Assessment for larvicidal potentiality of encapsulated plant based nanopesticide against mosquito vectors

Protection of our present and future generations from vector borne diseases chiefly mosquito borne diseases, malaria, encephalitis, dengue fever, chickengunia, elephantiasis, etc., requires an efficient and ecobenevolent approach urgently. Out of these mosquito borne diseases, the diseases transmitted by *Anopheles stephensi* and *Culex quinquefasciatus* are the prominent in South East Asian region.

Half of the world's population is at risk of malaria and an estimated 243 million cases led to nearly 8,63,000 deaths in 2008 (W.H.O., 2009). India accounts approximately two-thirds of the confirmed malaria cases reported in South-East Asian region. In 2008, 96 million slides were examined from which 1.5 million cases of malaria were confirmed (World Malaria Report, 2009). There has been an increasing trend in reported incidence of dengue from 2003. Indonesia, Myanmar and Thailand contributed more than 75% of the total reported cases of dengue in 2008 (W.H.O., 2009). Chikungunya affected at least 213 districts in 15 states of India during the year 2006. The total population at risk of Chikungunya infection was 565.41 million and the number of fever/suspected chikungunya cases were as high as 1.39 million and ranged between 35 cases in Lakshadweep and 7,62,026 cases in Karnataka (Krishnamoorthy, 2009). Lymphatic filariasis (LF) is endemic in 81 countries with 1.3 billion people at risk of acquiring the infection. It has been estimated that 1100 million people are exposed to filarial infection living in areas endemic for this disease and there are about 120 million cases are microfilaria carriers. About half (49.2%) of the 120 million estimated cases are in South East Asian region and another 34.1% of cases are in the African region (Pani, et al., 2005). The vectors transmitting these diseases, therefore, must be controlled. For this objective, we have to adopt new technologies after study their sustainability.

Nanotechnology is one of the most active emerging research area in modern science. Nanotechnology is the production, manipulation and application of materials with size ranging from less than a micron to that of individual atoms. Nanomaterials are synthesized chemically but now a days are also synthesized by using biological materials. Nanoparticles of biological origin are of great interest due to their unusual optical (Krolikowska et al. 2003), chemical (Kumar et al. 2003), photoelectrochemical (Chandrasekharan and Kamat, 2000), and electronical (Peto et al. 2002) activities. Bio-based nanoparticles can be used as
novel pesticides, drug carriers (Paciotti et al. 2004), antioxidants, sensors (Yanez-Sadeno & Pingarron, 2005; Puckett et al. 2005; Liu & Lu, 2004), etc.

In recent years, it is emphasized on the application of nanotechnology in insect pest management. Technologies like encapsulation and controlled release system (CRS) have, therefore, revolutionized the application of biocides. Nanotechnology based pesticides are formulated by several companies. These formulations comprise nanoparticles of size 120-250 nm size range being more effectively water soluble as compared to existing pesticides. Certain pesticidal industries have manufactured nanoemulsions which are either water or oil based uniform suspensions of biocidal nanoparticles with range of 200-400 nm. Such biocides are categorised as nanopesticides. The nanopesticides can easily be incorporated in different media and used as creams, gels, liquids, etc. Therefore, they can be more rapidly taken up by the target organisms. Further, nanopesticides have more shelf life and killing capacity of the chemical optimized.

In India, the application of nanotechnology in pest management has just started in the last decade. Pesticides encapsulated with nanoparticles like Karate and Matador are being successfully developed by some companies like Monsanto, Syngenta and BASF and marketed. These pesticides develop from the synthetic pesticides and used against insect pests. However, very scanty record is available regarding the application of nanopesticides against vectors. Further, encapsulated plant based nanopesticide research is in infancy. The encapsulated plant based nanopesticides have following advantages:

- They can be **easily taken** by target organisms.
- They are **more active in action** as compared to normal pesticides.
- Unlike, synthetic nanopesticides, the plant based nanopesticides are ecofriendly being **biodegradable**.
- Generally, pests fail to develop resistance against the crude plant based pesticides (Prakash, 1997). Pests/vectors, therefore, may also **fail to develop resistance** against plant based nanopesticides.
- They are **economical** being required in small quantity.
- Nanoencapsulated pesticides are with control release have **long shelf life** and **causing less pollution**.
The encapsulated plant based nanopesticides as compared to synthetic based nanopesticides, therefore, attract more attention and sincere efforts should be made for the development of plant based nanopesticides.

**REVIEW OF THE LITERATURE**

As the nanopesticides are recently developed and most of the relevant work is being done after 2000, therefore, they have limited historical background.

In recent years, nanoparticles have been isolated from plants by several workers. Lamb et al., (2001) studied the induced accumulation of gold in the plants *Brassica juncea*, *Berkheya coddii* and Chicory. Formation and growth of gold nanoparticles inside live *Medicago sativa* was observed by Torresday et al., (2002). Ahmad et al., (2003) studied the intracellular synthesis of gold nanoparticles by a novel alkalotolerant actinomycete, *Rhodococcus* species. Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* was synthesized by Prathap et al., (2006). Huang et al., (2007) introduced the biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaves. Gonzalez-Melendi et al., (2008), introduced the assessment of different microscopic techniques for their visualization of nanoparticles in plant tissues. Differential adsorpsion of silver nanoparticles to the inner and outer cuticular surfaces of *Agave americana* was observed by Marciano and Chefetz (2008). Barik et al. (2008) observed nanosilica from medicine to control pest.

in a microemulsion. Paula et al., (2009) introduced the preparation and characterization of cashew gum nanoparticles loaded with natural larvicide from *Moringa oleifera* seeds. The development of plant based nanopesticide, therefore, requires further research. Thus, sincere attempt has been made for developing encapsulated plant based nanopesticide.

**HYPOTHESIS**

The proposed research proposal is based on the hypothesis that nanoparticles containing garlic/garlic vine essential oil prepared with biocompatible appropriate polymer (AP) using the melt-dispersion method (Peng et al., 2008). This is based on the advantages of nanoparticles of size ranging from 200-400 nm over the microparticles. These particles after encapsulation are introduced to the target organisms, mosquito larvae. These encapsulated phytonanoparticles are active phytonanopesticide being actively transported in the tissue of the vectors and finally killing them.

**OBJECTIVES**

- Biosequestration of phytolarvicides from garlic/garlic vine and screening their bioefficacy against *Anopheles stephensi* and *Culex quinquefasciatus*.
- Separations of the different fractions present in the most potent phytolarvicide by chromatographic methods and their bioefficacy studies aiming towards the most potent fraction.
- Encapsulation of nanoparticles from the most potent fraction of *Pseudocalymma alliaceum* (Garlic vine)/ *Allium sativum* (Garlic)/essential oils Peng et al., method.
- Assessment of the bioefficacy of the most potent encapsulated phytonanoparticles against the target organisms.
- Assessment of the different environmental conditions (light, temperature and pH) on the nanoparticles loaded phytolarvicide to establish the suitable environmental condition for effective application.
- Determination of the effect of the nanoparticles loaded phytolarvicide against aquatic non-target organisms to aiming towards their probability for field application.
- Determination of shelf life of the nanoparticles loaded phytolarvicide.
MATERIALS & METHODS

A. MATERIALS

a) Target organisms: *Anopheles stephensi* and *Culex quinquefasciatus* larvae.

b) Plants selected:

- **Pseudocalymma alliaceum** (Garlic vine, garlic creeper), **Family**: Bignoniace.

  This plant is also known as *Adenocalymma alliaceum* (Lam.), *Adenocalymma obovatum* (Urb.), *Adenocalymma pachypus*, *Adenocalymma sagotti*, *Bignonia alliacea* (Lam.), *Pachyptera alliacea*, *Pseudocalymma pachypus*, *Pseudocalymma macrocarpum* (Donn. Sm.) and *Pseudocalymma sagotti* (Bur. and Schum.).

  Garlic vine, a dazzling ornamental vine with garlic-like smell, is a native of Amazon Rainforest. The plant is a decorative evergreen vine, 6-8 feet tall, with opposite leaves divided into two ovate leaflets. The vine bears clusters of funnel shaped, purple to pale or white flowers which become lighter on ageing. Full bloom with large number of floral bunches, is by November-December.

  Garlic vine has a long history of herbal medical systems in Peru and Brazil as an analgesic, anti-inflammatory and anti-rheumatic. Indian tribes in Amazon basin use the poultice of its barks on bumps, swellings and inflammatory conditions of the skin. An infusion or leaves in decoction is used for rheumatism, arthritis, uterine disorders and epilepsy. Leaves are also used as a common remedy for cold, flu, pneumonia, coughs, fever and headache.

- **Allium sativum** (Lehsun, garlic), **Family**: Liliaceae.

  Lahsun is cultivated in Central Asia, Southern Europe, U.S.A, and India. In India, it is found in almost all the states and cultivated as a spice or a condiment crop. Garlic is cultivated in well-drained moderately clay loamy soil. It needs cool moist climatic conditions during the growth and dry period during maturity. It is a hardy perennial with narrow flat leaves and bears white small flowers and bul-bils. The cultivation of drug is done by planting bulbs generally in the month of September to late in October.
Garlic is used as carminative, aphrodisiac, expectorant, stimulant, and disinfectant in the treatment of pulmonary ailments. Garlic cloves are also used in controlling the cholesterol. Oil of garlic is used as anthelmintic and rubefacient. Allicin is antibacterial. Garlic oil is useful in high blood pressure and atherosclerosis. Fresh garlic is prophylactic against amoebic dysentery. It is largely used as condiment.

B. METHODOLOGY

a) Bioassay of plant extracts & their fractions

I. Extraction of plant extracts & Preparation of test concentrations

The leaves of *Pseudocalymma alliaceum* are collected from the botanical garden of the Dayalbagh Educational Institute, Agra and *Allium sativum* cloves from the local market.

The leaves of *Pseudocalymma alliaceum* are washed with running tap water and dried in shade. The bulbils of *Allium sativum* are taken and are peeled their covers. Then, the bulbils are also dried in shade.

The weighed and dried materials are extracted separately in petroleum ether, hexane, and methanol in a Soxhlet Apparatus for 72 hours. Extracts were separated from the solvents by Vacuum Rotary Evaporator to get pure residues and extracts are finally weighed.

Residues obtained above are dissolved in ethanol and to get stock solutions. Different desired test concentrations are prepared by diluting these stocks in ethanol. A range of working test concentrations is prepared for each extract from the prepared test concentrations in 500 ml capacity of glass beakers containing 199 ml of tap water and 1ml of test concentration.

II. Bioassay

Twenty, 3rd instar mosquito larvae after 24 hrs. of acclimatization in lab condition are separately exposed to each working concentrations. Three replicates are arranged with control experiments and are run in parallel. Mortality observations are made after 24 and 48 hrs. of exposure period respectively. The experiments are set according to WHO Standard Procedure (1975) techniques. Bioassay tests showing more than 20% mortality in control are discarded.
and on less than 20%, values are corrected by the application of Abott’s formula (Abbot, 1925). LC\textsubscript{50} and LC\textsubscript{90} values are calculated by Probit analysis (Finney, 1971).

### III. Chromatographic fractionation of the most potent extract & Bioassay

The separation of the compounds present in the most potent extract will be carried out through column chromatography. The glass column filled with appropriate solvent and 100-200 mesh of silica gel activated at 105° C for half an hour for removal of moisture. The extract will be dissolved in appropriate solvent and packed the silica gel with gentle taping the column to ensure that all the air bubbles expelled from slurry. The column will never allowed drying out and ensuring that previous solvent is completely eluted. The column will run with non polar solvent by which low polar fractions was eluted first by increasing the polarity of eluent accordingly as required for getting the other increased polar fractions in the extract. The fractions obtained from column chromatography were tested to thin layer chromatography for confirmation of purity of compounds. For TLC, Silica gel-G (for TLC) will used to prepare the silica plates on which solvent will run. Iodine will used for visibility of the bands after running the solvent system on TLC plates.

Each recovered fraction will test for the larvicidal efficacy as per previously discussed methodology.

**b) Bioassay of garlic/garlic vine essential oils**

### I. Extraction of essential oils and preparation of test concentrations

The bulbils of garlic and leaves of garlic vine will be subjected to hydrodistillation in Clevenger type apparatus for eight hours to obtained essential oils. The oils thus obtained are dehydrated over anhydrous magnesium sulphate to extract the oils and stored at room temperature for further use (Prajapati et al., 2005).

The stock solutions of both garlic and garlic vine essential oils will be prepared independently by dissolving of essential oil in ethyl alcohol/acetone. A range of different test concentration will be prepared by dilution of stock solutions independently in 500 ml capacity of glass beakers in triplicates to expose the mosquito larvae.
II. Bioassay

Bioassay of garlic/garlic vine essential oils is determined as mentioned earlier.

c) Preparation of encapsulated plant based nanopesticide

I. Encapsulation of the most potent extract

Appropriate polymer (AP) nanoparticles as carrier loaded with garlic/garlic vine extracts are prepared by using the melt-dispersion method (Peng et al., 2008). AP is heated separately at proper temperature. The essential oils of garlic/garlic vine are mixed separately in different quantities with melted AP and stirred lightly with a glass rod to ensure even distribution of the mixture. The mixture is grounded completely in a mortar box after cooled naturally at 25 °C and then sieved using a sieve mesh 200. The powder thus obtained is placed in airtight, self sealable polyethylene pouches and stored at 25 °C in desiccators containing calcium chloride to prevent moisture absorption prior to further use.

II. Encapsulation of the most potent essential oil

Encapsulation of the most potent essential oil will be done as per previously discussed methodology.

III. Garlic/Garlic vine extract /essential oil – loading efficiency

The standard curve of concentration versus absorbency for garlic/garlic vine extract /essential oil is drawn according to the method of Peng et al. (2008). Aliquots of garlic/garlic vine essential oil are diluted in absolute ethanol by serial dilution method to obtain a series of concentrations. The calorimetric assays will be carried out for the absorbency of the respective concentration using a UV-visible spectrophotometer which is used to drawn the standard curve.

Nanoparticle samples containing different quantities of garlic/garlic vine extract /essential oils are dissolved separately in 2 ml of absolute ethanol. The mixture of nanoparticles and ethanol is heated in a closed centrifugal tube for 5-6 hours in a hot water heater (40 °C) until completely dissolved. The absorbency of the solution is then determined by UV-visible spectrophotometer, and the result is compared to that of the standard curve. The loading efficiency (LE) of the extract /oil-loaded nanoparticles is calculated according to Yang et al., (2009).
IV. Bioassay of encapsulated plant based nanoparticles

The stock solutions of both garlic and garlic vine essential oils encapsulated by AP will be prepared independently by dissolving of encapsulated nanoparticles in water (w/v). A range of different test concentrations will be prepared by dilution of stock solutions independently in 500 ml capacity of glass beakers containing 199 ml of water and 1 ml of test stock solution in triplicates to expose the mosquito larvae. Bioassay of encapsulated plant based nanoparticles is determined as mentioned earlier.

V. Physicochemical parameters

The most potent encapsulated phytonanolarvicide will be subjected for the effect of different physicochemical parameters, light, pH and temperature on their larvicidal potency as per standard WHO procedure discussed earlier.

VI. Bioassay of plant based nanoparticles against non-target aquatic organisms

Bioassay of encapsulated plant based nanoparticles will conduct according to the previously mentioned methodology.

VII. Shelf life of nanoparticles loaded phytolarvicide

The shelf life of the phytolarvicide loaded nanoparticles will done storing the phytolarvicide loaded nanoparticles in shelf at room temperature and followed by conducting their larvicidal biassay after the interval period of every three months.

BACKGROUND OF THE PROPOSED WORK

The different plant extracts of Pseudocalymma alliaceum leaves and Allium sativum were prepared separately in petroleum ether, hexane and methanol solvents and their bioefficacy against Culex quinquefasciatus were tested and screened out the most potent extract.