3.1. MATERIALS

3.1.1) TARGET ORGANISMS: (*Anopheles stephensi* and *Culex quinquefasciatus*):

Mosquitoes (Order: Diptera) comprising approximately 3500 species, are found beyond the tropical and subtropical regions of the world. They are true flies with a single pair of narrow wings that have a fringe of scales on the margins and veins. They have three pairs of long, slender legs and mouthparts are an elongate beak. The genera selected for the present study vector human disease-causing pathogens are *Anopheles* (malaria) and *Culex* (Japanese encephalitis, filariasis) are distributed globally.

3.1.1.1) MOSQUITO BIOLOGY:

Mosquitoes are small, primitive flies that breed in standing water. During their life span, they pass through four distinct stages: egg, larva, pupa, and adult. The eggs are deposited individually or in bunches called rafts in or near water, hatch into larvae (wrigglers) within a few days. They feed on microscopic plant and animals. They live in water and cannot survive long out of it and get air at the water surface through a snorkel-like device. When disturbed, larva actively moves in an s-shape motion which gives them their common name "wriggler." They molt four times and become comma-shaped pupae (tumblers). If disturbed, they tumble downward to escape hence also called tumblers. They do not feed. Pupae then subsequently turn into adults. The entire process for some species can take place in as short a time as 7 to 10 days. Adult’s behavior varies depending upon the species. Only the females seek blood, which they need as a protein source to produce eggs whereas adult males feed on flower nectar or plant juices for energy.

3.1.1.2) IDENTIFICATION OF ANOPHELINE AND CULICINE MOSQUITOES AND THEIR DEVELOPMENTAL STAGES:

There are some morphological differences between the developmental stages of Anopheline and Culicine mosquitoes.
Anopheline laid their eggs individually over the surface of water, each egg with lateral air floats to keep it floating while Culicine mosquitoes lay their eggs in a group of boat-shaped mass referred to as an egg raft. In case of larval stage anopheline larvae rests in a horizontal position on the surface of the water and for breathing spiracles are present on the eighth abdominal segment while culicine larvae have the spiracles at the end of a tubular organ called the siphon, which extends from the eighth abdominal segment and larvae hang down from the water surface by the tip of its siphon in order to breathe. In case of adult anopheline wing is generally patterned with dark and pale areas whereas the culicine wing is unpatterned and has a uniformly plain appearance. Another visual distinction is that, at rest, the body of an anopheline mosquito forms an angle of 45° with the surface while that of a culicine mosquito lies almost parallel to the surface.

3.1.1.3) WHY LARVAE?
The ideal method to control the mosquito infestation is the use of larvicides as it can control the “mosquitoes by reducing mosquito breeding” (Gluber, 1989). Adulticides can only temporarily reduce the adult populations. Therefore, for controlling mosquitoes the overall applications of pesticides are reduced by using this method (Dharmgadda, 2007).

3.1.2) SYNTHETIC PESTICIDES:
3.1.2.1) Temephos:

Chemical structure of Temephos
Chemical name: O,O'-(thiodi-4,1-phenylene)bis(O,O-dimethyl phosphorothioate)

Chemical formula: C_{16}H_{20}O_6P_2S_3

Chemical Family: Organophosphate Insecticide


Available formulation: 50% emulsifiable concentrates, 50% wettable powder, and up to 5% granular forms.

Used formulation: 50% EC

Some common trade name: Abate (BASF Corporation), Abathion (BASF Corporation), Acibate (Agro Chemicals Industries Ltd.), Biothion (discontinued by American Cyanamid Co.), Bithion, Difenphos, Ecopro, Nimitox (BASF Corporation), and Swebate (BASF Corporation).

**Abate** is the trade name of the temephos product used for larval mosquito control

Mammalian toxicity: **Oral** Acute oral LD \(_{50}\) for male and female rats 1226 to 13,000 mg/kg in rats and 460 to 4700 mg/kg in mice. **Toxicity class** General Use Pesticide (GUP). Slightly toxic compounds (EPA toxicity class III) that carry the Signal Word "WARNING" on their labels.
3.1.2.2) Imidacloprid:

![Chemical structure of Imidacloprid](image)

**Chemical structure of Imidacloprid**

**Chemical name:** \( N-[1-[(6\text{-Chloro-3-pyridyl})\text{methyl}]-4,5\text{-dihydroimidazol-2-yl}]\text{nitramide} \)

**Chemical Family:** Neonicotinoid Insecticide

**Technical product:** Colourless crystals with a weak characteristic odour

**Available formulation:** 95%TC, 98%TC, 10%WP, 25%WP, 70%WP, 70%WS, 70%WDG, 2%EC, 20%SL, 35%SC

**Used formulation:** 97.6% SL

**Some common trade name:** Kohinor, Admire, Advantage, Merit, Confidor, Hachikusan, Premise, Prothor, and Winner

**Mammalian toxicity:**

- **Oral** Acute oral LD\(_{50}\) for male and female rats 450 mg/kg. **Skin and eye** Non-irritating to eyes and skin (rabbits). Not a skin sensitiser. **Inhalation** LC\(_{50}\) (4 h) for rats >5323 mg/m\(^3\) dust, 69 mg/m\(^3\) air (aerosol).

**Other** Not mutagenic or teratogenic.

**Toxicity class** WHO (a.i.) II; EPA (formulation) II
3.1.3) PLANT MATERIALS:

3.1.3.1) *Solanum xanthocarpum* (Ver. Hin: Kantkari, Choti katheri or Kateli):

Family: Solanaceae. Synonym: *Solanum surattense*

It is cosmopolitan herb in India and often found in waste places, on roadsides and in open scrub lands. It is prickly, usually spreading or diffused perennial. It has much branched stem and younger ones are clothed with dense, stellate and tomentose hairs. The branches are zigzag, spread close to the ground and are covered with yellow, sharp, shining prickles, about 1.5 cm long. These prickles are compressed straight, glabrous and shining, often 1-3 cm long. Leaves are up to 10 cm in length and are hairy or spiny on both sides. Petiole is long, stellately hairy and prickly. Flowers are about 2 cm long, purple and are in cymes or sometimes reduced as solitary. The fruits are glabrous, globular drooping berries, 1.5-2 cm, yellow or pale with green veins. Kantkari is one of the dashamula roots (10 roots) in ayurveda. Hence, it is an important herb in medicinal system. The roots, fruits and the whole plant are used for medicinal purpose (Plate 1).

3.1.3.2) *Cuscuta reflexa* (Roxb.) (Ver. Hin: Amar bel /Akashbel; Eng: Doddar):

Family: Convolvulaceae (Morning glory family)

Amar bel (meaning, immortal vine) is an unusual parasitic vine/ an angiosperm parasite and occurs throughout the plains of India. It usually grows in a prolific manner over host plants (or other support) with inter-twined stems, giving it a common name of Devils Hair. It is a leafless and rootless plant. Initially the starter plant would have some roots and within a few days of germination, the plant, which is touch sensitive, finds a host or dies. After establishing on a host body, it draws nutrition from the host as a stem parasite. “The twining stem develops haustoria which are root like and which abstracts organic and inorganic solutes principally from the host phloem” (Jeschke et al., 1994). The flowers are small, white having a perfect bell shape attached directly to the stem nodes. In Ayurvedic medicine, the plant is considered to be useful in diseases of eye and heart (Chopra et al., 1958) (Plate 2).
3.1.4) FUNGI:

3.1.4.1) *Aspergillus flavus* (Order: Eurotiales  Family: Trichocomaceae):

**Diversity:** It is a sporophyte and a haploid filamentous fungus. It is found all over the world, mainly in the places of warm temperature and is also abundant in areas with temperate climates during warm drought years. It grows in temperature of 25° – 42° C and the optimum temperature for its growth is 37° C. It is also known as “Mold” and like other molds it also grows by producing hyphae. It can only reproduce asexually.

**Identification:** It is yellow-green mold with distinctive conidiophore composed of a long stalk supporting an inflated vesicle. Conidiogenous cells present on the vesicle produce the conidia (Plate. 3).

3.1.4.2) *Fusarium sporotrichioides* (Order: Hypocreales  Family: Hypocreaceae):

**Diversity:** It is a parasitic fungus which has cosmopolitan occurrence and kills its host. It is widely distributed filamentous fungus on the seeds, grasses and cereals such as wheat, maize and in the soil.

**Identification:** It produces the off-white color colonies with a powdery, cotton-like appearance, turned from light yellow to peach in five days (Plate. 4).

3.1.5) AQUATIC NON -TARGET ORGANISMS:

3.1.5.1) *Cypris* (Class: Crustacea, Order: Podocopa)

**Diversity:** It is a small ostracodan crustacean, commonly known as “mussel shrimp”. It is free swimming and occurs in large numbers in stagnant pools.

**Identification:** The unsegmented body is laterally compressed and completely enclosed in a bivalve carapace. The anterior end of the body bears a large median eye. The antennules and the antennae are large and the latter helps in swimming. They swim with first pair of legs.

**Feeding habits:** They are omnivorous, feeding on small organisms and detritus etc.
3.1.5.2) *Daphnia magna* (Class: Branchiopoda, Order: Cladocera, Family: Daphniidae):

**Diversity:** It is also one of the most common genera of freshwater crustaceans commonly called as water flea.

**Identification:** Small to medium-sized crustaceans. “The head is fused, and is generally bent down toward the body with a visible notch separating the two. In most species, the rest of the body is covered by a carapace, with a ventral gap in which the five or six pairs of legs lie. The most prominent features are the compound eye, second pair of antennae, and a pair of abdominal setae”.

**Feeding habits:** “They prey on tiny crustaceans and rotifers, but most are filter feeders, ingesting mainly unicellular algae and various sorts of organic detritus including protists and bacteria”.

### 3.1.6) REAGENTS:
1. Ethanol
2. Brewer’s yeast
3. Petroleum ether (60°-80° C)
4. Hexane
5. Methanol
6. 10% Glucose
7. Tween- 80 (Poly-oxyethylene sorbitan mono-oleate/palmitate)
8. Polyethylene glycol 6000 (PEG)
9. Alcohol (ethanol) series (30-100%)
10. Eosin (stain)
11. DPX (Distrene-80 Dibutyl Pthalate xylene)
12. Peptones
13. Dextrose
14. Agar
15. Sucrose
16. NaNO₃ (Sodium Nitrate)
17. K₂HPO₄ (Potassium Hydrogen Phosphate)
18. KCl (Potassium Chloride)
19. MgSO₄.7H₂O (Magnesium Sulphate)
20. FeSO₄.7H₂O (Ferrous Sulphate)
21. Yeast Extract
22. Filter Paper (Whatman No.1)
23. Chloroamphenicol

### 3.1.7) GLASSWARES AND EQUIPMENTS
1. Beakers (Borosil)
2. Erlenmeyer (Conical) flasks
3. Petri plates
4. Dropper
5. Pipette
6. Sieve
7. Slides
8. Cover slips
9. Glass tubes
10. Microscope
11. BOD
12. Laminar flow
13. Hot plate
14. Soxhlet apparatus
15. Vaccum rotatory evaporator
16. Refrigerator
17. Autoclave
18. Nanozetasizer (Malvern)
19. TEM
3.2) METHODOLOGY

3.2.1) COLLECTION AND REARING OF MOSQUITOES:

Detailed information regarding the mosquito life cycle is an important parameter for understanding the biology of mosquito vectors. Such kind of studies can be obtained from field observations; however, information is only partly or rarely available, owing to the difficulty of obtaining immature stages from breeding habitats. Therefore, colonization in the laboratory represents the most feasible and accessible source of information on these life stages.

The eggs of *An. stephensi* and *Cx. quinquefasciatus* were collected from the Malaria Research Centre, Delhi to start the colony. These were then transferred into enamel trays containing water and allowed to hatch. Mosquito larvae were reared under control laboratory conditions of 27±2°C and 85% relative humidity in a normal 14:10 (L/D) photoperiod. The Eggs were hatched when exposed to water, into the first instar larvae in 1-2 days. There are 4 larval instars and the total larval period is 7-9 days. The larvae were fed on finely grounded dog biscuits and brewer’s yeast in a 3:1 ratio. After several molting these larvae molted into pupae and then collected and transferred manually with the help of a glass dropper into a 500-ml beaker containing water. The beaker was then placed inside 1’×1’×1’ adult cages for adult emergence. The pupal stage lasts for about 2-3 days and it is a nonfeeding stage. To retain emerging adults cotton soaked in 10% glucose solution was provided to adult mosquitoes. On 2nd day of post emergence, female mosquitoes were fed on Albino rabbits for blood feeding. Shaved dorsal side of the rabbit was positioned over the mosquito cage and held in this position overnight. Glass Petri dishes lined with moist filter paper containing 50 mL of water were placed inside the cage for oviposition by female mosquitoes. This eggs were then immersed in larval basins (30 cm diameter) containing water for the maintenance of the colony.

3.2.2) COLLECTION AND EXTRACTION OF PLANT MATERIALS:

The roots of plant, *S. xanthocarpum* and stems of *C. reflexa* were collected from the availability area of Agra. The roots and the stems were separated from the plant and washed in running tap water and were dried in the shade.
Dried roots were chopped in small pieces of about 1 cm size using a Falcon-Stem Cutter. The roots and stems were subjected to extraction with petroleum ether, hexane and methanol subsequently in a soxhlet apparatus for 72 hours. Extracts were separated from their respective solvents by vacuum rotatory evaporator to get pure residues. The extracts were finally weighed and kept in refrigerator below 5°C until further use.

3.2.3) COLLECTION AND CULTURE OF FUNGI:

3.2.3.1) CULTURE OF FUNGI:

The strains of A. flavus and F. sporotrichioides were obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India (MTCC No.- 1973 and 1894) and stored at 4°C. Prior to testing on mosquito larvae they were cultured on PDA- peptones (20g/l), dextrose (40g/l), agar (20g/l) plate’s separately and incubated in BOD (Biochemical Oxygen Demand) at 28°C for 7 days. After seven days both were visually characterized. A. flavus and F. sporotrichioides isolates were then subcultured on Czepak solution agar media (sucrose-30g/l, agar-15g/l, NaNO₃-2g/l, K₂HPO₄-1g/l, KCl-0.5g/l, MgSO₄.7H₂O-0.5g/l, FeSO₄.7H₂O-0.01g/l at pH-7.3± 0.2) to obtain pure cultures. Species were determined using microscopic features (Klich and Pitt, 1994). All fungal isolates were kept at 4°C for further analysis. (Plate-5)

3.2.3.2) EXTRACTION OF MYCOTOXINS:

Isolates of A. flavus and F. sporotrichioides were cultured in a 250 mL flask containing 100 ml of sterile YES (Yeast Extract Sucrose) liquid medium (20% sucrose and 5% yeast extract) and incubated separately for 7-10 days in the dark at 27-30°C without agitation. Toxins were recovered from the mycelium by adding 25 mL of chloroform because of lysing of cells by adding chloroform and agitating on a rotary shaker for 10 min. The flask contents were filtered (Whatman No. 1) and the filtrate were used for extraction of toxins. For extraction the filtrate were transferred quantitatively to a separatory funnel and extracted successive with 100-mL volumes of chloroform for separation of the chloroform and aqueous layer. This extraction procedure was repeated three times and the lower transparent chloroform phases were collected to a new flask. The chloroform was evaporated by vacuum rotary evaporator under reduced pressure and residue was kept in refrigerator below 5°C until further use (Mallek et al., 1993).
3.2.4) BIOASSAY AND ENCAPSULATION:

Bioassay is the combination of two words: Bios-life; Assay-determination. Thus, bioassay stands for determination of relative toxicity of insecticides by studying and examining their effects on living organisms. The principle of bioassay is to compare the responses of insects from treated samples with a series of standards under the same conditions. The response of insects may be based on knock down, mortality etc. Following steps were taken in calculating the lethal dose of insecticides which is capable of killing 50 percent of the test population.

3.2.4.1) BIOASSAY OF SYNTHETIC PESTICIDES:

3.2.4.1.1) PREPARATION OF STOCKS AND TEST CONCENTRATIONS:

Imidacloprid were purchased from the local market and Temephos were taken from the District Malaria Office, Agra. The stock solutions of desired concentrations were prepared by diluting the pesticides independently in dechlorinated tap water (Table 1). Different test concentrations were prepared for the exposure of mosquito larvae by further diluting these stocks. One milliliter of these test concentrations was added into 249 mL of water in 500 mL capacity of glass beakers to obtained different working test concentration for the exposure of mosquito larvae.

**Table 1:** Stock preparation of different synthetic sesticides selected

<table>
<thead>
<tr>
<th>Synthetic Pesticides</th>
<th>Amount used (mL)</th>
<th>Stock medium</th>
<th>Conc. of stock (mL/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>0.01 mL</td>
<td>Deionised water</td>
<td>10</td>
</tr>
<tr>
<td>Temephos</td>
<td>0.1 mL</td>
<td>Deionised water</td>
<td>100</td>
</tr>
</tbody>
</table>

3.2.4.1.2) BIOASSAY:

After 24 hrs of acclimatization in lab condition twenty, 3rd instar mosquito larvae, *An. stephensi* and *Cx. quinquefasciatus* were collected and exposed to each working concentration independently. Each experiment was arranged in triplicates to minimize error with a control at an ambient temperature 27±2°C and 70–80% relative humidity. The larvae were supplied with small aliquot of powdered brewer’s yeast for nutrition. Loss of water was adjusted by adding required quantity of tap water upto the marked level of test.
3.2.4.1.3) ENCAPSULATION:
The synthesis of nanopesticides were done by using melt-dispersion method (Peng et al., 2008). PEG (6000) was heated in different five parts (49.5, 49.0, 48.0, and 46.0 g) separately at 65ºC. After melting, 1%, 2%, 4% and 8% of pesticide were mixed separately with the different parts of melted PEG and stirred gently with the glass rod to ensure even distribution of the mixture. The mixture was cooled at room temperature and the mixture was grounded completely in a mortar and sieved using a 200 mesh sieve. The powders are placed in airtight container, self-sealable polyethylene pouches and stored at 25ºC in desiccators containing calcium chloride to prevent moisture absorption prior to further experiment (Plate-6).

3.2.4.1.4) BIOEFFICACY OF ENCAPSULATED SYNTHETIC PESTICIDES:
The encapsulated nanopesticides were dissolved in deionised water (w/v) to make stock solutions of nanopesticides independently (Table 2). A range of different test concentrations were prepared by dilution of stock solutions independently in 500 mL capacity glass beakers containing 249 mL of water and 1 mL of test stock solutions in triplicates to expose the mosquito larvae. Bioassay of encapsulated synthetic pesticide based nanopesticides was depicted as mentioned earlier.

Table 2: Stock preparation of different encapsulated synthetic pesticides nanoformulations

3.2.4.2) BIOASSAY OF PLANT EXTRACTS:

<table>
<thead>
<tr>
<th>Synthetic Pesticides</th>
<th>Amount used (gms)</th>
<th>Stock medium</th>
<th>Conc. of stock (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>0.025</td>
<td>Deionised water</td>
<td>500</td>
</tr>
<tr>
<td>Temephos</td>
<td>0.025</td>
<td>Deionised water</td>
<td>500</td>
</tr>
</tbody>
</table>

3.2.4.2.1) PREPARATION OF STOCKS AND TEST CONCENTRATIONS:
The pure residues were dissolved in ethanol to get stock solutions. Different desired test concentrations were prepared by diluting these stocks in ethanol (Table 3).
A range of working test concentrations were prepared for each extract from the prepared test concentrations in 500 mL capacity of glass beakers containing 249 mL of tap water and 1 mL of test concentration.

**Table 3:** Stock preparation of different selected plants in different solvents

<table>
<thead>
<tr>
<th>Plants</th>
<th>Dry Plant Material (Kg)</th>
<th>Solvents for extraction</th>
<th>Amount of extracts obtained (gms)</th>
<th>Solvents for stocks</th>
<th>Conc. of stocks (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Solanum xanthocarpum</em></td>
<td>1</td>
<td>Petroleum Ether</td>
<td>5.4</td>
<td>Ethanol</td>
<td>50,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexane</td>
<td>0.8</td>
<td>Ethanol</td>
<td>50,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>15</td>
<td>Ethanol</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Cuscuta reflexa</em></td>
<td>1</td>
<td>Petroleum Ether</td>
<td>17.8</td>
<td>Ethanol</td>
<td>50,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexane</td>
<td>1.5</td>
<td>Ethanol</td>
<td>50,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>20</td>
<td>Acetone</td>
<td>50,000</td>
</tr>
</tbody>
</table>

**3.2.4.2.2) BIOASSAY:**

Methodology of Bioassay of plant extracts were same as conducted for the synthetic pesticides.

**3.2.4.2.3) ENCAPSULATION:**

The encapsulation of plant extracts were same as conducted for the synthetic pesticides.

**3.2.4.2.4) BIOEFFECTICACY OF ENCAPSULATED PLANT EXTRACTS:**

Bioassay of encapsulated plant extracts were conducted according to previous methodology Stocks prepared for each encapsulated phytonanopesticide is depicted in Table 4.

**Table 4:** Stock preparation of different encapsulated plant extract nanoformulations

<table>
<thead>
<tr>
<th>Plant Extracts (PEE)</th>
<th>Amount used for stock (gms)</th>
<th>Stock medium</th>
<th>Conc. of stock (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Solanum xanthocarpum</em></td>
<td>5</td>
<td>Deionised water</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Cuscuta reflexa</em></td>
<td>5</td>
<td>Deionised water</td>
<td>50,000</td>
</tr>
</tbody>
</table>
3.2.4.3) BIOASSAY OF FUNGAL EXTRACTS:

3.2.4.3.1) PREPARATION OF STOCKS AND TEST CONCENTRATIONS:
The fungal residues were dissolved in ethanol independently to prepare stock solutions and different test concentrations were made by further dilution of these stocks (Table 5). One milliliter of these test concentrations were added into 249 mL of water in 500 mL capacity of glass beakers to obtain working test concentration.

Table 5: Stock preparation of different fungi selected

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Amount of extracts obtained (gms)</th>
<th>Amount used for stock (gms)</th>
<th>Stock medium</th>
<th>Conc. of stock (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>1.110</td>
<td>1</td>
<td>Ethanol</td>
<td>20,000</td>
</tr>
<tr>
<td>F. sporotrichioides</td>
<td>2.84</td>
<td>1</td>
<td>Ethanol</td>
<td>20,000</td>
</tr>
</tbody>
</table>

3.2.4.3.2) BIOASSAY:
Bioassays of fungal extracts were conducted as per the previous methodology.

3.2.4.3.3) ENCAPSULATION:
Encapsulation of fungal extracts was conducted according to previous methodology.

3.2.4.3.4) BIOEFFICACY OF ENCAPSULATED FUNGAL EXTRACTS:
The bioassays of encapsulated fungal extracts were conducted according to previous methodology. Their desired stock preparation is shown in Table 6.

Table 6: Stock preparation of different encapsulated fungal extract nanoformualtions

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Amount used for stock (gms)</th>
<th>Stock medium</th>
<th>Conc. of stock (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>1</td>
<td>Deionised water</td>
<td>20,000</td>
</tr>
<tr>
<td>F. sporotrichioides</td>
<td>1</td>
<td>Deionised water</td>
<td>20,000</td>
</tr>
</tbody>
</table>

3.2.4.4) BIOEFFICACY OF THE COMBINATIONS IN FREE AND THEIR ENCAPSULATED FORMS:

3.2.4.4.1) COMBINATION OF THE MOST POTENT SYNTHETIC PESTICIDE AND THE PLANT EXTRACT:
Keeping synthetic pesticide as standard, the stock was mixed with the stock of phyto and fungal extract in ratios of 1:1, 1:2 and 1:4 individually. Test concentrations for each mixed formulated ratio were prepared by further diluting the combination mixture in water.
3.2.4.4.2) COMBINATION OF THE MOST POTENT PLANT EXTRACT AND THE Fungal Extract:
The combination and its bioassay were conducted according to the previous methodology.

3.2.4.4.3) COMBINATION OF THE MOST POTENT SYNTHETIC PESTICIDE AND THE Fungal Extract:
The combination and its bioassay were conducted according to the previous methodology.

3.2.4.4.4) COMBINATION OF THE MOST POTENT SYNTHETIC PESTICIDE, PLANT EXTRACT AND FUNGAL EXTRACT:
Most potent combination was kept as standard stock and was mixed with fungal extract in ratios, 1:1:1, 1:1:2 and 1:1:4. Experiments were conducted same as depicted above. The combination and its bioassay will be conducted according to the previous methodology.

3.2.4.4.5) ENCAPSULATION OF THE MOST POTENT COMBINATION:
All the most potent ratios among bipartite and tripartite combination were encapsulated in different nanoformulations and tested against target organisms as with the previous methodology.

3.2.5) PHYSICAL PARAMETERS:
Experiments conducting for testing the photo and thermal sensation phenomenon of the most potent encapsulated nanopesticide were same except for observing its toxicity in different parameters (light and temperature).

3.2.5.1) LIGHT:
The Photosensitivity of the most potent nanopesticide were observed under fluorescent (Visible range), in dark and under UV radiation.

3.2.5.1.1) FLUORESCENT LIGHT:
To observe the effect of visible range of light the experiments were exposed to visible radiations of the tube light in the UV chamber (UV tube switched off). The experiments were conducted in triplicates and mortality data were noted after 24, 48 and 72 hrs.
3.2.5.1.2) DARK:

The above experiment was repeated same except for the replacement of visible radiations to totally dark conditions by covering the UV chamber with black paper. The mortality data were recorded after 24, 48 and 72 hrs.

3.2.5.1.3) UV RADIATION:

Similar experiments were arranged in the presence of UV radiation. The period for the exposure of UV radiation was 1 hr, 2hrs, and 4 hrs respectively. After the required UV exposure, the UV radiations were replaced by normal conditions. The mortality data were recorded after 24, 48 and 72 hrs.

3.2.5.2) TEMPERATURE:

To observe Thermal sensitivity of the most potent nanopesticide, the experiments were conducted at different temperatures viz. 15°C, 20°C, 30°C and 35°C. All the experiments were set in triplicates with control and were kept in BOD.

3.2.6) BIOASSAY AGAINST AQUATIC NON-TARGET ORGANISMS:

For the Ecological risk assessment of nano pesticides their toxicity on aquatic non-target species, Cypris and Daphnia magna were observed. Experiments were set same as above said procedure.

3.2.7) COLLECTION OF DATA:

Mortality observations were recorded after 24, 48 and 72 hrs of exposure. All the experiments were devised according to WHO standard procedure (2005). The dead and moribund larvae were recorded as larval mortality. The mortality of larvae was determined by observing the movement of the larvae after the treatment period. The larvae were touched gently with the help of a glass rod and considered dead if they showed no sign of movements. While the larvae were considered moribund if they moved a little but did not show any kind of swimming movement. The moribund larvae were considered dead as these larvae could never revive (Macedo et al., 1997). The mortality was also recorded in the control. The experiment was discarded if the larval mortality exceeded 20 % in control and repeated again so as to keep the mortality in control below 20 %.
3.2.8) CALCULATIONS AND STATISTICAL ANALYSIS:

3.2.8.1) BIOEFFICACY:

Recorded mortality data were then subjected for calculation of lethal concentration 50 (LC$_{50}$) and 90 (LC$_{90}$) according to Probit analysis (Finney, 1971). Prior to LC$_{50}$ & LC$_{90}$ calculation, Abbott's formula was applied if the percent mortality in control ranged between 5 - 20% to percent mortality. If the percent larval mortality was below 5%, then these were treated as non-significant and thus no correction was required (Abbot, 1925).

Corrected % Mortality = [(T-C) / (100-C)]×100

Where, T = % mortality in test concentration.

C = % mortality in Control

3.2.8.2) COMBINATORIAL STUDIES:

Co-toxicity coefficient = \[ \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with plant/fungal extract}} \] \times 100

Synergistic Factor (SF) = \[ \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with plant/fungal extract}} \]

SF value > 1; indicates synergism and
SF value < 1; indicates antagonism.

3.2.9) PREPARATION OF WHOLE MOUNT AND MORPHOMETRIC STUDIES:

Dehydrate the material by transferring it through 30%, 50%, 70% and 90% alcohol. Leave for 5-6 minutes in each solution. Stain in Eosin and leave for 10-15 minutes depending on the thickness of material. De-stain the material by using acid alcohol (1% HCL in 70% alcohol). Further, the dehydration was done by the help of 90% alcohol for removal of water content if any and then to absolute alcohol and keep for 10 minutes in each. Remove alcohol by clearing with xylol/1-2 dimethylbenzene (xylene) for 10 minutes.
Finally, for permanent mounting the material was kept on slide in a drop of DPX mount and cover slip was lowered slightly over it.

For morphometric studies deformities produced in the exposed larvae of both species and both aquatic non-tagerts were noticed. Same observations were studied for control on the anopheline, culicine larvae and both aquatic non-tagerts kept in water with PEG in each case.

3.2.10) CHARACTERIZATION OF NANOPARTICLES:

3.2.10.1) NANOPARTICLE SIZE AND DISTRIBUTION:
The nanoparticle size and distribution of the most potent nanopesticide was analyzed with the Nanozetasizer (Malvern). Sample was diluted with deionised water with 0.5 g in 50 mL and filtered through a millipore filter to avoid any contamination. Each measurement was performed in triplicate.

3.2.10.2) TRANSMISSION ELECTRON MICROSCOPY:
The morphology of the most potent nanopesticide was determined by Transmission Electron Microscopy (TEM). For TEM studies, a small amount of nanopesticide was dissolved in deionised water. A drop of this solution was placed on a copper grid and allowed to dry in vaccum.