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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 macrophylla*) and there effects on IInd 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. IInd 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 LC_{50} values of 3.50 to 7.01 ppm against IInd instar and 4.44 to 8.74 ppm against IVth instar larvae of *A. aegypti* and 5.90 to 8.04 ppm for IInd instar, 4.90 to 9.55 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; LC_{50}; 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]. Phytosynthesized silver nanoparticles as a mosquito larvicidal 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 macrophylla*) are reported in the literature for their medicinal, biocidal applications as well as presence

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

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

of active chemical constituents (Table 1) and found abundantly in local site. Due to these reasons, we select these species for study. In the present paper, a simple, one-step ecofriendly method of silver nanoparticles synthesis using aqueous leaves extracts of *J. gossypifolia*, *E. tirucalli*, *P. tithymaloides,* and *A. macrophylla* is reported.

Furthermore, mosquito larvicidal potential of the synthesized AgNPs was carried out against IInd and IVth instar larvae of *A. aegypti* and *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 (*J. gossypifolia*, *E. tirucalli*, *P. tithymaloides* and *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 $AgNO_3$ 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^{-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 *A. aegypti* and *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, IInd and IVth instar larvae of *A. aegypti* and *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 *A. aegypti* and *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.

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].

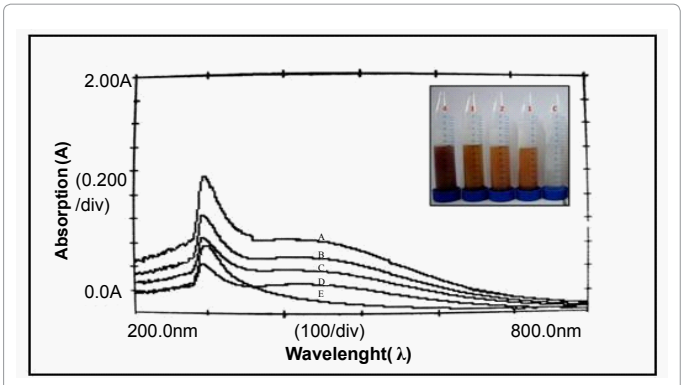

Figure 1: Absorption spectrum of silver nanoparticles synthesized from leaves extracts of A. *Jatropha gossypifolia*, B. *Euphorbia tirucalli*, C. *Pedilanthus tithymaloides, D. Alstonia macrophylla* and E. Silver nitrate solution (Inset- Reddish brown colour of AgNPs synthesized from left to right 1. *Jatropha gossypifolia*, 2. *Euphorbia tirucalli*, 3. *Pedilanthus tithymaloides,* 4. *Alstonia macrophylla* and 5. Silver nitrate solution).

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 extracts of all four plants under compared with standard AgNPs powder. Distinct peaks were obtained in AgNPs synthesized from other plant extract accounting presence of biomolecules with AgNPs (Figure 3).

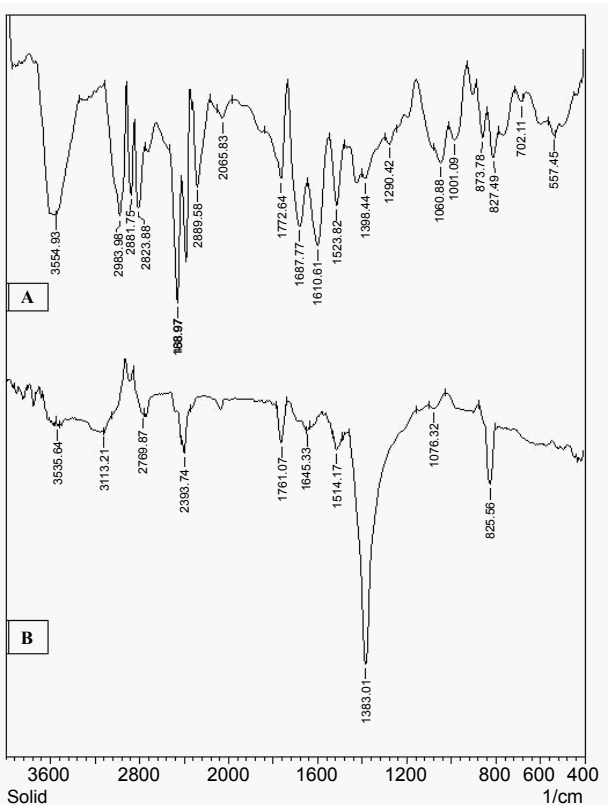

Figure 2: FT-IR spectrum overlay- (**A)** leaves extract of *J. gossypifolia* (**B)** AgNPs synthesized by leaves extract of *J. gossypifolia.*

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

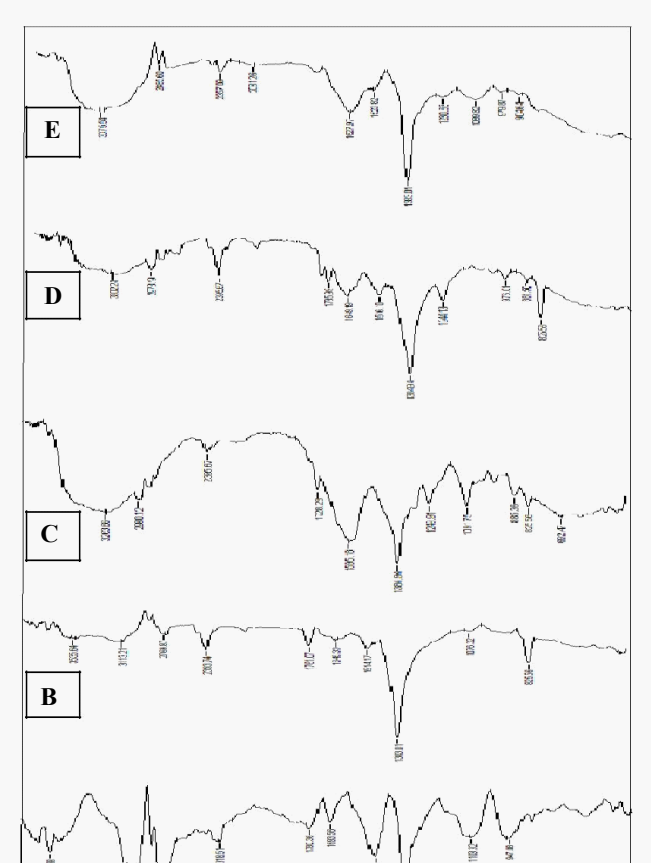

Figure 3: FT-IR spectrum overlay- (**A**) standard AgNPs powder; AgNPs synthesized using (**B**) *J. gossypifolia*, (**C**) *A. macrophylla*, (**D**) *P. tithymaloides* and (**E**) *E. tirucalli*.

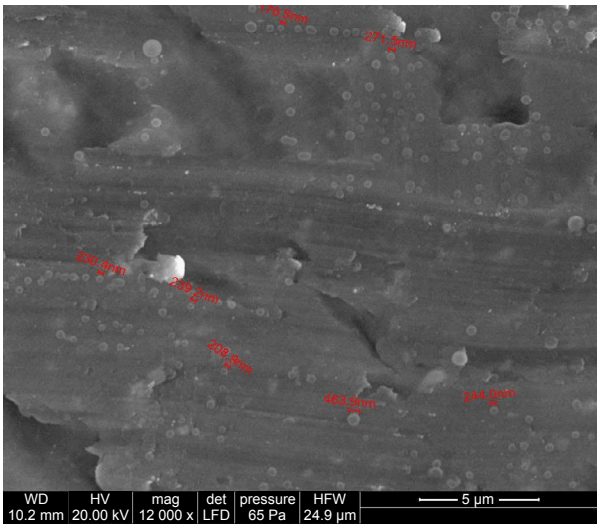

Figure 4: SEM image of spherical AgNPs synthesized by *J. gossypifolia*.

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 [51].

Larvicidal assay

The results of larvicidal bioassays of synthesized AgNPs performed on IInd and IVth instars larvae of *A. aegypti* and *A. 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 *J. gossypifolia* were highly effective against IInd instar larvae of both *A. aegypti* and *A. stephensi* with LC_{50} of 3.50 and 5.90 ppm, respectively and LC_{50}=4.44 and 4.90 ppm against IVth instars *A. aegypti* and *A. stephensi*. High larvicidal activity of *J. 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 [52].

The order of effectiveness decreased from *J. gossypifolia>P. tithymaloides>E. tirucalli>A. macrophylla* against IInd instars of *A. stephensi* and *J. gossypifolia>E. tirucalli>P. tithymaloides>A. macrophylla* against IInd instars of *A. aegypti*. For IVth instars of *A. aegypti* and A. *stephensi* effectiveness found in order of *J. gossypifolia>P. tithymaloides >E. tirucalli>P. 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. [53] reviewed that the varying

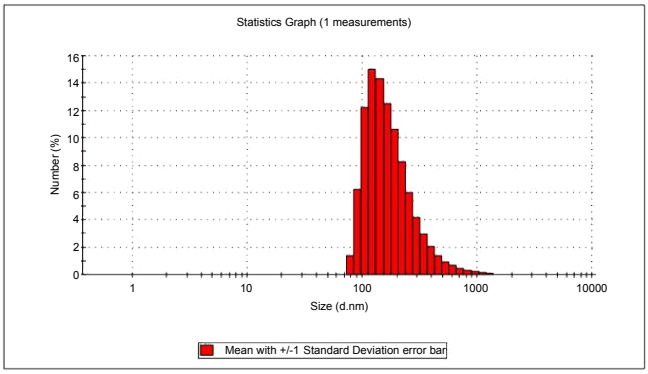

Figure 5: Particle size histogram of AgNPs synthesized using plant leaves extracts of *J. gossypifolia*.

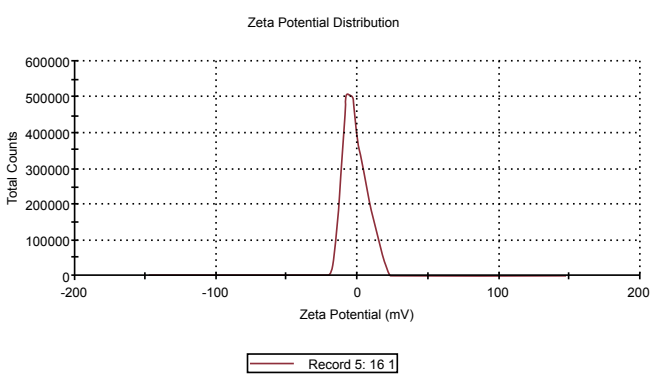

Figure 6: Zeta potential of AgNPs synthesized using leaves extracts of *J. gossypifolia*.

Mosquito species	Plant AgNPs	LC_{50} ±SE (mg lit-1)	95% Fiducial limits	LC_{90}±SE	95% Fiducial limits	Regression equation
Anopheles stephensi	*J. gossypifolia*	5.90 ± 0.42	3.21-4.89	5.17 ± 0.99	10.69-13.71	Y=5.85+0.99X
	E. tirucalli	7.21 ± 0.48	6.30-8.22	17.45 ± 1.11	15.55-20.02	Y=4.78+0.980 X
	P. tithymaloides	6.26 ± 0.45	5.40-7.19	16.08 ± 1.04	14.31-18.50	Y=5.68+0.975 X
	A. macrophylla	8.04 ± 0.54	7.02-9.19	19.80 ± 1.29	17.60-22.80	Y=4.76+0.920 X
Aedes aegypti	*J. gossypifolia*	3.50 ± 0.30	2.89-4.11	9.95 ± 0.69	8.78-1.60	Y= 8.53+ 0.960 X
	E. tirucalli	3.63 ± 0.35	2.92-4.32	11.21 ± 0.82	9.88-13.05	Y= 7.83+ 0.978 X
	P. tithymaloides	4.06 ± 0.35	3.35-4.77	11.73 ± 0.79	10.38-13.58	Y= 7.83+ 0.978 X
	A. macrophylla	7.01 ± 0.40	6.27-7.86	14.55 ± 0.86	13.07-16.52	Y= 3.29+ 1.14 X

Y mortality rate (significant at P<0.05 level), X concentration (significant at P<0.05 level), LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, SE standard error (all values are mean of four replicates), LCL lower confidence limit, UCL upper confidence limit.

Table 2: Larvicidal activity of plant extract synthesized AgNPs against IInd instars larvae of *Aedes aegypti* and *Anopheles stephensi.*

Mosquito species	Plant AgNPs	LC_{50} ±SE (mg lit-1)	95% Fiducial limits	LC_{90}±SE	95% Fiducial limits	Regression equation
Aedes aegypti	*J. gossypifolia*	4.44 ± 0.26	3.93-5.00	9.52 ± 0.58	8.52-10.89	Y=5.81+1.12X
	E. tirucalli	6.75 ± 0.44	5.91-7.69	15.96 ± 1.04	14.18-18.40	Y=4.79+1.00X
	P. tithymaloides	6.75 ± 0.44	5.91-7.69	15.96 ± 1.04	14.18-18.40	Y=4.79+1.00X
	A. macrophylla	8.74 ± 0.44	7.93-9.69	15.94 ± 0.88	14.42-17.95	Y= 0.892+1.20X
Anopheles stephensi	*J. gossypifolia*	4.90 ± 0.36	4.21-5.65	12.60 ± 0.85	11.16-14.59	Y=6.59+1.01X
	E. tirucalli	8.18 ± 0.43	7.38-9.12	15.76 ± 0.90	14.19-17.83	Y=1.86+1.16X
	P. tithymaloides	6.46 ± 0.41	5.68-7.34	14.94 ± 0.95	13.30-17.16	Y=4.69+1.04X
	A. macrophylla	9.55 ± 0.48	8.67-10.59	17.41 ± 0.95	15.76-19.57	Y=0.617+1.16X

Y mortality rate (significant at P<0.05 level), X concentration (significant at P<0.05 level), LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, SE standard error (all values are mean of four replicates), LCL lower confidence limit, UCL upper confidence limit.

Table 3: Larvicidal activity of plant extract synthesized AgNPs against IVth instars larvae of *Aedes aegypti* and *Anopheles stephensi*.

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 (LC_{50}=0.69 ppm, LC_{90}=2.15 ppm) against *A. subpictus* and *C. quinquefasciatus* (LC_{50}=1.10 ppm, LC_{90}=3.59 ppm) [15]. Marimuthu et al. reported bioactivity of synthesized AgNPs against the larvae of *A. subpictus*, *C. quinquefasciatus*, and *R. microplus* (LC_{50}=13.90, 11.73, and 8.98 ppm), respectively [32]. Larvicidal activity of synthesized AgNPs utilizing an aqueous extract from *Eclipta prostrate* was observed in crude aqueous, and synthesized AgNPs against *Cu. quinquefasciatus* (LC_{50}=27.49 and 4.56 ppm; LC_{90}=70.38 and 13.14 ppm) and against *An. subpictus* (LC_{50}=27.85 and 5.14 ppm; LC_{90}=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 be active compounds catalyzing AgNPs synthesis [57]. The plants used in the present study are reported to contain proteins, alkaloids, flavonoids, triterpenes, lectins etc. Proteins present in leaves extract may be responsible for reduction of silver. However, further detailed studies about role of proteins or cumulative action of different metabolites in AgNPs synthesis will be needed to reveal the exact mechanism of nanoparticles formation. The larvicidal property of AgNPs may be accounted for its effect on digestive tract enzymes, structural deformation in DNA, generation of reactive oxygen species [10,58,59]. Increased larvicidal spectrum may also be due to synergistic combination of AgNPs and proteins and other secondary metabolites adhering on AgNPs surface during reduction and stabilization and 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. An attempt has been made to evaluate the potential mosquito larvicidal activity of aqueous leaves extract synthesized AgNPs. The results reported in this study open the possibility of further investigations on the efficacy of the larvicidal, insecticidal properties of biologically synthesized AgNPs. Purification of different compounds from lyophilized extracts and detail characterization of active bioorganic compound of leaves extract catalyzing AgNPs synthesis and stabilization is further part of study.

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