Comparative Efficacy of Synthesized Mono- & Bi-Metallic Nanoparticles on Selected Mosquito Larvae and Non-Target Organisms

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<tr>
<td>AgNO₃</td>
<td>Silver Nitrate</td>
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<tr>
<td>HAuCl₄</td>
<td>Tetrachloroauric acid</td>
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<tr>
<td>PdCl₂</td>
<td>Palladium chloride</td>
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**Introduction**

Mosquito can indeed cause a lot of damage in terms of public health and wellbeing. These mechanical vectors of public health concern are abundant in nature due to the presence of versatile water bodies that serves as mosquito breeding habitats. Zoonotic transmission of diseases from animal to human and human to human by mosquito vectors is another reason for public health concern.

WHO 2015 reported nearly 214 million cases and 438000 deaths worldwide caused by the transmission of malaria parasite through Anopheline species, however from India 0.85 million cases and 316 deaths were reported (NVBDCP annual report, 2014-15). WHO also acknowledged a recent study according that has estimated 390 million cases and 96 million clinical manifestation caused by *Aedes* mosquito that transmit dengue viruses, however from India over 33320 cases and 86 deaths were reported (NVBDCP annual report, 2014-15).

Over 1379788 suspected cases and 191 deaths due to chikungunya transmission by *Aedes* mosquito were reported from USA, Caribbean islands and Latin American countries as reported by WHO (2015), while from India about 12694 suspected cases were reported (NVBDCP annual report, 2014-15), while recently 12,255 cases of chikungunya were reported from all over India till 31 August 2016, with the highest number of cases from Karnataka (8,941 cases). In India about 650 million people are at the risk of lymphatic filariasis transmitted by *Culex quinquefasciatus*, a neglected tropical disease (NVBDCP annual report, 2014-15).

In 2015, a report from Brazil has confirmed and association between Zika virus infection to a pregnant women and microcephaly birth defect caused to new born child. Recently 4 deaths due to dengue and as many as 1158 dengue cases and 1057 chikungunya cases till September 10 (2016) were reported from national capital New Delhi.

WHO’s Integrated Vector Management (IVM) aims at the formulation of new vector control tools that provide improved efficacy that sustainably control disease vector, should be cost-effective,
safe for ecosystem and encourages the synergistic approach of such tools for multi-disease control approach. IVM is focused on the use of appropriate measures for vector control after the critically analyzing the pattern of disease transmission, its severity, and need. These measures also aid to reduce further risk to increases insecticide resistance species in mosquito population.

Firstly IVM favors environmental management strategies to reduce the breeding habitats of mosquito, secondly it employs the use of biological control method such as the use of bacterial larvicide, larvivorous fish. Lastly it employs the use of costly synthetic and toxic chemical insecticides through indoor residual spray (IRS), Long-lasting insecticidal nets (LLINs) chemical larvicides and adulticides. In recent years a lot of work has been carried out on green synthesis and application of metal nanoparticles such as Nanosilver, nanogold, nanocopper, and nanozinc as mosquito larvicide.

Nanomaterials are composed of $n$ number of ions of mono, bi or multi types of metal that makeup the particle size to exist in nanometer range i.e. from 1nm to 100nm. Optical, mechanical, and electrical property of nanometal changes at nano-scale, nanoparticle of such metal reacts differently than the one which exists in macroscopic scale.

In comparison with synthetic insecticides nanometal particles also gives promising results in terms of vector control. These nanomets and nanometal oxides are synthesized from noble and transition metals most preferably silver, gold, platinum, titanium, palladium, copper, zinc and iron. Nanosilver has been used as algaecide and registered for its use in swimming pools with the Environmental Protection Agency (EPA). Nanosilver is also known to cause antimicrobial activity in a broad spectrum. The fact behind the anti-microbial property of silver nanoparticles is the release of silver ions that are responsible for killing bacteria but nanoparticles itself. Nanomaterials like nanopalladium, and nanoiron are now applied in bioremediation process for heavy metals, solid waste, hydrocarbons, waste water and uranium remediation.

Nanoparticles composed of two different metal elements show novel properties from monometallic nanoparticles. Bimetallic nanoparticles could show not only the combination of the
properties related to the presence of two individual metals, but also new properties due to a synergy between two metals. Nanoiron can remove silver from contaminate water and also other pollutants like mercury, arsenic, cadmium etc., while bimetallic iron-nickel nanoparticles are known to degrade trichloroethylene (TCE). Nanometals like iron-palladium nanoparticles can dechlorinate as they degrade chlorinated organic compounds such as dichloroethylene (DCE), trichloroethylene (TCE), and Polychlorinatedbiphenyls (PCB) found in polluted water.

Nanometals can be synthesized with the help of precursor agent (i.e. metal salt), reducing agent, and stabilizing agent. These reducing and stabilizing agents can be synthetic or natural. Phytochemical extract of different plant tissues contains these reducing and capping agents in the form of natural polymers and other naturally occurring chemical compounds that plays an important role in nanoparticle formation are primary and secondary metabolites of plants like starches, alkaloids, tannins, polyphenols, terpenoids, saponins, polypeptides, lectins etc.

Nanoparticles have been synthesized from a wide range of polymers. A polymer is made up of $n$ number of monomer subunit bound together through chemical bonds thus forming a polymer. Thus a natural polymer can be a macromolecule with high molecular mass and mostly formed by condensation polymerization with the elimination of water molecules. Natural polymer such as polysaccharides, polypeptides, and polynucleotides has been exploited for their use nanoparticle synthesis for biomedical application.

The applications of nanoparticles in biomedical science are immense and significant. Nanogold is known for its biocompatibility, and non-toxicity in humans. Application of nanogold colloids involves in $in-vivo$ imaging technique for diagnosis of biomedical disorders. Nanogold plays a significant role in therapeutic treatment for cancer as a crucial component of magic bullets in drug vectorization to target tissue selective anti-tumor drug delivery. Apart from synthetic polymer nanogold can be synthesized with the help of chitosan a natural amino polysaccharide for effective drug delivery as it is non-toxic for its application in human and environment and nanogold can eagerly bind with amino group of chitosan.
Nanoparticles has also been used to extent the self-life of the clothes, and apparels which can be extend to provide longevity and strength of materials along with the integrated home of anti-microbial and mosquito repellents.
Réviéw of Literature

I. Bimetallic Au-Ag (Gold-Silver) nanoparticles synthesis using plant tissue extract:

Shankar et al. (2004) synthesized Au, Ag and bimetallic Au core-Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf broth. Senapati et al. (2005) performed extracellular synthesis of alloy bimetallic Au-Ag nanoparticle with the help of fungal biomass of *Fusarium oxysporum*, and concluded that NADH released by fungus plays an important role in formation of alloy nanoparticles.

Raveendran et al. (2006) reported a green method for the synthesis of Au, Ag and Au-Ag alloy bimetallic nanoparticles using with the help of soluble starch and anhydrous D-glucose as reducing agent and capping agent. Song and Kim (2008) synthesized bimetallic Au/Ag nanoparticles using Persimmon (*Diopyros kaki*) leaf extract and characterized them with techniques such as energy dispersive X-ray spectroscopy (EDS), scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS).

Mondal et al. (2011) synthesized Ag, Au and bimetallic Au-Ag alloy nanoparticles using aqueous extract of Mahogany (*Swietenia mahogany* JACQ.) leaves and characterize them via UV–visible spectroscopic and TEM. Sheny et al. (2011) synthesized Au, Ag and Au-Ag bimetallic nanoparticles using aqueous extract and dried leaf of *Anacardium occidentale and the* nanoparticles are characterized using UV–vis and FTIR spectroscopies, XRD, HRTEM and SAED analyses.

Zhang et al. (2013) reported the synthesis of Au-Ag bimetallic nanoparticles using Cacumen *Platycladi* leaf extract at 90 °C and characterization study was performed using X-ray diffraction, transmission electron microscopy, energy-dispersive X-ray spectroscopy, and X-ray photoelectron spectroscopy. AbdelHamid et al. (2013) synthesized Ag-, Au- monometallic nanoparticles and Au-Ag bimetallic nanoparticles with the help of Aqueous Extract of Sago Pondweed (*Potamogeton pectinatus* L.).
II. Bimetallic Ag-Pd (Silver-Palladium) nanoparticles synthesis using plant tissue extract:

Lu et al. (2014) synthesized silver – palladium alloy nanoparticle with the help of *Cacumen platycladi* leaf extract. Farhadi et al. (2015), synthesized Ag core – Pd shell nanoparticles with the help of plant biomass (extract of sap root and Astraglmanna).

III. Bimetallic Au-Pd (Gold-Palladium) nanoparticles synthesis using plant tissue extract:

Huang et al. (2010) reported the synthesis of Au@Pd core–shell nanoparticles with tunable structure using Bayberry tannin (BT), a natural plant polyphenol, for the one-step synthesis of Au@Pd core–shell nanoparticles (Au@Pd NPs) in aqueous solution at room temperature and characterized by TEM, HAADF-STEM, EDS mapping, an EDS line scan, and EDS point scan.

Zhang et al. (2011) reported the synthesis of Au-Pd bimetallic nanoparticles with the help of single step bioreduction using plant leaf extract of *Cacumen Platycladi*.

Sun et al. (2014) synthesized biogenic flower-shaped Au–Pd nanoparticles with the help of ascorbic acid and *Cacumen Platycladi* leaf extract at room temperature and the characterization techniques, such as transmission electron microscopy, energy-dispersive X-ray spectroscopy, and X-ray diffraction, were employed to confirm that the as-synthesized nanoparticles were alloys.

Alshatwi et al. (2015) synthesized gold-platinum alloy nanoparticles using the total tea polyphenols (TPPs) and reported its application on proliferation inhibition and apoptosis induction in human cervical cancer cell.

IV. Monometallic Ag nanoparticles synthesis using plant tissue extract and mosquito larvicidal bioassay:

Salunkhe et al., (2011) tested the efficacy of silver nanoparticles derived from the extract of fungus *Cochliobolus lunatus* against the second, third and fourth instar larvae of *Aedes aegypti* and *Anopheles stephensi*.

Marimuthu et al. (2010) synthesized silver nanoparticles using aqueous leaf extract of *Mimosa pudica*, and tested larvae of mosquitoes and ticks, and concluded that low doses of silver nanoparticles were more efficient in compare antiparasitic efficacies of silver nanoparticles and
aqueous leaf extract of *Mimosa pudica* at varying concentration, on to high doses of aqueous leaf extract alone.

Priyadarshini et al., (2012) reported the highest mortality in first to fourth larvae of *Anopheles stephensi*, when perform bioassay using silver nanoparticle derived from the leaf extract of *Euphorbia hirta* against these larvae. Sareen et al., (2012) tested the larvicidal efficacy of silver nanoparticles synthesized from the leaf extract of *Hibiscus rosasinensis* against the larvae of dengue vector *Aedes albopictus* to control its population.

Vankar and Shukla (2012) synthesized silver nanoparticles with the help of aqueous extract of leaves of *Citrus limon*, and used these nanoparticles to coat cotton and silk fabric for durable textile finish and have tested the anti-microbial activity of the coated fabric.

Haldar et al., (2013) reported highly stable, and reproducible silver nanoparticle derived from the dried green fruits of *Drypetes roxburghii* (wall), tested its efficacy on *Anopheles stephensi* and *Culex quinquefasciatus*. Karuppiah and Rajmohan (2013), synthesized silver nanoparticles with the help of aqueous leaf extract of *Ixora coccinea* L.

V. **Monometallic Au nanoparticles synthesis and mosquito larvicidal bioassay:**

Babu (2010), reported the synthesis of hexagonal gold nanoparticles with the help of ethanol extract of leaves of *Mentha arvensis*. Soni and Prakash (2011) examined the potential efficacy of pathogenic keratinophilic fungus *Chrysosporium tropicum* mediated nanosilver and nanogold particles, against all instars of the dengue vector *Aedes aegypti*, and described the susceptibility of second instar larvae to silver nanoparticle followed by third, first and fourth instar.

Soni and Prakash (2013) reported silver and gold nanoparticle as novel nanoparticle which were synthesized from cell free extract of fungus *Fusarium oxysporum* f.sp. pisi, and these fungal derived gold and silver nanoparticles were found to be effective in killing larvae and pupae of *Aedes aegypti* followed by *Culex quinquefasciatus*, and *Anopheles stephensi*. Soni and Prakash (2014) reported an ecofriendly green approach for the synthesis of gold and silver nanoparticles from the bark of *Cinnamomum zeylanicum* for mosquito vector control, and tested its larvicidal
efficacy on larvae of malaria vector *Anopheles stephensi* and filariasis vector *Culex quinquefasciatus*.

Sadeghi (2015), synthesized gold nanoparticles with the help of *Ziziphus mauritiana* and tested its anti-bacterial activity against gram positive bacteria (*S. aureus*).

VI. **Monometallic Pd nanoparticles:**

Khan et al. (2013) synthesized palladium (Pd) nanoparticles using an aqueous solution of *Pulicaria glutinosa* and characterization was done by UV-Vis spectroscopy, powder X-ray diffraction (XRD), transmission electron microscopy (TEM), energy-dispersive X-ray spectroscopy (EDX), and Fourier transform-infrared spectroscopy (FT-IR).

Adams et al. (2014) synthesized palladium nanoparticles by chemical methods using Palladium acetate \([\text{Pd}_3(\text{OAc})_6]\), Dodecyl sulfide and absolute ethanol with three different sizes i.e. 2.0±0.1 nm, 2.5±0.2 nm and 3.1±0.2 nm. They reported antibacterial activity of palladium nanoparticles on *Escherichia coli* and *Staphylococcus aureus*.

Nasrollahzadeh et al (2015) synthesized Pd nanoparticles using *Hippophae rhamnoides* Linn leaf extract and applied them as heterogeneous catalysts for the Suzuki–Miyaura coupling in water, the synthesized nanoparticles are characterized by XRD, SEM, TEM and UV–vis techniques.

Nasrollahzadeh and Sajadi (2016) synthesized Pd nanoparticles using extract of the plant of *Euphorbia granulate* and concluded the presence of flavonoid and phenolics acids in the extract could be responsible for the reduction of Pd\(^{2+}\)ions and formation of the corresponding Pd nanoparticles.

VII. **Effects of nanoparticles on non-target organisms:**

Murugan et al. (2015), synthesized carbon and silver nanoparticles and evaluated larvicidal and pupicidal efficacies of mosquito vector, also reported no harmful impact of nanoparticle doses on behaviour of natural biological control agent water bug (*Lethocerus indicus*) and reported that carbon nanoparticles has moderate evidences of genotoxicity against non-target goldfish, (*Carassius auratus*).
VIII. Coating of nanoparticles on cotton fabric:

Nateri et al. (2011) coated nanosilver on cotton fabric by “Simultaneous Synthesizing and Finishing Process”, by first treating the cotton fabric with reducing agent like D-(+)-glucose monohydrate with continuous stirring in the solution and the added molar solutions of AgNO₃, to the solution that contain reducing agent and cloth for treatment with AgNO₃ followed by centrifugation and oven drying. They confirmed antibacterial activity of the fabric and characterized the nanoparticles coated cloth with SEM.

IX. Work done in our laboratory on proposed research work:

In our, “Environmental and Advanced Parasitology and Vector Control Biotechnology Laboratory”, there is a significant work done on nanoparticle mediated mosquito vector control since 2011 – 2016.

Soni and Prakash (2011) examined the potential efficacy of pathogenic keratinophilic fungus *Chrysosporium tropicum* mediated nanosilver and nanogold particles, against all instars of the dengue vector *Aedes aegypti*, and described the susceptibility of second instar larvae to silver nanoparticle followed by third, first and fourth instar.

Soni and Prakash (2012) verified the comparative efficacy of silver nanoparticles synthesized separately with three fungus namely, *Chrysosporium keratinophilum*, *Verticillium lecanii*, and *Fusarium oxysporum* f.sp. pisi, and the bioassay was performed against the adult mosquito of filariasis vector *Culex quinquefasciatus*. Soni and Prakash (2012) synthesized gold nanoparticles with the help of *Aspergillus niger*, and tested its larvicidal efficacy on *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* and reported that *Culex quinquefasciatus* larvae were susceptible to gold nanoparticle synthesized by *Aspergillus niger*, followed by *Anopheles stephensi* and *Aedes aegypti* larvae.

Soni and Prakash (2013) reported silver and gold nanoparticle as novel nanoparticle which were synthesized from cell free extract of fungus *Fusarium oxysporum* f.sp. pisi, and these fungal derived gold and silver nanoparticles were found to be effective in killing larvae and pupae of *Aedes aegypti* followed by *Culex quinquefasciatus*, and *Anopheles stephensi*. Soni and Prakash
(2014) reported an ecofriendly green approach for the synthesis of gold and silver nanoparticles from the bark of *Cinnamon zeylanicum* for mosquito vector control, and tested its larvicidal efficacy on larvae of malaria vector *Anopheles stephensi* and filariasis vector *Culex quinquefasciatus*. Soni and Prakash (2014) synthesized biogenic silver nanoparticles using, leaf and bark aqueous extracts of *Azadirachta indica* (Neem), and preformed the insecticidal efficacy on the larvae, pupae and adult imago of malaria vector *Anopheles stephensi* and filariasis vector *Culex quinquefasciatus*. Soni and Prakash (2015), synthesized silver nanoparticles with five different fungal strains i.e. *Chrysosporium tropicum*, *Chrysosporium keratinophilum*, *Fusarium oxysporum*, *Aspergillus niger*, and *Verticillium lecanii*. They examined efficacy of different dual combination of silver nanoparticles produced by these fungal broth in 1:1 ratio, on all the four instars of *Culex quinquefasciatus* and *Anopheles stephensi*, and proposed that combinatorial approach can give better results.

In our laboratory we have tested Cu-Zn bimetallic nanoparticles against 3rd instar larvae of *Culex quinquefasciatus* mosquito and have encouraging results (Savy-2015), and a comparative analysis was done between Cu-Zn bimetallic nanoparticles and Ag-Cu bimetallic nanoparticles against third instar larvae of *Anopheles stephensi*, here Ag-Cu nanoparticles colloids were proved to give higher larvicidal efficacy at lower concentration as compare to Cu-Zn bimetallic nanoparticles (Savy-2016).
**Objectives**

1. To synthesize monometallic and bimetallic nanoparticles (Ag, Au, Pd, Au-Ag, Au-Pd, and Ag-Pd) from aqueous extract of the leaves of the selected plant.

2. To characterize synthesized monometallic and bimetallic nanoparticles using characterization techniques.

3. To check comparative mosquito larvicidal efficacy of selected synthesized bimetallic and monometallic nanoparticles on the larvae of the following mosquitoes:
   a. *Anopheles stephensi* (Liston)
   b. *Aedes aegypti* (Linn.)

4. To evaluate efficacies on the non-target organisms like Dragon fly nymphs and Damselfly nymphs.

5. Coating and characterization of nanoparticles coated cotton yarn/fabric to integrate mosquito repellent properties
**Material and Methods**

1. **Material:**
   1. **Chemicals and solvents**
      
      For all experimental studies pure and analytical grade chemicals will be used, like Silver Nitrate (AgNO$_3$) and Tetrachloroauric acid (HAuCl$_4$), Palladium Chloride (PdCl$_2$), NaOH, D-(-)-Glucose, DI water, DD water.

   2. **Selection of plant leaves for nanoparticles synthesis**
      
      We have selected the leaves of *Citrus limon* (common name – Nimbu (hindi), Lemon (English)) and if required, *Ixora coccinea* (common name –Rugmini (hindi), Jungle flame (English)) only as a substitute.

   3. **Mosquito larvae collection**
      
      Larvae will be collected from the local breeding sites in DEI campus, urban and semi-urban sites in Dayalbagh region of Agra.

   4. **Non-target Organism Collection**
      
      They will be collected from the local sites in DEI campus, urban and semi-urban sites in Dayalbagh region of Agra. The non-target organisms Dragonfly nymphs and Damselfly nymphs are selected because they are found in common habitat as the selected mosquito species. The effect of nanoparticles concentrations will be checked time to time i.e. after 4 h, 6 h, 24 h, 32 h etc.

   5. **Laboratory Instruments**
      
      U.V. –Vis Spectrophotometer, Laminar hood, Autoclave, Flash Chromatograph, Nanodrop Spectrophotometer, Soxhlet apparatus, Rotary evaporator, oven, Magnetic stirrer cum hot plate etc.
II. Methodology:

1) Preparation of aqueous infusion of phytochemical components of the leaves of selected plant (Soni and Prakash, 2014)

Any type of dirt and contamination will be removed through washing, the leaves will then be dried. Phytochemical aqueous infusion of selected plant will then be done on hot plate. The dried leaves of the selected plant will be chopped/blended and dissolved in DI water and the components will be extracted via heating on hot plate for 1-2 h at 60-65°C. The preparation will then be filtered using whatman filter paper no. 1 (pore size 11μm) and after it attain room temperature, it will be stored in refrigerator.

2) Preparation of monometallic and bimetallic nanoparticle from aqueous extract (Raveendran et al. (2006) and Zhan et al. (2011), with some modifications)

The molar solution containing metal salt will be subjected to heating at 90°C with continuous stirring, a reducing agent like D-Glucose or NaOH will be added drop by drop into this solution. After 5 min. the solution containing the metal salt and reducing agent will be cool down and to this solution plant leaf extract will be added which will work as both reducing and capping agent.

- For monometallic nanoparticles, molar solution of AgNO₃, PdCl₂ and HAuCl₄ will be prepared, to this solution plant extract will be added,
- For bimetallic nanoparticles, the plant extract will be added to mixed molar solution of AgNO₃, PdCl₂ and HAuCl₄.

Any change in color of the colloidal solution will indicate metal ion reduction into nanoparticles. These preparations will kept in dark for over-night and then it will be characterized and used for bioassay.

The molar strength of bimetallic nanoparticles and monometallic nanoparticle will be kept equal to compare their efficacy in bioassay.
3) **Characterization of nanoparticle colloids**

Characterization will be done via different techniques like

a. **U.V. Vis. Spectroscopy** Most precursors exhibit color in solution and change color when they are decomposed or reduced. UV-Vis spectroscopy has been used to monitor this transition to confirm the completion of reduction. Noble metal nanoparticles, especially Au and Ag nanoparticles, have well-defined absorbance peaks in UV-Vis spectroscopy due to surface plasmon resonance. The measurements are used to provide information on the size and quality of nanoparticles, because the shape and absorbance of the peaks are related to the uniformity, shape and size of nanoparticles.

b. **Electron microscopy** is a powerful technique for the characterization of metallic nanostructures, and is capable of providing information for visual analysis to determine shape and size distribution of nanoparticles. It includes **Transmission Electron Microscopy (TEM)** which gives two dimensional image of the sample and **Scanning Electron Microscopy (SEM)** which gives three dimensional image of the sample.

c. **Energy dispersive X-ray Spectroscopy (EDX),** a technique that allows the chemical composition of a material to be determined.

d. **Fourier transform infrared spectroscopy (FT-IR)** will be employed to investigate the surface ligand binding to nanostructures, since the ligands are mostly organic molecules. It provides information of the degree of order and relative orientation of surface-bound ligands and also plays an important role in confirming the completion of a ligand exchange reaction. The chemical shifts before and after the binding help determining the binding functional group and the binding pattern.

e. **Dynamic light scattering (DLS)** is used to determine the particle size by measuring the random changes in the intensity of light scattered from a suspension or solution.
4) **Maintenance and acclimatization of mosquito larvae in laboratory**  
   (Vyas et al. 2007)  
   Mosquito larvae will be twice washed and then acclimatized in a glass tub containing distilled water with glucose and yeast as nutrition, in laboratory condition. Larvae of different stages will be then isolated for bioassay.

5) **Identification of mosquito species**  
   Identification of the laboratory maintained mosquito will be done by following identification keys by Barraud (1934), Singh (2009).

6) **Maintenance and acclimatization of non-target organisms in laboratory**  
   (Vyas et al. 2007)  
   Non-target organisms such as Damselfly nymphs and Dragonfly nymphs etc. will be acclimatized in glass tub containing suspension media of Spirulina, Hydrophytes, and some mosquito larvae as food source, in laboratory condition.

7) **Bioassay on selected mosquito vectors**  
   Larvicidal efficacy of prepared bimetallic (Au-Ag, Au-Pd, Ag-Pd) nanoparticles and monometallic (Ag, Pd, Au) nanoparticles in laboratory will be assessed as per W.H.O.’s guidelines for laboratory testing of mosquito larvicides (2005), on selected mosquito vectors namely *Anopheles stephensi* (Liston), and *Aedes aegypti* (Linn.). Controls like positive control and negative control will be maintained during bioassay.

8) **Data analysis**  
   Obtained data on efficacy testing will be subjected to corrected % mortality, using Abbott’s formula only when the mortality in positive control is more than 5%. (Abbott, 1925). Then the data will subjected to probit analysis (Finney, 1971). The relationship between probit and log concentrations will be plotted on graph in MS Excel, from which probit regression line and probit equations will be obtained and LC values will be determined. Software like SPSS can be used to calculate other parametric and non-parametric tests.
9) **Effect on non-target organisms**

The obtained lethal concentration (LC\(_{50}\)) will be selected to test their effect on selected non-target mosquito larvivorous organisms, by calculating the predator efficiency as done by Murugan et al. (2015). Three replicates containing mosquito larvae will be assigned with two predators each as a blank and three replicates of test concentrations containing nanoparticle treated mosquito larvae with two predators. The predator efficiency of the selected predator in comparison with blank will be assessed according to the following formula:

\[
Predator \ Efficiency = \left( \frac{\text{No. of consumed mosquito larvae}}{\text{No. of predators}} \right) \times \frac{1}{\text{Total no. of mosquito larvae}} \times 100
\]

10) **Cotton fabric coating method of nanoparticle composites**

There are several chemical and physical methods for coating nanoparticles on cotton fabric but we will be following the method performed by Nateri et al. (2011) “Simultaneous Synthesizing and Finishing Process”, by first treating the cotton fabric with reducing agent like D-(+)-glucose monohydrate and then treating it with molar solutions like AgNO\(_3\), HAuCl\(_4\) and PdCl\(_2\), followed by centrifugation and oven drying of nanoparticles coated cotton fabric. Different reducing agents and methods will be adapted for nanosilver and nanogold coating on cotton.

11) **Characterization of monometallic and bimetallic nanoparticle coated cotton fabric** (Nateri et al. 2011)

The nanoparticle coated cotton fabric will be characterization through SEM and TEM.

12) **Repellency of nanoparticles coated cotton fabric against selected mosquitoes**

Mosquito repellency of nanoparticles coated cotton fabric will be assessed by following WHO guidelines for efficacy testing of mosquito repellents for human skin (2009).

**PLAN OF ACTION**
1. Synthesis of nanoparticles from aqueous/ethanol phytochemical extract and selection of best combination of reducing agent and nanoparticles formed for further study:

- Preparation of aqueous extract from the leaves of the selected plant

- Synthesis of Ag, Au, Pd, Au-Pd, Ag-Pd and Au-Ag nanoparticles

- Characterization (U.V. – Vis. spectroscopy, EM, EDX, DLS, FT-IR)

- Bioassay on mosquito larvae

  Effect of LC$_{50}$ conc. obtained from bioassay on non-target organism

2. Coating of nanoparticles on cotton fabric and repellence testing
Preparation of Ag, Au, Pd, Au-Pd, Ag-Pd and Au-Ag nanoparticles coated cotton fabric using different techniques and reducing agents

Centrifugation and oven drying of coated cotton fabric

Characterization (TEM, SEM)

Mosquito Repellency test
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