Phyto-Synthesized Silver Nanoparticles: A Potent Mosquito Biolarvicidal Agent

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Abstract
Mosquito transmit diseases like malaria, dengue accounted for global mortality and morbidity with increased resistance to common insecticides. In the present study silver nanoparticles (AgNPs) were synthesized from aqueous leaves extracts of four plant species (Jatropha gossypifolia, Euphorbia tirucalli, Pedilanthus tithymaloides and Alstonia macrophyla) and there effects on II²nd and IVth instars larvae of Aedes aegypti and Anopheles stephensi were evaluated. Synthesized AgNPs were characterized by UV-Vis spectroscopy, fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), particle size distribution and zeta potential analysis. II²nd and IVth instars larvae of A. aegypti and A. stephensi were exposed to varying concentrations of AgNPs synthesized from plants under investigation (0.625 to 20 ppm) for 24 hours, which revealed larvicidal activity of AgNPs with LC₅₀ values of 3.50 to 7.01 ppm against II²nd instar and 4.44 to 8.74 ppm against IVth instar larvae of A. aegypti and 5.90 to 8.04 ppm for II²nd instar, 4.90 to 9.56 ppm against IVth instar of A. stephensi. Results obtained from this study present biosynthesized silver nanoparticles as novel biolarvicidal agent and can be used along with traditional insecticides as approach of Integrated Pest Management (IPM).

Keywords: Silver nanoparticles; Biolarvicide; LC₅₀; Malaria

Introduction
Silver nanoparticles (AgNPs) synthesis has been reported by chemical, physical and biological methods [1-6]. Currently, chemical and physical methods are mostly employed for nanoparticles synthesis at industrial level but use of toxic reducing and capping agents for synthesis, high temperature and pressure protocols, concerns for use in biomedical applications raise difficulties in utility of these methods. In view of shortcomings associated with chemical and physical methods, the interest is shifted towards utilizing potential of biological agents (living cells and their extracts) for nanoparticles production [7-9]. Among different nanomaterials, silver nanoparticles (AgNPs) are most commercialized [7] and its applications ranges from antimicrobial [10,11], biomedical [12,13], insecticidal [14,15], agriculture [16], biosensor [17] and water purification [18] to name a few.

Mosquito species A. aegypti and A. stephensi attracted considerable attention in medical and social region. They are vectors of many diseases accounting for huge mortality and morbidity worldwide. A. aegypti is carrier of Dengue Fever Virus (DENV) causing dengue fever, chikungunya fever, and dengue hemorrhagic fever [19]. According to WHO (2009) report of year 2009, two fifth of world population is under risk of dengue infection [20] and in year 2010, 28,292 cases of infection and 108 deaths were reported to be caused by dengue in India [21]. A. stephensi is vector of Plasmodium genus (protozoa) responsible for causing malaria. Figure of malaria is much higher than dengue affecting 225 million and 7,81,000 deaths worldwide in 2009 [20]. 1.49 million Infection and 767 deaths were reported in India in 2010 [22].

Control of these diseases carrying vector is need of our as they are the major public health concern at global level. Once the person infected with malaria, then it involves typical medical treatment and there is report of resurgence of malaria after eradication in many countries [23]. The better strategy to lower the incidence of mosquito-transmitted diseases and to avoid further complication is to avoid biting of mosquito’s using repellents and target larval stage of mosquito. Because, in larval stage they are having less mobility in breeding habitat so devising control measures at this stage involves comparatively easy [24]. Current practice to control mosquito larvae is the use of insecticides like carbamate, organophosphate and pyrethroids. Insecticides in there early days of use showed success in reducing vector population but Frequent and blind use of insecticides increases selection pressure on mosquitoes creating resistance to commonly used insecticides [20,25]. Varying amount of resistance to commonly used insecticides like temephos, fenthion, malathion and dichlorodiphenyltrichloroethane (DDT) is reported by Tikar et al. [26]. Moreover, chemical insecticides associated with many concerns like harm to nontarget species [27], long persistence in environment, entry in food chain [28]. In view of these facts, insect control agents from biological sources can be considered as safe and effective alternative.

Several plants are screened successfully for silver nanoparticles synthesis like Azadirachta indica [29], Aloe vera [1], Plumeria rubra [6], Nelumbo nucifera [15] and Emblica officinalis [30], Medicago sativa sprouts [31]. Phyto-synthesized silver nanoparticles as a mosquito larvicalid agent are gaining importance instead of chemical insecticide because of their safety, less harmful effect to non-targeted species, novelty in mechanism of action [6,32]. The plants used in the present study for AgNPs synthesis (Jatropha gossypifolia, Euphorbia tirucalli, Pedilanthus tithymaloides and Alstonia macrophyla) are reported in the literature for their medicinal, biocidal applications as well as presence

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Therefore, plant extracts from under this study can be promising as the synthesized AgNPs show ideal eco-friendly larvicidal activity. Under study is capable of synthesizing stable AgNPs at rapid rate and maintained under same conditions, separately.

Furthermore, mosquito larvicidal potential of the synthesized AgNPs was carried out against II\textsuperscript{nd} and IV\textsuperscript{th} instar larvae of \textit{A. aegypti} and \textit{A. stephensi}. Present study showed that leaves extract of plants under study is capable of synthesizing stable AgNPs at rapid rate and the synthesized AgNPs show ideal eco-friendly larvicidal activity. Therefore, plant extracts from under this study can be promising candidates for synthesis of mosquito larvicidal nanoparticles.

**Materials and Methods**

**Chemicals and reagents**

Silver nitrate and other chemicals were purchased from HiMedia and GlaxoSmithKline, India.

**Plant materials**

Plants used in the present study (\textit{J. gossypifolia}, \textit{E. tirucalli}, \textit{P. tithymaloides}, and \textit{A. macrophylla}) were collected from vicinity of Jalgaon district (210 00′ 24.5° N, 750 29′ 45.5° E, elevation 218 msl). Fresh leaves from the plants were collected, surface sterilized using Tween 20 and washed several times with distilled water. Ten gram of leaves were cut in fine pieces and mixed in 100 mL distilled water. The mixture was stirred for 5 hrs at 50°C. The solution was filtered through Whatman number 1 filter paper and filtrate was lyophilized, stored at 4°C and used as stock solution of plant extract for AgNPs synthesis.

**Synthesis of silver nanoparticles**

10 mg of lyophilized plant extract was added into 100 mL of silver nitrate (100 ppm) solution. The flask was incubated at 28°C without shaking. Simultaneously, controls with ten mg plant extract dissolved in Milli-Q deionised water and silver nitrate solution (100 ppm) were maintained under same conditions, separately.

**Characterization of silver nanoparticles**

**UV-Vis spectroscopy:** Leaves extract were challenged to 100 ppm AgNO\textsubscript{3} solution. The mixture were observed visually for any colour change and one mL of reaction mixture were withdrawn periodically for analysis of surface Plasmon resonance of silver nanoparticles using a UV-Vis spectrophotometer (Shimadzu 1601 model, Japan) at the resolution of 1 nm in range of 200 to 800 nm.

**FT-IR analysis:** FT-IR analyses were performed using Shimadzu FT-IR model number 8400. Approximately three mg of lyophilized leaves extract under study was mixed with 300 mg of dried KBr, crushed well in mortar and pestle to prepare thin pellet for analysis. Same procedure was performed for synthesized AgNPs using leaves extract. 16 scans per sample were taken in range of 400-4000 cm\textsuperscript{-1}.

**Scanning electron microscopy (SEM)**

A drop of aqueous solution containing purified silver nanomaterials obtained after repetitive centrifugation was placed on the carbon coated copper grids and dried under infrared lamp for characterization of their morphology using FEI Quanta 200 Scanning electron microscope at accelerating voltage of 20 KeV.

**Particle size analysis and Zeta potential**

Particle size and zeta potential of silver nanoparticles was analyzed on particle size analyzer system (Zeta sizer, Malvern Instruments Ltd., USA). In short, zeta potential cell were washed with ethanol and deionized water followed by AgNPs sample. The average distribution of nanoparticles based on intensity, volume, and number weighting was studied comparatively.

**Mosquito larvae**

Larval strains of \textit{A. aegypti} and \textit{A. stephensi} were collected from larval habitat in surrounding area such as small ponds near river, ponds below water storage tanks, pots and water collected in junkyard materials like tyres. The strains collected thus were identified from district malaria control Department, Jalgaon. Larvae were maintained in enamel tray containing dechlorinated tap water mixed with preparation of dog biscuits and yeast extracts (1:3) at 28 ± 2°C and 75–85% relative humidity under 14:10 light and dark.

**Mosquito larvicidal bioassay**

For bioassay test, II\textsuperscript{nd} and IV\textsuperscript{th} instar larvae of \textit{A. aegypti} and \textit{A. stephensi} were taken in four batches of 25 larvae in 249 mL of water and 1.0 mL of the desired concentration of AgNPs solution were added in each batch. The control was set up with dechlorinated tap water. The numbers of dead larvae were counted after 24 h of exposure, and the percentage mortality was recorded for the average of four replicates.

**Dose response bioassay**

Based on the preliminary screening results, synthesized AgNPs were subjected to dose–response bioassay for larvicidal activity against the larvae of \textit{A. aegypti} and \textit{A. stephensi}. Different concentrations of synthesized AgNPs ranging from 0.625 to 20 ppm were prepared for larvicidal activity of mosquitoes. The numbers of dead larvae were counted after 24 h of exposure and the percentage of mortality was reported from the average of four replicates.

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**Table 1:** Plants tested for AgNPs synthesis along with their reported medicinal properties.

| Botanical Name | Common Name (vernacular name) | Family | Medicinal Property | Chemical Constituents | References |
|---------------|-------------------------------|--------|-------------------|----------------------|------------|
| \textit{Jatropha gossypifolia} | Rataniyoli | Euphorbiaceae | Stomachache, veneral disease, Hemostatic agent | Alkaloid (jatrophone), lignan (jatroiden), proteins | [33,34] |
| \textit{Euphorbia tirucalli} | Konpal (thor) Indian tree spurge | Euphorbiaceae | Anticancerous, cure skin problem, antiasthemi, against snake bite, scorpion sting. | Coumarins, flavonoids, alkaloids, triterpenes | [35] |
| \textit{Pedilanthus tithymaloides} | Nagdaman (vayali sher) | Euphorbiaceae | Antiprotozoa and antimicrobial. | Euphorbol (terpene), beta-sitosterol, lectin | [36,37,38] |
| \textit{Alstonia macrophylla} | Satliun(saptaparni) | Apocynaceae | Antipyretic, antimaterial, antifungal and antiinflammatory | Indole alkaloids beta-sitosterol, ursolic acid. | [39] |

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Prepared by: Ratanjyoti Euphorbiaceae (gossypifolia) — Alstonia spurge (tirucalli) — Indian tree Nagdamon (thor) Euphorbiaceae (vilayati sher) Euphorbia (E, elevation 218 msl).
Statistical analysis

Mortality was calculated using Abbott's formula [40]. The dose-response data were subjected to probit regression analysis [41]. The lethal concentrations in parts per million (LC_{50}, LC_{90}) and the 95% confidence intervals of LC_{50} (upper confidence limit) and (lower confidence limit) were calculated.

Results and Discussion

UV-Vis spectroscopy

Leaves extracts from all plants under study (J. gossypifolia, E. tirucalli, P. tithymaloides and A. macrophylla) showed rapid conversion of silver nitrate into silver nanoparticles indicated by color changes from colorless to red brown within few minutes of extract addition in 100 ppm AgNO_3 solution. A representative scheme of biosynthesis and UV-Vis spectrum is given in Figure 1. Synthesized silver nanoparticles primarily characterized by UV-visible spectroscopy. AgNPs give typical spectrum having maximum absorption in range of 420-450 nm. This absorption is unique property of metal nanoparticles called SPR (surface Plasmon resonance) arises due to conduction of electrons on surface of AgNPs. SPR for different metal nanoparticles were reported in previous studies, for gold nanoparticles it is around 540 nm [42], 315 nm for Zinc sulphide (ZnS) nanoparticles [43]. After adding leaves extract in AgNO_3 solution, the biomolecules are stabilized in medium, interact with each other, and with silver salt, after initial interaction silver salt are consumed and the process of nucleation, reduction and capping starts leading nanoparticles synthesis. Similar observations were also reported by other researchers [1,44-47].

FT-IR

Typical IR spectrum of lyophilized powder of J. gossypifolia leaves extract showed presence of C-H bending vibrations at 827.49 cm^{-1}. C-O stretching at 1060.88 cm^{-1} may be due to alcohol, carboxylic acid and esters, peaks at 1398.44 cm^{-1} and 1523.82 cm^{-1} suggest presence of nitro compounds, C=O cm^{-1} stretching at 1772.64 cm^{-1} attributed to aldehyde, ketones and carboxylic acid, while the peak at 3554.93 cm^{-1} arises due to N-H (amines) present in proteins. IR of lyophilized AgNPs showed interesting observations. The intense peak at 1383.01 cm^{-1} is due to NO_3 which is very similar with observation reported by Begum et al. [48]. The peak at 3554.93 is nearly disappeared in spectrum of AgNPs suggesting role of protein in reduction and capping around formed nanoparticles. Previous studies also show role of proteins as reducing and capping agents [49,50]. Similarly, several other peaks are disappeared, change in transmission value and decreased in intensity after AgNPs synthesis (1060.88 cm^{-1}; 1523.82 cm^{-1}). Finding from FT-IR clearly suggest involvement of proteins and other bioorganic compounds from leaves extract in the formation and stabilization of AgNPs. An overlay spectrum of leaves extract of J. gossypifolia and AgNPs synthesized from J. gossypifolia leaves extract shown in Figure 2. FT-IR of AgNPs synthesized from leaves extract of all four plants under study (J. gossypifolia, E. tirucalli, P. tithymaloides, D. Alstonia macrophylla) showed rapid conversion of silver nitrate into silver nanoparticles indicated by color changes from colorless to red brown within few minutes of extract addition in 100 ppm AgNO_3 solution. A representative scheme of biosynthesis and UV-Vis spectrum is given in Figure 1. Synthesized silver nanoparticles primarily characterized by UV-visible spectroscopy. AgNPs give typical spectrum having maximum absorption in range of 420-450 nm. This absorption is unique property of metal nanoparticles called SPR (surface Plasmon resonance) arises due to conduction of electrons on surface of AgNPs. SPR for different metal nanoparticles were reported in previous studies, for gold nanoparticles it is around 540 nm [42], 315 nm for Zinc sulphide (ZnS) nanoparticles [43]. After adding leaves extract in AgNO_3 solution, the biomolecules are stabilized in medium, interact with each other, and with silver salt, after initial interaction silver salt are consumed and the process of nucleation, reduction and capping starts leading nanoparticles synthesis. Similar observations were also reported by other researchers [1,44-47].

Scanning electron microscopy (SEM), particle size analysis and Zeta potential

Characterization of plant nanoparticles under the study by SEM. Particle size analysis and zeta potential revealed that nanoparticles formed by J. gossypifolia are spherical in shape, average size of 163 nm with negative zeta potential i.e., -1.38 mv compared to other plants (Figures 4-6). AgNPs synthesized by other plant extract also showed spherical shape with larger size than that of J. gossypifolia synthesized AgNPs (data not shown). The zeta potential of all AgNPs show negative values thus strongly supporting long time stability of AgNPs. We observed that AgNPs are stable up to four months without agglomeration (Data not shown). The negative zeta potential contributes to stability of AgNPs. Different organophosphates and
Larvicidal assay

The results of larvicidal bioassays of synthesized AgNPs performed on IIrd and IVth instars larvae of \textit{A}. \textit{aegypti} and \textit{A}. \textit{stephensi} are presented in Tables 2 and 3. All synthesized AgNPs showed the larvicidal efficacy within 24 hr of exposure. Mortality rate \((Y)\) is positively related to the concentration of dose \((X)\) indicating that mortality increases with the increasing dose.

Among the AgNPs tested, the AgNPs of \textit{J}. \textit{gossypifolia} were highly effective against IIrd instar larvae of both \textit{A}. \textit{aegypti} and \textit{A}. \textit{stephensi} with LC$_{50}$ of 3.50 and 5.90 ppm, respectively and LC$_{50}$=4.44 and 4.90 ppm against IVth instars \textit{A}. \textit{aegypti} and \textit{A}. \textit{stephensi}. High larvicidal activity of \textit{J}. \textit{gossypifolia} mediated AgNPs can be correlated with its lower particle size than other AgNPs from different plants. Smaller particle size increase surface area to volume ratio and thus increases its action against larvae. Sosenkova and Egorova give similar results of effect of particle size and shape on antibacterial application \cite{52}.

The order of effectiveness decreased from \textit{J}. \textit{gossypifolia}>\textit{P}. \textit{tithymaloides} >\textit{E}. \textit{tirucalli} >\textit{P}. \textit{tithymaloides} against IIrd instars of \textit{A}. \textit{stephensi} and \textit{J}. \textit{gossypifolia}>\textit{E}. \textit{tirucalli}>\textit{P}. \textit{tithymaloides}>\textit{A}. \textit{macrophylla} against IIrd instars of \textit{A}. \textit{aegypti}. For IVth instars of \textit{A}. \textit{aegypti} and \textit{A}. \textit{stephensi} effectiveness found in order of \textit{J}. \textit{gossypifolia}>\textit{P}. \textit{tithymaloides} >\textit{E}. \textit{tirucalli}>\textit{P}. \textit{tithymaloides} (Tables 2 and 3).

All plants used in the present study showed LC$_{50}$ values less than 13 ppm, which would be important factor in design of promising and practical larvicidal dose. Shaalan et al. \cite{53} reviewed that the varying polymers were used to increase zeta potential of iron nanoparticles but in our case, it does not require any external stabilizers thus further eliminating use of synthetic compounds \cite{51}. 

Figure 3: FT-IR spectrum overlay- (A) standard AgNPs powder; AgNPs synthesized using (B) \textit{J}. \textit{gossypifolia}, (C) \textit{A}. \textit{macrophylla}, (D) \textit{P}. \textit{tithymaloides} and (E) \textit{E}. \textit{tirucalli}.

Figure 4: SEM image of spherical AgNPs synthesized by \textit{J}. \textit{gossypifolia}.

Figure 5: Particle size histogram of AgNPs synthesized using plant leaves extracts of \textit{J}. \textit{gossypifolia}.

Figure 6: Zeta potential of AgNPs synthesized using leaves extracts of \textit{J}. \textit{gossypifolia}.
results obtained in lethal concentration were probably due to the differences in the levels of toxicity among the insecticidal ingredients of each plant and the effect of plant extracts can vary significantly depending on plant species, plant part, age of plant part, solvent of extraction and mosquito species. The higher mortality rates at lower doses are comparable with earlier reports of AgNPs produced by plant *Nelumbo nucifera* leaf extracts (LC₅₀=0.69 ppm, LC₉₀=2.15 ppm) against *A. subpictus* and *C. quinquefasciatus* (LC₅₀=1.10 ppm, LC₉₀=3.59 ppm) [15]. Marimuthu et al. reported bioactivity of synthesized AgNPs against the larvae of *A. subpictus* and *C. quinquefasciatus*, and *R. microplus* (LC₅₀=13.90, 11.73, and 8.98 ppm, respectively) [32]. Larvicidal activity of synthesized AgNPs utilizing an aqueous extract from *Eclipta prostrata* was observed in crude aqueous, and synthesized AgNPs against *Cu. quinquefasciatus* (LC₅₀=27.49 and 4.56 ppm; LC₉₀=70.38 and 13.14 ppm) and against *An. subpictus* (LC₅₀=27.85 and 5.14 ppm; LC₉₀=71.45 and 25.68 ppm) respectively [54].

Previous studies have demonstrated the involvement of proteins, polyphenols, carbohydrates in AgNPs synthesis [32,55,56]. Shankar et al. [29] suggested role of protein and terpenoids from *Azadirachta indica* (Neem) leaf broth in AgNPs synthesis. Allicin and other carbohydrates from *Allium sativum* (garlic) extract were shown to catalyzing AgNPs synthesis and stabilization is further part of study. The results recorded from UV-Vis spectrum, FT-IR, SEM, Particle size analysis and zeta potential supports the biosynthesis of AgNPs. Different researchers [60,61] reported similar reports of binding of protein and carbohydrate on silver and gold nanoparticles surface.

**Conclusion**

The results recorded from UV-Vis spectrum, FT-IR, SEM, Particle size analysis and zeta potential supports the biosynthesis of AgNPs. It is therefore, suggested that leaves extract of plants (*J. gossypifolia, E. tirucalli, P. tithymaloides* and *A. macrophylla*) can be applied as an ideal potential source for synthesis of AgNPs. AgNPs can be used in the development of novel therapeutic agents with the potential to control vector-borne disease. AgNPs have been reported to have substantial potential in the development of new drugs against *P. falciparum* and *P. vivax* infections [12,22]. In order to extend the application of AgNPs as new drug formulation, further studies are needed to explore the pharmacological activity of AgNPs.

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