitoes were exposed to different doses at double time interval viz. 2,4,8,16,32, 64 min. etc. to obtain 5- 100 % mortality. Regression line was established on standard log-probit paper, maintaining the time interval on X- axis and mortality on Y- axis. The percent mortality was corrected by Abbott's formula (when the control morality in control group was 5-20 %,) and probit percentage was drawn from table of Transformation of Percentage to Probit (Finney, 1964).

Mortality rate of adults:

In all the experiments, the mortality was measured at 24 hours post exposure. A mosquito was considered if it was immobile or unable to stand or fly in a coordinated way. Mosquitoes were considered as alive if they were able to fly even a small distance.

Normal population: This is a population that never subjected to insecticidal pressure and in which resistant individuals are rare. Exposure of such population to different concentration of insecticide should yield a straight line relationship between the logarithm of concentration and probit mortality. From such a data, it is possible to predict by extrapolation that particular concentration that causes 100% mortality of the susceptible population.

Discriminating concentration or diagnostic concentration: Double of that concentration (that killed 100%) would be highly lethal to the population and lacking the resistant gene. This concentration is called as diagnostic concentration or discriminating concentration. After repeated exposure to this diagnostic concentration, the population was checked frequently for presence of any tolerant individuals.

12. Establishment of base line susceptibility for larvae:

For all insecticides, batches of mosquito larvae were exposed to different concentrations of insecticides using a series of double dosages viz. 2,4,8,16,32,64 mg/l

(ppm) at double time interval viz. 2,4,8,16,32, 64 min. etc. to obtain 5- 100 % mortality. Regression line was established on standard log-probit paper, maintaining the time interval on X- axis and mortality on Y- axis. The percent mortality was corrected by Abbott's formula (when the control morality in control group was 5-20 %,) and probit percentage was drawn from table of Transformation of Percentage to Probit (Finney, 1964). The tests were done in cool place at about 25° C (not below 20 °C or above 30°C). The control groups were treated with alcohol only.

Mortality rate of larvae:

After 24 hours of exposure, the percentage of mortality was counted for each concentration after taking into account the moribund (incapable to climb up to surface within a reasonable time or showing characteristic diving attitudes (tremors, rigour, in coordination etc.) and dead larvae (did not show symptoms of movements even after proving with a needle in siphon or cervical region under dissection microscope) in samples of both the replicates.

13. Interpretation of results of susceptibility test (WHO 2013 recommendation**):**

a**. Adults:**

	Mortality %	**Indicative**	**Confirmatory path**	**Interpretation**
1.	98 – 100	Susceptibility.	-	-
2.	<98%	Suggest the possibility of resistance	Needs further investigation	—
3.	Between 90-97%	Suggestive of confirmation of resistance gene in that population.	The confirmation of resistance may be obtained by performing two additional bioassay tests with the same insecticide on same population on their progeny of survived populations.	If the two additional tests consistently show mortality below 98%, then resistance is confirmed.
4.	Less than 90			Existence of resistance gene(s)

b. **Larvae**

	Mortality %	**Indicative**	**Confirmatory path**	**Interpretation**
1.	98 – 100	Susceptibility.	-	-
2.	<98%	Indicates tolerance ie. suggest the possibility of resistance.	Needs further investigation	_
3.	Between 90-97%	Presence of resistance gene in that population.	The confirmation of resistance may be obtained by performing two additional bioassay tests with the same insecticide on same population on their progeny of survived populations.	If he two additional tests consistently show mortality below 98%, then resistance is confirmed.
4.	Less than 90%			Existence of resistance gene(s)

14. Probit analysis:

Probit analysis was done as per method of Miller and Tainter (1944) using standard logathrim – probit paper. The corrected % of death at 0% and 100 % level were calculated by using formulae viz. 100x(0.25/n) and 100x(n-0.25)/n, respectively. In a probit transformation, the sigmoid curve becomes a straight line. Bliss (1934) first proposed the name probit for modification of his Gaddum's normal equivalent deviate, by increasing 5 to simplify the arithmetical procedure by avoiding negative values.

15. Preservation of developmental stages of mosquitoes:

Larvae and pupae were first killed in very hot water which fixed the proteins that prevents from darkening the specimens in later. Subsequently they were placed in 80% ethyl alcohol which was replaced twice to remove the water.

16. Mounting adult mosquitoes:

Adult mosquitoes were killed using the ethyl acetate. In a glass tube, one third was filled with dried plaster of paris and saturated with ethyl acetate with out leaving any excess fluid over the plaster of paris. It was found easier to mount the mosquitoes that were killed in ethyl acetate than those allowed to die in rearing tube. The killed mosquitoes were either directly mounted temporarily in mounting medium (Canada Balsam) or they were digested initially in potassium hydroxide followed by ascending grade of alcohols and then mounting in DPX or Canada Balsam.

Herbal seeds, leaves, petals and oils

The aquatic extracts obtained after boiling of different herbs (viz. fruits, flowers and seeds of neem (*Azadirachta indica*), seed of *Abrus precatorius*, (Fig.36,37 & 38), leaves of *Nicotina tabacum* (Fig.39), datura seeds (*Datura inoxia*), seeds of ripen papaya (*Carica papaya*) and patels of Merry gold (*Calendula officinalis*) were tried against developmental stages of mosquitoes. Similarly effects of different oils of herbs, purchased from the local market and Ooty (Tamil Nadu) (Fig.29) viz castor oil (*Ricinus communis*), eucalyptus oil (Eucalyptus globus) , neem oil (*Azadirachta indica*), Mahua oil (*Madhuca indica*), black sed oil (Nigella sativa), mastered oil (*Sinapis alba / Brassica nigra*), pongam oil (*Millettia pinnata*) were also tried on larvae and pupa of mosquitoes of genera *Anopheles, Aedes* and *Culex* .

RESULTS

1. Effect of bile of ruminants and fishes on larvae and pupa of mosquitoes:

The larvae and pupae of different species of genus *Anopheles*, *Aedes* and *Culex* were treated with bile of sheep, goat, cattle and bile of two sweet water fishes (*Labeo rohita* and *Catla catla*) without adding any preservatives. Higher concentrations of bile (viz. 20%, 10%, 5%, and 4%) of *Labeo rohita* were found highly effective against larvae of all the three genera of mosquitoes and yielded cent percent mortality after 24 hr but 3% concentration resulted only 60% mortality. However, 16% mortality was recorded at minimal concentration of 2 % and no mortality was recorded at 1% concentration, after 24 hr (Table 1). Similarly, higher concentrations of bile of *Catla catla* (Table 2) also showed similar result , producing cent percent mortality upto 4% concentration. Following the *in vitro* test, no any significant difference in efficacy of bile of *Labeo rohita* (rahu) and *Catla catla* (catla) was recorded. Similarly, there was no significant difference in the efficacy of bile of fish and ruminants (Table 3). However, no mortality was recorded up to 48 hrs at some minimal concentrations viz. 0.5%, 1% .Only 16 % mortality was recorded by 2 % concentration of fish bile and few survived ones showed some tremors for short duration and was disappeared after 24 hours and subsequently moulted to next stages (i.e. pupa) without any abnormalities in behaviour or morphology. Similar result was also recorded for bile of ruminants (Table-3). At freezing temperature, the efficacy of bile of both ruminants and fish was recorded up to 30 days of collection with out any preservative. Since mortality of test group was cent percent at 3% concentration , therefore, Abbot's formula was not applicable to correct the mortality percentage of test group, in spite of having 8% mortality in control group. At 2% concentration of bile of *Catla catla* resulted 4.34 % mortality after correction by Abbot's formula since control mortality was 8% (Table 2). The effect of bile of ruminants on larvae had been represented in Table -3. Bile of ruminants upto 3% concentration yielded cent percent mortality of larvae and Abbott's corrections for 2% concentration for cattle and sheep were 8.69% and 13.04%, respectively. The LD50 of bile of rohu (*Labeo rohita)* on larvae of *Anopheles* was estimated as 0.13±0.007 ml/5ml water and LD90=0.23±0.007 ml/5ml water (Table 1A). The LD50 of bile of catla (*Catla catla*) on larvae of mosquitoes was estimated as 0.13±0.005 ml/5ml water and

LD90=0.25±0.029 ml/5ml water (Table 2A). Since almost all the concentrations of bile of ruminants (cattle, sheep and goat) caused death of larvae of mosquitoes and cent percent mortality was observed at 0.20 ml concentrations and lower degree of mortality were 64%, 80%, 32% and 32% respectively at 0.10 ml/5ml concentration, therefore , LD50 values could not be determined with two adjacent limits values (extreme upper and extreme lower values).

The effect of bile of ruminants and fish on pupae of mosquitoes were depicted in Table 4a, 4b, 4c, 4d and 4e. The results revealed that bile of ruminants were more effective in comparison to bile of sweet water fish. Among the bile of ruminants, cattle bile was found more toxic to pupae of mosquitoes than the bile of sheep and goats. Combination of bile of sheep and cattle with water (1:1) at 0.05 ml of concentration led to cent percent mortality after 60 and 70 min interval, respectively. No cent percent mortality was achieved after I hr. by bile of fishes (Catla and Rohu).

2. Aquatic extracts of seeds and petals of flowers :

The effect of aquatic extracts of seeds and petals of plants were depicted in Table -5. The aquatic extracts obtained after boiling of seeds of jequirity or rosary pea or crab's eye, *Abrus precatorius*, neem seed (*Azadirachta indica*), datura seeds (*Datura inoxia*), seeds of ripen papaya (*Carica papaya*) and patels of Merry gold (*Calendula officinalis*) had no effect on larvae of mosquitoes of any of the most common genera (viz. *Anopheles, Aedes, Culex*), even after 24 hrs to 48 hours of exposure at the room temperature (28 °C± 2°C) in month of January. After 48 hours of exposure of fourth stage larvae of mosquitoes, the moulting rate of exposed larvae was ranging from 24 – 39.13 %. Highest moulting was observes in aquatic extract of *Azadirachta indica* treated larvae (39.13 %) followed by 36 % (seeds of *Carica papaya*) , 32 % (seeds of *Abrus precatorious* and petals of *Calendula officinalis*) and least was 24 % for seeds of *Datura inoxia*. In control group the moulting of larvae to pupae after 48 hr was ranged from 40 – 60 % and mortality of larvae in control group was very minimal (only 4%), therefore, Abbott's formula was nullified.

3. Ether extracts of seeds and patels of flowers :

The impact of ether extract of different plants on larvae of mosquitoes were depicted in Table - 5A which revealed that seeds of *Azadirachta indica* was lethal to larvae of mosquitoes and cent percent mortality resulted after 48hr of exposure followed by seeds of Datura (*Datura inoxia)* (60%), seeds of *Abrus precatorious* (28%), Patels of *Calendula officinalis* (12%) and least (8%) was for seeds of *Carica papaya.* Subsequent moulting to pupae was highest for seeds of *Carica papaya* (52%) followed by seeds of *Abrus precatorious* (44%), seeds of Datura (*Datura inoxia)* (36%), and Petals of *Calendula officinalis* (36%).

4. Effect of oils of herbs on developmental stages of mosquitoes

The effects of different oils of herbs, purchased from the local market and Ooty (Tamil Nadu) viz castor oil (*Ricinus communis*), eucalyptus oil (*Eucalyptus globus*) , neem oil ((*Azadirachta indica*), Mahua oil (*Madhuca indica*), black sed oil (*Nigella sativa*)**,** mastered oil (*Sinapis alba / Brassica nigra*), pongam oil (*Millettia pinnata*) on larvae and pupa of mosquitoes of genera *Anopheles, Aedes* and *Culex* were depicted in Table 6. Out of eight oils tried, eucalyptus oil was found very effective against larvae and pupae of *Anopheles, Aedes* and *Culex* spp. and killed both larvae and pupae in shortest period of time comparison to other oils. In many occasion it was observed that larvae particularly , became curled and then extended just before the death (eucalyptus oil, castor oil, neem oil etc.). Maximum time was taken by bhringani oil followed by neem oil, mahua oil, and black seed oils.

5. WHO larval resistance monitoring tests:

The results of larval resistance monitoring tests by WHO kits to monitor the resistant population of mosquitoes of genera *Aedes, culex* and *Anopheles*, had been depicted in Table 7, 8 & 9 representing the results of replicates of three for each concentration. The cent percent mortality was observed for each concentration of malathion (0.25 mg/lit, 0.125 mg/lit,0.625 mg/lit, 3.125mg/lit) after 24 hr of exposure but the highest concentration (3.125 mg/ lit) was found highly effective for all the three genera. since mortality rate of control group was less than range of 5-20 %, therefore Abbots' correction was null and void. The mortality rate of all the three genera of

mosquitoes at different concentrations of malathion at different time interval viz. 2 hr, 4 hr, 6 hr, 8 hr, 12 hr, 24 hr had no significant difference.

At 2hr and 12 hr post exposure the mortality rate of *Aedes* was 0 %,3%,8%,16% and 36%, 68%, 73%,and 78% respectively indicating that 3.125 mg/ lit concentration was more effective as per as time interval was concerned. Similarly, the mortality rates of *Culex* spp at 2 and 12 hr of post exposure were 0%, 3%, 6% ,9% and 45 %, 58%, 61%, 78%, respectively. Mortalities of *Anopheles* spp were 0%,6%, 8% ,18% and 49 %, 66%, 73%, 89% respectively after 2 and 12 hours of exposure to different concentrations of malathion.

Since at all the concentrations of malathion (0.025%,0.125%,0.625%, 3.125% - supplied by WHO) led to none survived situation after 24 hr of exposure, therefore, probit analysis was null and void.

6. Test for resistance/susceptibility of adult mosquitoes following exposure to different insecticides impregnated papers using WHO kit

The result of WHO resistance monitoring test for adult mosquitoes using insecticide impregnated papers using WHO kit had been represented in Table 10 . In Table 11, the results of susceptibility of adults mosquitoes of different genera of mosquitoes (*Anopheles, Culex* and *Aedes*) were depicted, where papers were impregnated in the laboratory with locally purchased different insecticides viz. monocrotophos 36%, chlorpyriphos 20% and deltamethrin (12.5 mg/ml). The results revealed that all the three genus of mosquitoes viz. *Anopheles, Culex* and *Aedes* were highly susceptible to dieldrin 4%, malathion 5%, propoxur 0.1%, cyflumethrin 0.15% that revealed that field population of mosquitoes were not resistant to these insecticides. In comparison to Dieldrin 4% and Malathion 5%, the 0.1% Propoxur and 0.15% cyflumethrin were found more effective and killed the adults mosquitoes of genera *Anopheles* (11-12 hr), *Aedes* (11-12 hrs) and *Culex* (12-14 hr) in shorter time span. The cent percent mortality with malathion at concentration of 5% was obtained after 16 hrs for adults of *Anopheles, Culex* and *Aedes* but same was achieved with dieldrin at 4% concentration after 13- 15 hr.

The results of table 11 revealed that monocrotophos 36%, chlorpyriphos 20% and deltamethrin (12.5 mg/ml) were highly effective and led cent percent mortality of all the adult mosquitoes of genera *Anopheles, Culex* and *Aedes* but 36% monocrotophos and 20% chlorpyriphos were more effective in comparision to deltamethrin (12.5 mg/ml). All the adults of *Aedes* and *Anopheles* mosquitoes died after 1 hour following exposure to monocrotopos 36% but all adults of *Culex* sp died after 2 hr of exposure with same compound. The deltamethrin (12.5 mg/ml) caused cent % mortality of adults of all the three genera within 5-6 hours.

7: Bottle bio assay

The effect of deltamethrin by bottle bio assay against adult mosquitoes of genera *Aedes, Anopheles* and *Culex* was represented in Table 12. The results revealed that 1.25 mg/ml of concentration of deltamethrin was highly effective to cause 100% mortality (called as diagnostic dose) after 5 min of exposure in the bottle (called as diagnostic time). No significance difference was observed in the mortality rate of different genera of mosquitoes (viz. *Aedes, Anopheles* and *Culex*). At the interval of 3 min of post exposure, mortality was 85%, 68% and 81% for *Aeds, Anopheles* and *Culex* spp, respectively but after 5 min of exposure no adult mosquitoes were survived.

In Table 13, the effect of chlorpyriphos 20%, (Pyron) against adult mosquitoes of genera *Aedes, Anopheles* and *Culex* was represented and results revealed that 0.02 part/0.1 ml was highly effective concentration (diagnostic dose) to cause cent percent mortalities of adults of all the three genera but diagnostic time was 1 min for adult mosquitoes of *Aedes* and 2 min for adult mosquitoes of *Anopheles* and *Culex*.

From the data of Table 14 it was revealed that monocrtotophos 36% (Target) was very effective and 0.036 parts was highly effective diagnostic dose to produce cent percent mortalities of all the three genera of mosquitoes (*Aedes, Anopheles* and *Culex*) in 1 min of time (diagnostic time).

Discussion

Using the chemical insecticides, vector control was very successful for many decades world wide to combat the animal and human diseases. For example, wide-scale house spraying of DDT in the 1950s and 1960s dramatically reduced prevalence of malaria in Asia (Phillips 1983). Similarly, the aerial spraying of temephos in riverine black fly breeding sites in West Africa as onchocercosis control program, nearly eliminated river blindness from certain regions during the 1970s and 1980s (Curtis, 1989). In 1947, the Pan American Health Organization, initiated a campaign in the Western Hemisphere to eradicate *A. aegypti* with DDT and by 1972 *A. aegypti* had been eradicated from 73% of the land area in 19 countries (Schliesman and Calheiros, 1974, and Gubler, 1989). But due to development of DDT resistance (Brown and Pal, 1971), the campaign ended in 1972 before achieving the goals.

Though insecticides are one of the major tools for controlling vector populations but now a days only few new insecticides being developed and marketed globally for control of vector. This is because the high cost involved in discovery of new insecticide, the recent downsizing of the agrochemical industry due to environmental hazard and the low profitability of the vector control market because the countries most in need of new insecticides for vector control have very limited resources to purchase insecticides (Paul *et. al*., 2006). As a consequence of these factors, coupled with the evolution of resistance in vector species, herbs and other bio products might be the new corridors for future strategic control of vectors.

Control of mosquito larvae becomes a very pertinent issue in controlling the rapid replication of mosquitoes in management of vector-borne diseases. Chemical insecticides are till today the primary means or tools to control mosquitoes of different genera in India for reducing the transmission of pathogens. Worldwide, mosquito control relies primarily on pyrethroids (e.g., permethrin, resmethrin, and phenothrin), organophosphates (e.g., temephos and chlorpyrifos), carbamates (e.g., propoxur and carbosulfan), insect growth regulators (IGRs, primarily methoprene), and biologicals (*Bacillus thuringiensis israelensis and Bacillus sphaericus*) (Paul *et al,* 2006).

Pyrethroids was found very effective for eradication of mosquitoes in last few decades (Nauen, 2007). But now a days vector control measures are facing challenges

due to the development of resistance in vector population against wide variety of insecticides (Chandre et al., 1999) through out the world, due to consistent use or/ and indiscriminate use of insecticides. To overcome that problem, use of herbal products may be the very useful and economical means to overcome the menace of mosquitoes. In ancient period bile of animals were used as traditional medicine in many countries but there is no literature available about use of fish bile for the use to control mosquitoes. In the present study, attempt had been made to use the bile of fish and ruminants and few herbal preparations against mosquitoes, besides the determination of resistant population of mosquitoes , if any in Puducherry, by WHO resistant monitoring kits for both adults and larvae of mosquitoes.

Bile

Bile is a complex fluid containing water, electrolytes and other organic molecules like bile acids, cholesterol, phospholipids and bilirubin that flows through the biliary tract into the small intestine. Bile contains 85% water, 10% bile salts, 3% mucus and pigments, 1% fats, and 0.7% inorganic salts. Bile acids are critical for digestion and absorption of fats and fat-soluble vitamins in the small intestine. Bile is alkaline in nature and neutralizes excess stomach acid before entering to duodenum. Bile salts also potent bactericides. Initially, hepatocytes secrete bile into canaliculi, from which it flows into bile ducts. Bile acids are derivatives of cholesterol synthesized in the hepatocyte. Cholesterol, ingested as part of the diet or derived from hepatic synthesis is converted into the bile acids cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (glycine or taurine) to yield the conjugated form that is actively secreted into cannaliculi. As bile flows through the bile ducts it is modified by addition of a watery, bicarbonate-rich secretion from ductal epithelial cells. The gall bladder stores and concentrates bile during the fasting state. Typically, bile is concentrated five-fold in the gall bladder by absorption of water and small electrolytes - virtually all of the organic molecules are retained. Bile of bears, cattle and pig are been used as traditional home remedies in many countries since the ancient period.

In Japan, bile is used as component of digestive medicine and in China, bear bile is used as traditional medicine for about 1300 years (Shiro and Koichi, 2012). Animal

bile is mainly composed of bile acids with highly hydrophobic such as glycine and taurine conjugates of cholic acids and deoxycholic acids (Watanebe, *et al.,* 2009), which are highly hydrophibic and cytotoxic in nature (Rode, *et al*., 1998 and Iwaki, *et al*., 2007).

Bile of ruminants and fish were found having toxic effect on the pupae of mosquitoes and sheep and cattle bile leads to cent percent mortality after 1hr of exposure. Bile may be used as an alternative control measure against mosquitoes, however, to observe its affectivity in natural breeding place needs further investigations in details before drawing a final conclusion about effectiveness of bile against pupae of mosquitoes.

Results of the present study revealed that bile of cattle, sheep and goats were equally effective and resulted cent percent mortality of larvae of mosquitoes at different concentrations viz. 1.0 ml, 0.5 ml, 0.25ml, 0.20 ml with minimum concentration of 0.15 ml/5ml water. However, more field studies are required before drawing a final conclusion because, mortality may also be influenced by the unseen environmental factors and adaptability. Similarly results also revealed that bile of rahu and catla were also effective to inhibit the further growth of developmental stages of mosquitoes. The LD 50 doses of bile of fish rohu and catla were almost same (0.13±0.007 ml/5ml water and 0.13±0.005 ml/5ml water, respectively). This indicates that bile of both the fishes were equally toxic and 50% mortality of larvae of mosquitoes were resulted from almost at same concentration of bile. However, LD 90 value of rohu and catla viz. 0.23±0.007 ml/5ml water and 0.25±0.029 ml/5ml water, respectively, though varied slightly but the values were found statistically non significant. Since bile of bile cattle, sheep and goat at almost all the concentrations causes death of larvae of mosquitoes with highest mortality percentage (100%) at 0.15 ml concentrations and lower degree of mortality were 64%, 80%, 32% and 32%, respectively at 0.10 ml/5ml concentration, therefore , LD50 values could not be determined with two adjacent limits values (extreme upper and extreme lower values).

Bile of ruminants and fish were found having toxic effect on the pupae of mosquitoes and sheep and cattle bile leads to cent percent mortality after 1hr of exposure. The results revealed that bile might be used as an alternative control measure against mosquitoes, however, to observe its affectivity in natural breeding place needs further

investigations in details before drawing a final conclusion about effectiveness of bile against pupae of mosquitoes.

Use of herbs against mosquitoes

Because of resistance to current insecticides and to environmental and health hazard, naturally occurring compounds and their derivatives are of increasing interest for the development of new insecticidal compounds against vectors of disease-causing pathogens. Boiled extracts of seeds of jequirity or rosary pea or crab's eye, *Abrus precotorius*, neem seed (*Azadiretcha indica*), datura seeds (*Datura inoxia*), seeds of ripen papaya (*Carica papaya*) and patels of Merry gold (*Calendula officinalis*) revealed no effective toxicity on larvae of mosquitoes of genera *Anopheles, Aedes, Culex*, even upto 48 hours of exposure at the room temperature and moulted to next stages (24 – 39.13 %). But the ether extract of neem seed was found highly effective to larval stages of mosquitoes in comparison to datura seeds (*Datura inoxia*), and patels of Merry gold (*Calendula officinalis.* The ether extract of neem seed was found highly effective to larval stages of mosquitoes in comparison to datura seeds (*Datura inoxia*), and patels of Merry gold (*Calendula officinalis.* The ether extract of seeds of ripen papaya (*Carica papaya*) was least effective. The highest mortality rate of larvae of mosquitoes by ether extract neem seed also might be due to the oily nature of the extract which may block the spiracles of the larvae and resulted into high mortality. An insecticide containing azadirachtin, a neem tree (*Azadirachta indica*) extract, was tested against mosquito larvae in the Islamic Republic of Iran under laboratory and field conditions and found very effective and the mortality in the pupal stage was significantly higher than the other stages. Prevention of adult emerged and pupal mortality was the main activity of this compounds and the maximum time of efficacy was 7 days at the highest concentration (2 L/hectare). Dhar *et. al.* (1996) demonstrated the inhibitory effect of neem oil volatiles on gonotropic cycle in An. stephensi and An. culicifacies. A neem oil formulation containing 32% neem seed oil (an equivalent of 0.03% azadirachtin), an emulsifier (5%) and 63% iso propanol (solvent) was investigated for its larvicidal activities against *A. gambiae* (Okumu *et.al.*2007). Repellent activity of *Eucalyptus* and *Azadirachta indica* seed oil against the filarial mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) in

West Bengal of India and *A. indica* seed oil provided 90.26% and 88.83% protection, and the *Eucalyptus* oil 93.37% and 92.04%, at concentrations 50% and 100% (v/v), respectively, with the protection time up to 240 min.(Mandal, 2011). Larvicidal activity of essential oils and different extracts of *Ocimum sanctum, O. basilicum and O. gratissimum* were compared by Rajamma *et.al.*(2011) on laboratory reared late third or early fourth instar larvae and field collected larvae of *Culex quinquefasciatus* for a period of 24 h observed that laboratory reared larvae were more sensitive than field collected larvae and *O. basilicum* was more active than the other two species. Neem or *Azadirectha indica* (syn. *Antelaea azadirectha* or *Melia azadirectha*) belongs to Meliaceae (mahogany) tree, is well known for its medicinal values for more than 2000 years in India. Ethanolic extracts from the kernels (fruit endocarps) of ripe fruits from the, *Azadirachta indica* were found effective against for third to fourth instar larvae of *Aedes aegypti*, at doses ranging from 0.0033 to 0.05 g% in an aqueous medium for 24 and 48 h, at 25 or 30 °C, with or without feeding of the larvae (Wandscheer et.al. 2004). Non-fed *A. aegypti* larvae were more susceptible to *Azadirachta* extracts but rates caused by the *Melia* extract were higher, although at 30 °C the extract of *Azadirachta* had an even higher lethality. Though similar result was also observed in our experiment by ethanolic extracts from the kernels of fruits of neem but aquatic extract obtained by boiling in water was not found very effective, probably it causes destruction of active principle of toxicity. Although a large number of compounds have been isolated from various parts of neem, Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *A. indica* (Biswas et. al. 2002).

3. Effect of oils of herbs on developmental stages of mosquitoes

The result revealed that all the eight oils were effective to kill the larvae and pupa of mosquitoes of genera *Anopheles, Aedes* and *Culex* spp but eucalyptus oil was highly effective that killed both larvae and pupae within 5-7 min time. The seeds from the castor bean plant, Ricinus communis, are poisonous to people, animals and insects. One of the main toxic proteins is "ricin", named by Stillmark in 1888 when he tested the beans' extract on red blood cells and saw them agglutinate. It is found highly toxic to aphids, European corn borer and the Southern corn rootworm larvae were killed when exposed to

feed painted with 2% ricin (Czapla, and Johnston, 1990 and Olafia *et.al.* 1991). Eucalyptol, one of the active chemicals found in eucalyptus oil, is thought to have antibacterial properties and probably it is also effective against developmental stages of mosquitoes but it needs further investigations. Volatile oils were derived principally from species that were rich in 1,8-cineol (eucalyptol, a monoterpene), such as *Eucalyptus globulus* Labillardiere (blue gum), *E. smithii,* or *E. fructicetorum. E. globulus* etc. The oil was found effective against dust mites (Tovey, and McDonald 1997), and it had repellent activity against some biting arthropods (Trigg and Hill ,1996). Azadirachtin the most active principle of neem oil is a good insect repellent and it also interfered with insect hormone systems, making it harder for insects to grow and lay eggs. Other components of neem oil kill insects by hindering their ability to feed. However, the exact role of every component is not known. The bio-chemicals found in the Mahua tree include semisolid fixed oil (50 – 55%), which had 40% oleic acid, 26% palmitic acid, 13% linoleic acid and 16% myrsitic acid. The seed cake from the tree contained moruin, a glycocidal saponin with toxic effect. Dried flowers of the tree contained 52% of invert sugars, 2% of sugar, 2% of albuminoids, 2% cellulose and 15% aqueous contents. The ash included salicylic acid, phosphoric acid, calcium, iron, potash and traces of soda apart from other compounds. It was used as medicinal plants in India against diabetic, ulcers, analgesic, anthelmintic etc, but its effect against mosquitoes needs further investigation. Black seed was used as medicine for over 2000 years and commonly used for headache, toothache, congestion, and intestinal worms, conjunctivitis, flu and parasites. There was some scientific evidence to suggest that black seed might help boost the immune system, fight cancer, prevent pregnancy, and lessen allergic reactions . *Nigella sativa* oil contained an abundance of conjugated linoleic (18:2) acid, thymoquinone, nigellone (dithymoquinone), melanthin, nigilline, damascenine, and tannins (Boskabady and Shirmohammadi, 2002). Melanthin was toxic in large doses and nigelline was paralytic in nature. Mustard oil, was rich in alpha - linolenic acid and other ingredients and had medicinal values. The pungency results when ground mustard seeds were mixed with water, vinegar, or other liquid (or even when chewed). Under these conditions, a chemical reaction between the enzyme myrosinase and a glucosinolate known as sinigrin from the seeds of black mustard (*Brassica nigra*) or brown Indian mustard

(*Brassica juncea*) produced allyl isothiocyanate. Volatile oil of mustard, containing more than 92% allyl isothiocyanate. The pungency of allyl isothiocyanate was due to the activation of the TRPA1 ion channel in sensory neurons. And allyl isothiocyanate, was toxic and irritated the skin and mucous membranes. This toxicity might be the reason for death of developmental stages of mosquitoes. While the oil and residue of the pongam oil tree are toxic and induced nausea and vomiting if ingested, the fruits and sprouts, along with the seeds, were used in many traditional remedies. Juices from the plant, as well as the oil, were antiseptic and resistant to pests. Oil made from the seeds, known as pongamia oil, is an important asset of this tree and had been used as lamp oil, in soap making, and as a lubricant for thousands of years. The oil had a high content of triglycerides, and its disagreeable taste and odor were due to bitter flavonoid constituents including karanjin, pongamol, tannin and karanjachromene.

Though the plant of *Abrus precotorius* was best known for its seeds which were used as beads and in percussion instruments, are very toxic (Davis, 1978) due to the presence of toxic substances called abrin. But its aquatic extract of seed was found not effective against larvae of mosquitoes. The toxin abrin was a dimer consisting of two protein subunits, termed A and B. The B chain facilitates abrin's entry into a cell by bonding to certain transport proteins on cell membranes, which then transport the toxin into the cell. Once inside the cell, the A chain prevented protein synthesis by inactivating the 26S subunit of the ribosome. One molecule of abrin would inactivate up to 1,500 ribosomes per second. For purification this seed had been used in sidha medicines for centuries which was done by boiling the seeds in milk and then drying them. The protein was denatured when subjected to high temperatures which removes its toxicity. Since the grinded seeds were boiled in water, so protein gets denatured and probably it lost its toxicity and it needs further investigation in long time scale.

4. Susceptibility of larvae of mosquitoes by WHO kits

The results revealed that larvae of mosquitoes of genera *Aedes, Anopheles* and *Culex* were highly susceptible to different concentrations of malathion

(0.025%,0.125%,0.625%, 3.125% - supplied by WHO) led to cent percent mortality. This cent percent mortality proves that larval population of mosquitoes of different genera viz. *Aedes, Anopheles* and *Culex* did not develop any resistance. However this preliminary work requires further investigation in more lower concentration than 0.025% concentration to rule out the minimal lavel of resistance at minute concentrations, since development of resistance following mutation in gene level is a very slow process. Since all the WHO recommended concentrations of malathion led 100% mortality of larvae and no survived population was available, so for plotting probit analysis was not applicable to determine the LD50, LD 90 doses.. Similarly the larvae of *An. stephensi*, in Iran exhibited 100% mortality for temephos and malathion when resistance status was monitored by WHO standard protocol (Vatandoost *et.al.*,2005). Larvae of *Aedes albopictus* obtained from dengue endemic areas in Selangor, Malaysia were evaluated for their susceptibility to operational dosage of temephos (1 mg/L). by WHO larval bioassays method and the 50% mortality lethal time (LT50) for *A. albopictus* tested against temephos ranged between 58.65 to 112.50 minutes,(Chen *et.al.* 2013).

5. Susceptibility of adult mosquitoes following exposure to different insecticide impregnated papers (WHO kit)

The results of table 7 and 8 revealed that dieldrin 4% and malathion 5%, the propoxur 0.1%, cyflumethrin 0.15% (supplied along with WHO kits) and monocrotophos 36%, chlorpyriphos 20% and deltamethrin (12.5 mg/ml) were highly effective to cause cent percent mortality of adult mosquitoes of genus *Anopheles, Culex* and *Aedes*. However, in comparison to dieldrin 4% and malathion 5%, the propoxur 0.1%, cyflumethrin 0.15%, the monocrotophos 36%, chlorpyriphos 20% and deltamethrin (12.5 mg/ml) were proved to be more effective in comparison to time required to bring cent percent mortality of the adult mosquitoes in Puducherry. However, it requires further investigations before drawing a conclusion, since mortality depends on age of adults and fed or unfed state of adults. All the investigations of the present study were done with unfed adult mosquitoes only. High mortality with the monocrotophos 36%, chlorpyriphos 20% and deltamethrin (12.5 mg/ml) might be due to the fact that monocrotophos and chlorpyriphos were commonly used against the agricultural pests by the farmers in

Puducherry but not specifically used against the mosquitoes. Probably mosquitoes were not previously exposed to deltamethrin in Puducherry and so it was proving its better efficacy than conventional insecticides. Moreover, deltamethrin was more frequently used in veterinary practice against tick and mites infestation on animals. Jahan and Mumtaz (2010) had evaluated the resistance for field population of *Aedes* mosquitoes from Lahore, Pakistan using 2.5% concentration of deltamethrin which was contradicted to our present reports . It was probably due to the fact that in India deltamethrin was marketed in high concentration (12.5 mg/ml) than the concentration used in Pakisthan (2.5%). In lower concentration genetic mutation took place very frequently in the subsequent generations of insects than high concentration.

6: Bottle bio assay

The effect of deltamethrin by bottle bio assay against adult mosquitoes of genera *Aedes, Anopheles* and *Culex* was represented in Table 11. The results revealed that 1.25 mg/ml of concentration of deltamethrin was highly effective to cause 100% mortality (called as diagnostic dose) after 5 min of exposure in the bottle (called as diagnostic time). No significance difference was observed in the mortality rate of different genera of mosquitoes (viz. *Aedes, Anopheles* and *Culex*). At the interval of 3 min of post exposure, mortality was 85 %, 65 % and 81% for Aedes, *Anopheles* and *Culex* spp, respectively but after 5 min of exposure no adult mosquitoes were survived. In Table 12, the effect of chlorpyriphos 20%, (pyron) against adult mosquitoes of genera *Aedes, Anopheles* and *Culex* was represented and results revealed that 0.02 part/0.1 ml was highly effective concentration (diagnostic dose) to cause cent percent mortalities of adults of all the three genera but diagnostic time was 1 min for adult mosquitoes of Aedes and 2 min for adult mosquitoes of Anopheles and Culex. From the data of Table 13, it was revealed that monocrtotophos 36% (target) was very effective and 0.036 parts was highly effective diagnostic dose to produce cent percent mortalities of all the three genera of mosquitoes (*Aedes, Anopheles* and *Culex*) in 1 min of time (diagnostic time).

7. Conclusion of results as per WHO recommendation using WHO Kits

In the present preliminary study, cent percent mortality of adult mosquitoes of genera *Anopheles, Culex* and *Aedes,* obtained using the WHO kits by different concentrations (WHO recommended) of malathin, indicated that probably mosquito population in Puducherry had not develop any resistance against malathion. However, it requires further investigations before drawing a final conclusion. As per WHO 2013 recommendation, if mortality rate percentage of mosquitoes ranged between 98-100 that indicated susceptibility of the population and <98% was suggestive of the possibility of resistance and mortality between 90-97% was the suggestive of confirmation of resistance gene in that population.

SUMMARY

The use of bile and herbs as alternative control strategy to control the mosquitoes was found very encouraging. The bile of cattle, sheep and goat, & bile of sweet water fish (*Labeo rohita*: Rahu and *Catla catla*: Catla) were found effective against larval stages of field strain of mosquitoes of genera *Anopheles, Culex* and *Aedes*, collected from in and around Puducherry city limit. Besides that, to monitor the resistance status of mosquitoes population in Puducherry, the resistance assays were carried out according to the method of WHO, using the insecticide resistance monitoring WHO kits for adults and larvae. The toxicity of dieldrin 4%, malathion 5%, propoxur 0.1% and cyfluthrin 0.15% (supplied by WHO) were tested against the field-collected population of adult mosquitoes of genera *Aedes, Culex* and *Anopheles* and cent percent mortality was obtained within 24 hours. In the laboratory, three more new chemical compounds, purchased from local market, viz. butox (deltamethrin, MSD Animal Health), pyricon (chlorpyriphos 20%, Jayakrishna Pesticides (P) Ltd.) and target (monocrotophos 36 %, Jayakrishna Pesticides (P) Ltd) were also tried for both adults and larvae following technique of WHO and these compounds were found highly susceptible and resulted cent percent mortality within 5-6 hours. For larval susceptibility test, different concentrations of WHO supplied compound i.e. malathion 781.25 mg/l, 156.25 mg/l,31.25 mg/l and 6.25 mg/l were also resulted cent percent mortality of larvae of mosquitoes. Out of locally purchased different herbal oils like Eucalyptus oil, Neem oil, Mahuwa oil, Punga oil, Black seed oil, Bhringa oil and Castor oil, the eucalyptus oil was found more effective. Similarly among the water or chloroform/ ether extracts of seeds of jequirity or rosary pea or crab's eye, *Abrus precotorius*, neem seeds (*Azadirachta indica*), Datura seeds (*Datura inoxia*), seeds of ripen papaya (*Carica papaya*) and patels of Merry gold (*Calendula officinalis*), leaves of neem (*Azadiracthta indica*), fruits of *Abrus precatorius*, flowers of Marry gold and neem, stem extract of Marry gold., the ether extract of neem (*Azadiracthta indica*), was more effective against larvae of mosquitoes in Puducherry.

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